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*Research Article*

## Effects of three pesticides used to control sea lice on the early development of *Choromytilus chorus*, *Sphaerechinus granularis*, and *Paracentrotus lividus*

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**ABSTRACT.** The high production levels of the Chilean salmon farming industry have resulted in the emergence of several diseases that affect all stages of development of cultured species. The parasitic copepod *Caligus rogercresseyi* is considered a potential vector of pathologies, and different chemical compounds such as pyrethroids and organophosphates have been used to prevent and control sea lice outbreaks. In this study, the effect of azametiphos, deltamethrin, and emamectin benzoate on the larval stages of the mussel *Choromytilus chorus* and the sea-urchins *Sphaerechinus granularis* and *Paracentrotus lividus* was explored. No effects were found in the final larval development of *C. chorus* (D larvae). However, the trochophore development seemed accelerated in the presence of pesticides compared to the control larvae. In the case of *P. lividus* and *S. granularis*, exposure to all pesticides caused an increase in the rate of abnormal larval development compared to the control.

**Keywords:** *Choromytilus chorus*, *Sphaerechinus granularis*, *Paracentrotus lividus*, salmon farming, embryo-larvae bioassay, pesticides.

### INTRODUCTION

Parasitic infections derived from copepods have been described in the Chilean salmon farming industry since its beginning. Reports of *Caligus teres* were followed by the appearance of *Caligus rogercresseyi* in 1997 (Reyes & Bravo, 1983a, 1983b; Boxshall & Bravo, 2000). *C. rogercresseyi* mainly affects two species of salmon; Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) (Oelckers *et al.*, 2015). In addition to causing epidermal lesions, sea lice can act as a vector for other diseases (*e.g.*, ISA) resulting in lower quality in the final product, low growth rates, and important economic losses (Bravo *et al.*, 2012, 2013, 2015a; Oelckers *et al.*, 2015).

Several pesticides, which were initially developed for the bovine and agricultural industry, have been used in order to keep this parasite under control. Currently, the pyrethroids deltamethrin (DELTA) and emamectin benzoate (EMA) are widely used in baths or supplied in salmon feeds in Chile and Norway. EMA binds the invertebrate glutamate regulated ion channels while DELTA causes paralysis by interfering with the sodium-potassium channels in nerve cells. Recently were introduced the organophosphate azametiphos (AZA) that has also been efficiently and which applies through of baths with this compound (Bravo *et al.*, 2015b; Nilsen *et al.*, 2017). Since treatments are carried out frequently in impacted areas, significant quantities of these compounds can potentially be released into the

aquatic environment by either dilution of bath treatments or as uneaten medicated food pellets, which can account for as much as 15% of total administered food (Chen *et al.*, 1999). These anti-lice compounds can cause deleterious effects on non-target species, regardless of the mode of action or administration.

Several studies have described the effects of some of these products (*i.e.*, cipermethrin, deltamethrin) on macrofauna, phytoplankton, and some species of economic importance (Ait *et al.*, 2011; Kumar *et al.*, 2011; Wang *et al.*, 2011; Shen *et al.*, 2012; Samuelson *et al.*, 2015). EMA, for instance, is known to cause premature molting in *Homarus americanus*, but lethal effects on this species have been discarded. However, the existing information on the effect of other compounds on the early stages of development of marine organisms is not conclusive and seems to vary depending on the tested compound.

The embryo-larvae bioassays have been widely used for evaluating toxicity because early stages of development are more sensitive to pollutants than adults. It is a quick and inexpensive method that allows testing the effect of chemotherapeutic drugs on the normal development of model species (Stebbing *et al.*, 1980). Mussels and sea urchins have been successfully used to evaluate the quality of water and the biological effects of contamination in marine environments (Liu & Lee, 1975; Dermeche *et al.*, 2012). *Choromytilus chorus*, commonly known as “choro zapato,” is a Chilean native mussel of commercial and ecological importance. It is found between Callao (Peru) and the Magellan Strait and Beagle Channel in southern Chile (Osorio, 2002). *Sphaerechinus granularis* and *Paracentrotus lividus*, on the other hand, are two species of echinoderms with a wide distribution in the Mediterranean Sea and the Atlantic Ocean which are also commonly used for toxicity tests (Young *et al.*, 1997; De Nicola *et al.*, 2003; Carballeira *et al.*, 2011). *P. lividus*, in particular, is considered an excellent indicator of environmental health (Dermeche *et al.*, 2012).

The experiments in this study were designed to describe the possible effects of the three pesticides AZA, DELTA, and EMA on the early development of the mussel *Choromytilus chorus* and the sea urchins *Sphaerechinus granularis* and *Paracentrotus lividus*.

## MATERIALS AND METHODS

### Effect of three pesticides on the early stages of mussel development

The experiments were carried out between September 1 and 12, 2017 at the Laboratory of Ecotoxicology at the University of Concepcion, Chile.

The experimental design included the evaluation of toxicity of AZA, DELTA, and EMA on the early stages of development of *Choromytilus chorus*. A negative control was systematically setup with no pesticide addition. Stock solutions of AZA (Dr. Ehrenstorfer, 98.5% purity), DELTA (Dr. Ehrenstorfer, 99.5% purity), and EMA (Dr. Ehrenstorfer, 91% purity) were prepared by dissolving the reagents in dimethylsulphoxide (DMSO, MERCK, 99.9% analytical grade) and then diluting the solution in four different concentrations for each pesticide as treatments (1, 10, 100, 1000  $\mu\text{g L}^{-1}$ ). These concentrations were defined in order to test increasing doses while keeping the final concentration within the range of values commonly used in the salmon industry. Pesticides used were of high purity and not the commercial version used in salmon farming, in an effort to avoid additive effects of chemical compounds used in the commercial version of the product.

