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Oregano oil as a therapeutic treatment in the production of mixotrophic larvae of the lion's paw scallop *Nodipecten subnodosus*

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ABSTRACT

Antibiotics have been used massively for aquatic organism production. However, their use should be limited given the problems of increased antibiotic resistance, and new alternatives should be attempted, such as the use of plants extracts with antibacterial properties. The effect of the essential oil of oregano (*Origanum vulgare*) compared with three antibiotics (enrofloxacin, florfenicol, and oxytetracycline) on embryogenesis and early larval stages were evaluated to obtain mixotrophic larvae of the scallop *Nodipecten subnudosus*. Incubation densities of 20 and 100 fertilized oocytes mL^{-1} were used. The highest yield of normal larvae (calcified D-larvae) occurred at high densities in an order of 300% (36–45 D-larvae mL^{-1}), with the highest values being obtained when the fertilized oocytes were treated with the oregano essential oil and with the antibiotic florfenicol, 44.8 ± 5.16 and 40.9 ± 12.06 D-larvae mL^{-1}, respectively. Nevertheless, the use of florfenicol produced larvae with shorter anteroposterior length, which strengthens the advantage of using oregano essential oil over the antibiotics for spat production of *N. subnudosus*.

1. Introduction

The bivalve lion's paw *Nodipecten subnodosus* (G. B. Sowerby I 1835) is one of the largest species of scallops, which can reach about 200 mm in anteroposterior length and a weight of almost 2 kg (Holguin-Quiñoñes and García-Domínguez, 2011). It is distributed in tropical and subtropical waters of the Eastern Pacific, from the coasts of Peru, in Paita (4.7° S) to Isla de Cedros (28.2° N) in Baja California Sur, Mexico (Coan and Valentich-Scott, 2012) and it is considered as one of the most promising species for aquaculture diversification in tropical and subtropical Pacific Ocean. Therefore, a series of studies in production technology have been performed which have led to the development of spat production under controlled conditions and cultivation in the sea (Maeda-Martínez and Lodeiros, 2011). Recently, the adaptability and optimization of these techniques are being tested in the tropical waters of Ecuador by CENAIM-ESPOL, with promising results. Thus, growth rates at sea culture have shown to be some of the highest observed for the species. However, more studies are required to optimize the spat production to reach an adequate technological package and to find new therapeutic treatments to control bacterial invasions (Revilla et al., 2016).

While the specific information related to optimal incubation density for embryos of marine bivalves is scarce, it is easy to find studies reporting embryos densities on early larval culture (Aarab et al., 2013). With regard to *N. subnodosus*, Mazón-Suástegui et al. (2011), concluded that the successful results obtained in Mexico in spat production are a consequence of the implementation of techniques developed for the Caribbean *Nodipecten nodosus* cultivation (De La Roche et al., 2002; Acosta, 2004; Rupp et al., 2004). However, none of these cases evaluated the effect of embryonic density on the production of D-larvae, being 30 embryos mL^{-1} the highest density reported for this species (Velasco et al., 2007). Some studies show that densities between 50 and 100 embryos mL^{-1} originate healthy veliger larvae in *Pinctada margaritifera*, *Pteria penguin* and *Pecten maximum* (Southgate et al., 1998; Wassnig and Southgate, 2011).

In the spat production of bivalve under controlled conditions, obtaining mixotrophic larvae (D larvae veliger) during the initial phase is a process highly sensitive to bacterial infections leading to low survival (Southgate, 2008). For the development of embryos and larval production in hatcheries, the water is usually filtered and irradiated with UV light before being used (Rose and Baker, 1994; Miranda et al., 2013; Rojas et al., 2015). Thus, the main entry way of contaminating agents in

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eggs or embryos is through vertical transmission, isolating bacteria harmless for adults but pathogenic for larvae (Sainz-Hernández and Maeda-Martínez, 2005; Prado et al., 2014). Thus, making microbiological control is difficult to establish at this point (Wassnig and Southgate, 2011; Dubert et al., 2016b). Additionally, the incubation of embryos at high density is a common practice to produce many mixotrophic larvae for massive spat production, which increases the risk of bacterial infections due to the increment of organic matter coming from the reproductive tissues and gametes (Riquelme et al., 1994; Jorquera et al., 2001). This situation compromises the normal development and survival of embryos (Gruffydd and Beaumont, 1970).

The tolerance of bivalve embryos has been a topic researched for decades (Calabrese and Davis, 1970; Tettelbach and Rhodes, 1981; Kurihara, 2008; Parker et al., 2009). Variations in the incubation density of the eggs have been related to embryonic survival and the formation of normal larvae (Sprung and Bayne, 1984, Clotteau and Dubé, 1993, Southgate et al., 1998, Galley et al., 2010; et al., Aarab et al., 2013). Also, there is evidence to explain the wide variations in the production of normal larvae of bivalves; among the main factors it can be included the physiological condition of the breeding stock, the proportion of gametes, the genetic variations, and the quality of eggs and the water (Utting and Helm, 1985; Doroudi and Southgate, 2003; Galley et al., 2010; Mazón-Suástegui et al., 2011; Angel-Dapa et al., 2015; González-Araya and Robert, 2018).

Antibiotics have been used massively for spat production of marine bivalve in hatcheries (Uriarte et al., 2001; Miranda et al., 2013; Dubert et al., 2015) alerting the scientific community to this trend. In recent years, the number of reports have increased indicating that the use of antibiotics leads to persistence, virulence and bacterial resistance through the induction of genetic repair (SOS responses) and generates a greater number of mutations (Harms et al., 2016). Thus, resistance factors have been identified in some pathogenic bacteria, not only affecting bivalves and cultured organisms (Kümmerer, 2004; Kitiyodom et al., 2010; Cabello et al., 2013), but also human health (Manchanda et al., 2010; Smith et al., 2016; Navajas-Benito et al., 2017). Consequently, it is imperative to complement the management practices that provide the best possible biosecurity (disinfection of the areas, materials and work personnel, filtering with UV light the water of the culture facility, among others) with the health management that limits the use of antibiotics. In this context, the use of natural products with bioactive properties is presented as a promising viable alternative (Citarasu, 2010; Randrianarivelo et al., 2010; Romero et al., 2012; Teixeira et al., 2014) against recurrent mortality episodes in the larval and post-larval production of bivalve culture (Dubert et al., 2015).

Among natural products, essential oils have stimulated interest given their antimicrobial properties (Mejlholm and Dalgaard, 2002; Shehata et al., 2013). In aquaculture organisms, the essential oil of species of the genus *Origanum* has shown some promising applications, like the essential oil of *Origanum vulgare* which has been applied in fish cultures and produced significant improvements in the performance of Nile tilapia juvenile (Zheng et al., 2009). Likewise, a study by García-