The experimental setup of these bioassays included the following steps: spawning induction or stripping/trip spawning, incubation (in the presence of DMSO and pesticides), sperm toxicity test, larval development count, and evaluation of possible malformations and mortality.

### Spawning induction

Adult specimens of *Choromytilus chorus* were obtained in a local culture facility at Coliumo Bay in Concepcion, Chile (37°31.317'S, 72°57.662'W).

Before spawning, mussels were cleaned of any attached biological materials and were separated by sex in individual trays at the laboratory. The female and male gametes were obtained using the “stripping/trip spawning” technique. Sperm was carefully added to the eggs, and the mix was gently stirred in order to allow fertilization. The proportion of eggs and spermatozooids for fertilization was 1:100, respectively.

Embryos were allowed to stand for 30 min in order to allow sedimentation of the embryos with higher lipid content and therefore better quality. The supernatant was discarded. Incubations were carried out in seawater at 14°C, pH 7.8-8.1, 30 to 35 of salinity and 8 mg L<sup>-1</sup> of dissolved oxygen. Larvae were not fed during the incubations.

### Evaluation of DMSO toxicity

Before testing pesticide toxicity, the potential toxicity of the solvent DMSO was evaluated on the development of larvae and the viability of the fecundation process via a sperm toxicity test. For the sperm toxicity test, 5 mL borosilicate test tubes were used in order to expose the sperm to seawater and DMSO (99.9%) at different concentrations (0.3, 0.7,

**Table 1.** Pesticide concentration ( $\mu\text{g L}^{-1}$ ) per cup in each treatment and control used in mussels' bioassay. a) Sperm toxicity test, b) larval development. SW: seawater, AZA: azametiphos, DELTA: deltamethrin, EMA: emamectin benzoate. NA: not applicable.

a)

| Treatment | Concentration ( $\mu\text{g L}^{-1}$ ) | SW (mL) | Pesticide dose (mL) | Ovules (mL) | Sperm (mL) |
|-----------|--|---------|---------------------|-------------|------------|
| Control   | NA                                     | 5       | 0                   | 0.1         | 10         |
| AZA       | 1-10-100-1000                          | 4.5     | 0.5                 | 0.1         | 10         |
| DELTA     | 1-10-100-1000                          | 4.5     | 0.5                 | 0.1         | 10         |
| EMA       | 1-10-100-1000                          | 4.5     | 0.5                 | 0.1         | 10         |

b)

| Treatment | Concentration ( $\mu\text{g L}^{-1}$ ) | SW (mL) | Pesticide dose (mL) | Embryos (mL) |
|-----------|--|---------|---------------------|--------------|
| Control   | NA                                     | 30      | 0                   | 1            |
| AZA       | 1-10-100-1000                          | 27      | 3                   | 1            |
| DELTA     | 1-10-100-1000                          | 27      | 3                   | 1            |
| EMA       | 1-10-100-1000                          | 27      | 3                   | 1            |

1%) for a 1 h period. Ovules were then added and left for 2 h for fecundation.

In order to evaluate the possible effects of DMSO in larval development, the embryos were exposed to a solution containing seawater and DMSO in the same concentrations used in the bioassays with sperm. Incubations lasted for 5 days. Bioassays were terminated by fixing the samples with 100  $\mu\text{L}$  of formalin at 10% and were observed under a microscope (Olympus CH2, 40 $\times$ ).

### Evaluation of pesticides toxicity

As in the previous section, a sperm toxicity test (Table 1a) was performed with the three pesticide solutions (AZA, DELTA, and EMA). Dilutions of pesticides were made with DMSO (99.9%) to obtain the final concentrations of 1, 10, 100, and 1000  $\mu\text{g L}^{-1}$  for every pesticide. Incubations lasted 3 h and began when male gametes were exposed for 1 h to the pesticides; ovules were later added and incubated for 2 h.

Toxicity during fertilization was tested during incubation (2 h) in 30 mL polypropylene cups. In total, 45 polypropylene cups were used, which were divided into four groups: three were used for the pesticides tests and one group was used as a negative control (all treatments were done in triplicate; Table 1b).

The fertilized samples in polypropylene cups were incubated in a cold chamber at 14°C. Three cups were fixed with 100  $\mu\text{L}$  (10% formalin) after 0 h (T0), 2.5 days, and 6 days of incubation. Embryos were counted and observed to determine possible deformities and malformations using an Olympus CH2 inverted microscope. Pictures of the larvae were taken at each sampling time.

Successful fecundation was verified with microscope examination, by verifying the presence of the polar corpuscle or cell divisions in the samples. Two hours after adding the sperm to the oocytes, the concentration of embryos and larvae were determined by counting 1 mL of the sample in a Neubauer chamber.

### Statistical analysis

The Dunnett's test was used with the statistical package TOXSTAT in order to determine the significant differences ( $P \leq 0.05$ ) among the treatments. Before this, the chi-square test and Hartley's test were applied for normality and homogeneity. No transformations were made for data processing.

### Effect of three pesticides on early stages of sea urchin larvae

The experiments were carried out between March 2 and April 9, 2015 at the Observatoire Océanologique de Banyuls sur Mer, France.

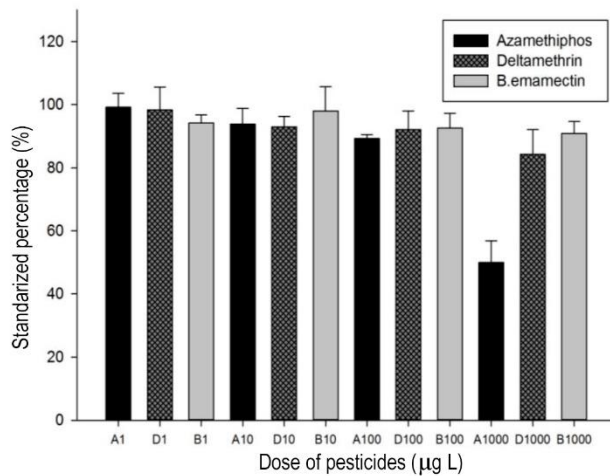
The effect of the three pesticides (AZA, DELTA, and EMA) was tested on the embryonic and larval stages of development of cultured sea urchins, *Sphaerechinus granularis* and *Paracentrotus lividus*. The initial solutions of AZA, DELTA, and EMA were prepared to a concentration of 10 g  $\text{L}^{-1}$  in a solution of dimethyl sulfoxide (DMSO, Sigma-Aldrich, 99.9%). Serial dilutions were made with DMSO 1% to obtain the final concentrations of 1000, 100, 10 and 1  $\mu\text{g L}^{-1}$ . The experimental setup includes three steps: spawning induction, fertilization, and incubation.

### Spawning induction

Specimens from each species (*S. granularis* and *P. lividus*) were shaken vigorously and deposited on a 200

**Table 2.** Results of Dunnett's test for sperm toxicity test (SP) and larval development (L. develop) at 2.5 days (T1) and 6 days (TF). DMSO in % and pesticides in  $\mu\text{g L}^{-1}$ . D larvae (LD), trochophore (TRO), and non-fertilized ovules (NFO). Significant values are highlighted in bold. NA: not applicable.

| Treatment  | P-value SP test | P-value (L. develop; T1)                      | P-value (L. develop; TF)  |
|------------|-----------------|---|---------------------------|
| DMSO 0.3   | >0.05           | NA  | LD > 0.05                 |
| DMSO 0.7   | <0.05           | NA  | LD > 0.05                 |
| DMSO 1     | < 0.05          | NA  | LD>0.05                   |
| AZA 1      | >0.05           | LD>0.05 TRO>0.05 NFO>0.05                     | LD>0.05 TRO>0.05 NFO>0.05 |
| AZA10      | >0.05           | LD>0.05 TRO>0.05 NFO>0.05                     | LD>0.05 TRO>0.05 NFO>0.05 |
| AZA100     | >0.05           | LD>0.05 TRO>0.05 NFO>0.05                     | LD>0.05 TRO>0.05 NFO>0.05 |
| AZA1000    | <0.05           | LD>0.05 TRO>0.05 NFO>0.05                     | LD>0.05 TRO>0.05 NFO>0.05 |
| DELTA 1    | >0.05           | LD>0.05 <b>TRO&lt;0.05</b> NFO>0.05           | LD>0.05 TRO>0.05 NFO>0.05 |
| DELTA 10   | >0.05           | LD>0.05 <b>TRO&lt;0.05</b> NFO>0.05           | LD>0.05 TRO>0.05 NFO>0.05 |
| DELTA 100  | >0.05           | LD>0.05 <b>TRO&lt;0.05</b> NFO>0.05           | LD>0.05 TRO>0.05 NFO>0.05 |
| DELTA 1000 | <0.05           | LD>0.05 <b>TRO&lt;0.05</b> NFO>0.05           | LD>0.05 TRO>0.05 NFO>0.05 |
| EMA 1      | >0.05           | LD>0.05 <b>TRO&lt;0.05</b> <b>NFO&lt;0.05</b> | LD>0.05 TRO>0.05 NFO>0.05 |
| EMA 10     | >0.05           | LD>0.05 <b>TRO&lt;0.05</b> <b>NFO&lt;0.05</b> | LD>0.05 TRO>0.05 NFO>0.05 |
| EMA 100    | >0.05           | LD>0.05 <b>TRO&lt;0.05</b> NFO>0.05           | LD>0.05 TRO>0.05 NFO>0.05 |
| EMA 1000   | >0.05           | LD>0.05 TRO>0.05 <b>NFO&lt;0.05</b>           | LD>0.05 TRO>0.05 NFO>0.05 |



**Figure 1.** Percentage of fertilized ovules of *Choromytilus chorus* submitted under different concentrations of pesticides. AZA (A), DELTA (D), and EMA (B).

mL plastic beaker filled with seawater to collect the eggs and sperm. Eggs were filtered through nylon 120  $\mu\text{m}$  mesh, washed with 0.22  $\mu\text{m}$  filtered seawater (FSW), and diluted to 2500 cells  $\text{mL}^{-1}$ . The eggs were fertilized with diluted sperm (1:100.000 FSW; 10  $\mu\text{L}$  of dry sperm per mL of FSW and 10  $\mu\text{L}$  of this solution to 10 mL of eggs).

**Incubations with added pesticides**

The incubations were carried out in three 96-well polystyrene flat-bottom microplates (Perkin Elmer). fertilization decreased with increasing DMSO concentrations, although losses did not exceed 20%. The percentage of fecundation with respect to the control

Each microplate was divided into four sections; three sections were used for the pesticides test (with five replicates for each concentration), and one section was used for a seawater control with 12 replicates. In each well treated with pesticide, 160  $\mu\text{L}$  of 0.22  $\mu\text{m}$  filtered seawater (0.22  $\mu\text{m}$  FSW) was added along with 20  $\mu\text{L}$  of pesticides solution and 20  $\mu\text{L}$  of embryos solution. In control wells, 180  $\mu\text{L}$  of 0.22  $\mu\text{m}$  FSW and 20  $\mu\text{L}$  of embryos solution were added. DMSO control was not included in this experiment because no impacts have been reported on sea-urchin development, especially in *P. lividus* (Sciarrino & Matranga, 1995).

The microplates were incubated at 17°C in dark conditions. Two microplates were fixed with formaldehyde 2% at 48, 72, and 96 h post-fertilization. Embryos in each well were observed using an Olympus IX70 inverted microscope.

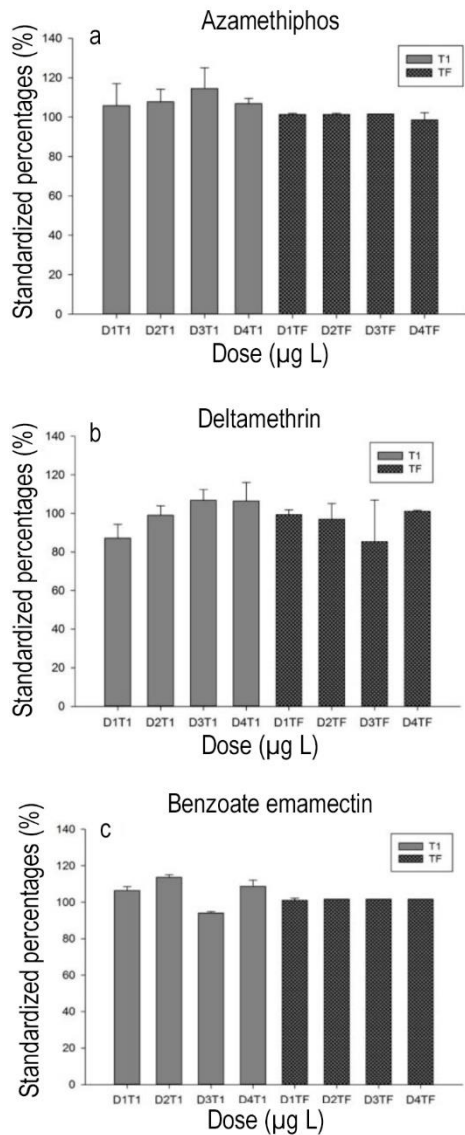
The embryos were classified as normal, delayed, or abnormal. Each embryo category was characterized using observations made by Young *et al.* (1997) as a reference for the species *S. granularis*, and reported descriptions for *P. lividus* (Carballeira *et al.*, 2012b).

**RESULTS**

**Effect of DMSO on sperm viability, fecundation and larval development of *Choromytilus chorus***

The toxicity of the reagent DMSO was tested at different concentrations (0.3, 0.7, 1%). The rate of fer-

was  $95 \pm 2\%$  at DMSO 0.3%,  $89 \pm 1\%$  at DMSO 0.7%, and  $83 \pm 6\%$  at DMSO 1%. Consequently, 0.7% of DMSO concentration was selected for the working solu-



**Figure 2.** Standardized percentages of larval development (Larva D) of *C. chorus* submitted to different concentrations of pesticides:  $1 \mu\text{g L}^{-1}$  (D1),  $10 \mu\text{g L}^{-1}$  (D),  $100 \mu\text{g L}^{-1}$  (D3),  $1000 \mu\text{g L}^{-1}$  (D4) plotted at different times. T1: 2 days and TF: 6 days.

tion, because the sperm toxicity test and larval development bioassays were higher than 80% and this would fall within the 70-90% accepted range of EPA norms (2002). Also, the  $P$ -value ( $>0.05$ ) was significant concerning the control (Table 2). Therefore, these percentages are considered acceptable and not indicative of the toxicity of the solvent.

There were no significant effects of DMSO on larval development (larvae D stage; Table 2). Larval development presented percentages of  $99 \pm 1$ ,  $100 \pm 1$  and  $98 \pm 2$  (standardized to the control) with 0.3, 0.7 and 1% of DMSO, respectively.

### Effect of pesticides in sperm viability and larval development of *Choromytilus chorus*

At T1 (2.5 d), embryo cells with normal development had experienced three cell divisions or less. The different treatments of the sperm toxicity test resulted in percentages of fertilization exceeding 84% (standardized to the control). Only the treatment with the highest dose of AZA ( $1000 \mu\text{g L}^{-1}$ ) showed a lower percentage of fertilization ( $50 \pm 7\%$ ;  $P < 0.05$ ). The decrease in fertilization success became more important as concentrations increased (Fig. 1).

During the larval development bioassays, most of the samples showed embryos reaching the D larval stage ( $>84\%$ ; Fig. 2) by the end of incubation. Treatments with AZA and EMA had a higher percentage of larval development ( $>97\%$  and  $99\%$ , respectively) for all concentrations. The treatment with DELTA had lower percentages for intermediate concentrations (such as  $100 \mu\text{g L}^{-1}$ ). At this concentration, around 85% of embryos reached larval D stage while only  $16 \pm 21\%$  arrived at the trochophore stage (the previous stage to larva D). On the other hand, only 1% of the D larvae had an irregular form.

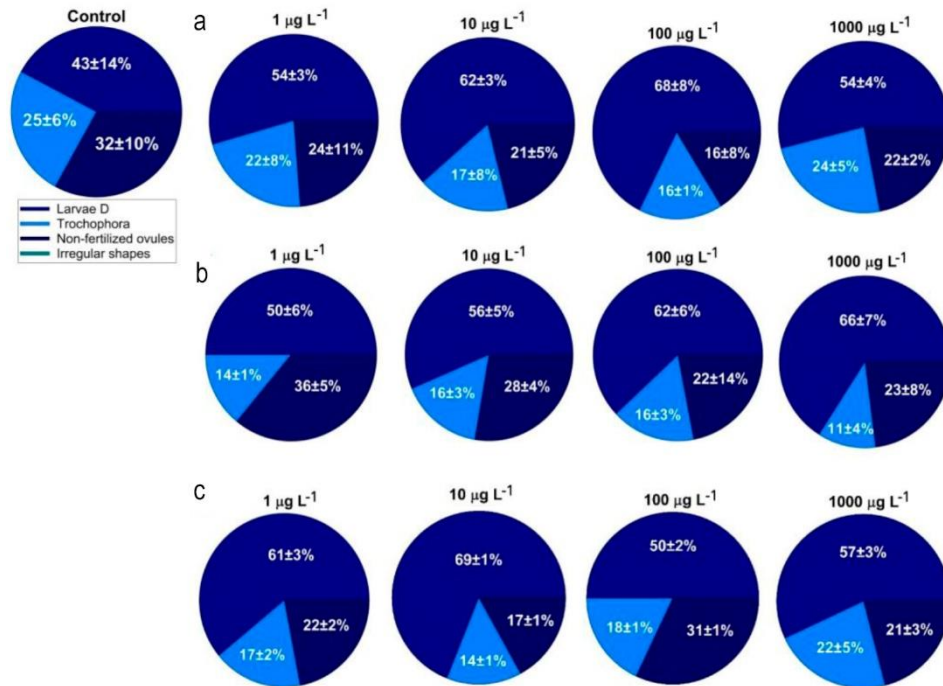
During the intermediate time (T1; 2.5 days; Fig. 3), the percentage of larval development of the bioassays with the three pesticides was higher than the control ( $43 \pm 14\%$ ). For AZA and DELTA treatments the abundance of trochophore larvae was higher compared to the control and remained high as the concentrations increased (up to  $68 \pm 18\%$  for the  $100 \mu\text{g L}^{-1}$  dose and  $66 \pm 7\%$  for the  $1000 \mu\text{g L}^{-1}$ , respectively). Only in the most concentrated dose of AZA ( $1000 \mu\text{g L}^{-1}$ ) was the number of D larvae similar to the  $1 \mu\text{g L}^{-1}$  treatment. In the case of EMA, the first two concentrations ( $1$  and  $10 \mu\text{g L}^{-1}$ ) showed percentages close to 60%, while the most concentrated treatment had percentages around 50%.

The percentages of the intermediate stages (trochophore) for the DELTA treatments were always lower than the control ( $P < 0.05$ ). The results for EMA, showed an increment with increasing concentrations ( $P < 0.05$ ), while with AZA the percentages were more variable, but with no significant differences compared to the control ( $P > 0.05$ ).

The counting of non-fertilized ovules, in general, decreased in the presence of the three pesticides, and only with  $1 \mu\text{g L}^{-1}$  of DELTA and  $100 \mu\text{g L}^{-1}$  of EMA were the percentages higher than 30% (Fig. 3).

### Effect of pesticides on sea urchin larvae

After the addition of pesticides, different stages of larval development were observed including normal, delayed, and malformed.



**Figure 3.** Pie charts of intermediate time T (2.5 days) with the percentages of the development stages of *C. chorús*, submitted to different concentrations of pesticides.

The *P. lividus* pluteus larvae with normal development 48 h post-fertilization (Fig. 4a) had a complete skeleton with a pointed aboral end, the whole formation of the first pairs of skeletal rods, and a second pair is growing. Delayed larvae had a late skeletal development, a skeleton with a rounded tip, the first pair of skeletal rods growing, and without evidence of a second pair of arms (Figs. 4b-4c).

Larvae with malformations showed different defects in the skeleton: the aboral end open or crossed (Figs. 4d-4f), the skeletal rods and aboral end gathered up (Fig. 4g), or ceased development of aboral end and skeletal rods (Fig. 4h). In some cases development completely stopped before gastrulation (Fig. 4i).

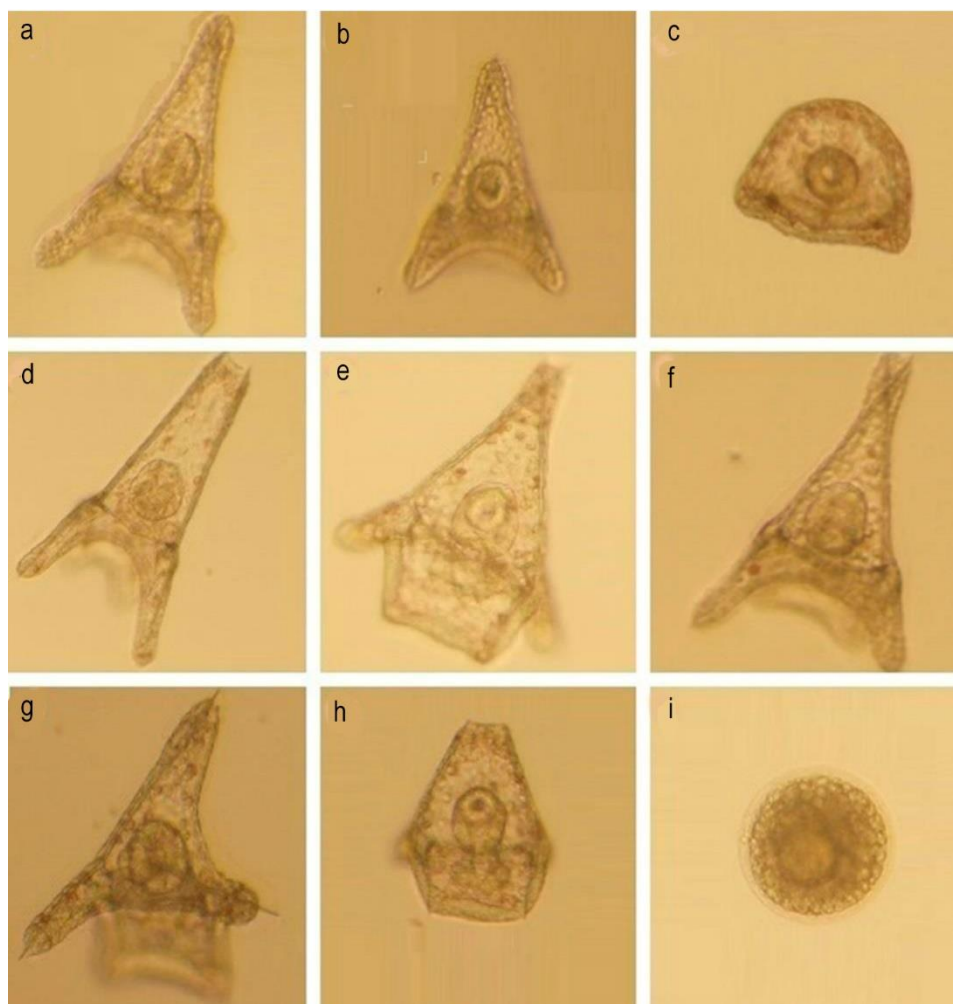
In control conditions, about 10% of *P. lividus* larvae showed a small delay in development (Figs. 4a-4c) and 20% had slight morphological anomalies. The frequency of abnormal development severely increased with pesticide concentration. The percentages of malformation reach 70% of the larvae in DELTA (1000 µg L<sup>-1</sup>), 66% in AZA (1000 µg L<sup>-1</sup>), and 70% in EMA (1000 µg L<sup>-1</sup>).

The *S. granularis* pluteus larvae with normal development 96 h post fertilization (Fig. 5a) had a developed skeleton, the whole formation of the first pair of skeletal rods, and a growing second pair. Larvae

in a delayed state showed a formed skeleton with the first pair of skeletal rods growing and without the second pair (Figs. 5b-5c). Skeletal malformations of the larvae were observed (Figs. 5d-5e) with the incorrect location of the skeletal bars, hyper-extended arms, fused tips into the aboral end, as well as aborted development (Fig. 5f).

The percentage of delayed larvae increased with pesticides concentration. At low pesticide concentrations (1 µg L<sup>-1</sup>), 15% of larvae treated with DELTA or AZA and 28% treated with EMA had delayed development. Those percentages reach respectively 20 to 25% in DELTA and AZA, and 35% in EMA treated eggs (1000 µg L<sup>-1</sup>; Fig. 6). While 25% of larvae presented deformities at low pesticides concentration (1 µg L<sup>-1</sup>), this percentage increased with the dose with a maximum of 50% larval abnormalities in EMA (1000 µg L<sup>-1</sup>) treated embryos.

Higher percentages of abnormal larvae were observed in *P. lividus* compared to *S. granularis*, suggesting different levels of sensitivity to xenobiotics in different species (Fig. 6), as already reported (Carballeira *et al.*, 2012a). Moreover, preliminary tests did not find effects of pesticides on the fertilization and early development divisions of *P. lividus* and *S. granularis*.



**Figure 4.** States of development of pluteus larvae of *P. lividus* after 48 h of incubation in the presence of pesticides. a) normal, b-c) larvae with delayed development, d-i) larvae with malformations. Scale: 10x.

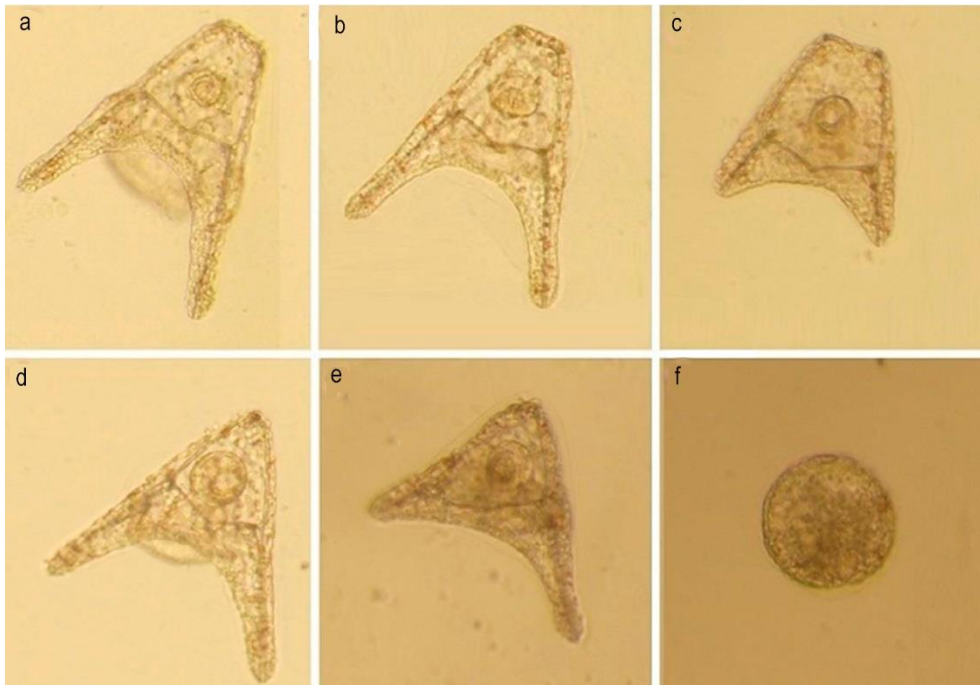
## DISCUSSION

The compounds tested in this study are commonly used by the salmon industry as treatments for lice infections in Chile (*C. rogercresseyi*) and Norway (*L. salmonis*) (Olesen *et al.*, 2011). Their intensive use is currently instrumental for decreasing losses due to diseases and parasites. While azametifos and deltamethrin can be applied as external bath treatments, emamectin benzoate is orally administrated as a food supplement and therefore is less absorbed by fish. However, it can be significantly transferred into the environment as uneaten food pellets and fecal pellets. Pesticides can also be added to the environment through plant feeds that have been recently developed. Mixtures of contaminants are therefore possible among neighboring farming facilities, with yet poorly characterized effects in non-target biota (Søfteland *et al.*, 2014).

This study explored the occurrence of developmental abnormalities resulting from the exposure to these compounds of three biological models with potential economic importance.

Mussel larvae development did not show significant abnormalities after exposure to AZA, EMA, or DELTA. Before the beginning of the toxicity experiment, the solvent DMSO was tested for possible effects on sperm viability and fertilization. Results showed no significant effect of DMSO on the larval development of sperm viability. On the other hand, other solvents like acetone have been observed to increase the percentage of undeveloped larvae of *M. chilensis* up to 60% (Tucca *et al.*, *unpubl. data*). Severe reduction of oxygen levels and the presence of acetone also have negative effects on development, as reported in previous studies with mussels (Liu & Lee, 1975; Wang & Widdows, 1991).





**Figure 5.** *S. granularis* pluteus larvae after 96 h of incubation in the presence of pesticides. States of development a) normal larvae, b-c) larvae with delayed development, d-f) larvae with malformations. Scale: 10x.

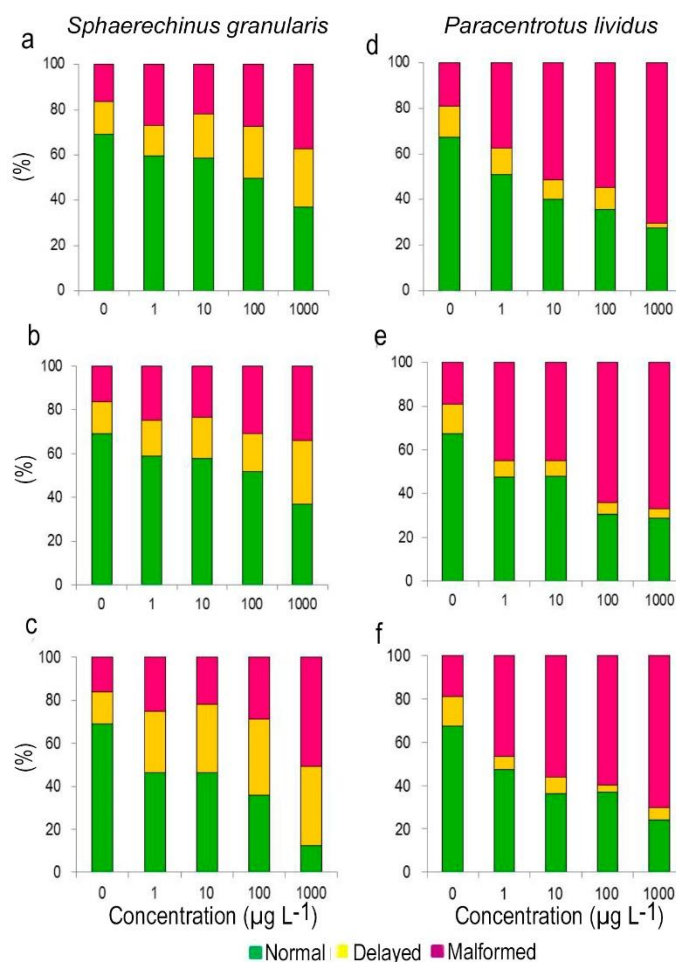
Although negative effects after exposure to pesticides such as pyrethroids and organic phosphorus compounds have been reported in adult and larval mussel stages (Gowland *et al.*, 2002; Renault, 2011), there are no studies that report the accelerated rate of intermediate stages of D larvae (trochophore) in *C. chorus* in the presence of pesticides (DELTA and EMA) as observed in this study. Adults of freshwater mussels (*Anodonta cygnea*) suffered altered filtration behavior after exposure to deltamethrin (Kontreczky *et al.*, 1997). For other organisms, such as American oyster and hardshell-clam, different effects were found for pesticides in different stages of development (Calabrese, 1972). Some pesticides affect primarily embryonic development compared to survival rate or growth of larvae. Likewise, a low concentration of a toxicant that had a low effect in embryonic development can delay the growth of fully developing larvae.

Concerning echinoderms, the exposure of embryos of *P. lividus* and *S. granularis* to the pesticides AZA, DELTA, and EMA altered their larval development, therefore compromising the viability of exposed individuals. Until now, there is no evidence of reversible effects after exposure to chemicals, so that alterations of embryonic and larval development can reduce the success of the adult individuals and the continuity of the population in natural environments (Carballeira *et al.*, 2012b).

Similar skeletal abnormalities have been already reported in *P. lividus* after exposure to the organophosphate Diazinon (Pesando *et al.*, 2003) with ruptures of the ectoderm and incomplete skeletal rods (Carballeira *et al.*, 2012b). Similarly, embryos and larvae of this species showed a high sensitivity to heavy metal exposure (Dermeche *et al.*, 2012). Carballeira *et al.* (2011) suggest that fertilization tests are less sensitive than the embryo-larval bioassays and that pollutants can disturb the larval development without affecting the fertilization process.

It is known that the distribution of chemical compounds (particularly of pharmaceutical use) in marine environments depends on the depth of the cage and local circulation (Langford *et al.*, 2014; Samuelsen *et al.*, 2015). In Norway, emamectin benzoate accumulates in high quantities in sediments (Langford *et al.*, 2014). Low currents can also enhance organic accumulations of pesticides (*i.e.*, teflubenzuron) below or near salmon cages and can limit its horizontal distribution beyond 500 m (Samuelsen *et al.*, 2015).

Wild fauna associated with salmon farming can also be affected, although studies on adult fish species have proved inconclusive. Accumulation of chemicals has been described (*e.g.*, teflubenzuron) but toxic testing revealed a low toxicity effect (Olsvik *et al.*, 2013; Samuelsen *et al.*, 2015). High accumulation but the low



**Figure 6.** Percentage of pluteus larvae of *S. granularis* (72 h) and *P. lividus* (48 h) with normal development, delayed development, and malformations when exposed to pesticides. a-d) Deltamethrine, b-e) azame-tiphos, c-f) emamectin benzoate.

lethal outcome has been reported for polychaetes, but high toxicity was observed in diverse species of crustaceans (Olsvik *et al.*, 2013; Samuelsen *et al.*, 2015). Unlike EMA, DELTA was not detected in natural samples in a study carried out in Norway (Langford *et al.*, 2014). EMA is mostly accumulated in sediments, and its incidence in blue mussel (*M. edulis*) has been reported as low or under detection limits during treatments (Telfer *et al.*, 2006; Langford *et al.*, 2014).

Transcriptional modifications partly cause resistance to drugs developed in sea lice. When occurring, resistance forces alternations in the use of specific products by the salmon farming industry. However, the use of chemical treatments for lice infections cannot be suspended until the still variable effectiveness of alternative treatments such as fresh-water, and biological methods are improved (Olesen *et al.*, 2011; Bravo *et al.*, 2015a). The recent increase in resistance

to emamectin benzoate has to lead to a decrease in its use while deltamethrin, cypermethrin, and other pyrethroids have presented increases in use, including the organophosphorus azametiphos. Azametiphos has been known to cause mortality and spawning damage in lobsters in confined conditions (Burrige *et al.*, 2008; Couillard & Burrige, 2015) and to alter some metabolic functions in *M. edulis* adult stages (Canty *et al.*, 2007).

The concentrations of veterinary pesticides used in the salmon industry are lower than those tested in this study. In contrast to the high purity products used in our experiments, bath treatments for salmon use azametiphos and deltamethrin at 50% and 1% of purity, respectively. In the case of emamectin benzoate supplied as food, a product with only 0.2% of purity is applied (IFOP, 2015). Therefore, the concentrations of compounds found in marine environments during treatments are likely to be lower than those used in the present study. Nevertheless, considering the frequency

of treatments and the rotation of compounds usually applied, and given the limited existing information regarding the amounts of these compounds regularly used, more studies are necessary for evaluating the effects of these compounds and their mixtures in organisms.

The results reported here suggest variable effects of anti-lice treatments for marine organisms, from an innocuous impact in the final larval development on *C. chorus* (D larvae) to the recruitment compromising effects on echinoderms. However, an interesting effect is observed during the intermediate times of the development stages before D larvae (as trochophore), as observed in the bioassays on *C. chorus*, where some counts were higher than the control. These responses imply that, although used in low concentrations, anti-lice drugs can affect non-target species of the benthic fauna in near-shore facilities. Although embryo-larval bioassays allow a simple evaluation of the effect of pollutants on organism development, further molecular tests will allow understanding of the regulation of toxicity in vulnerable species.

### CONCLUSIONS

The early stages of development of *C. chorus* embryos did not show a clear effect to pesticide exposure at the final time in the bioassays, but further studies are needed to explain the rapid development (compared to the control) observed during 2.5 days. It will be necessary to evaluate the later stages of organisms, as pesticides can eventually compromise their ability to feed or to fix to a substrate. The response of echinoderms in different developmental stages to the pesticides tested in this study was significant. The embryo and larvae development stages of *P. lividus* were more sensitive to pesticide exposure compared to *S. granularis*, with a higher percentage of abnormal embryo-larvae development or malformations.

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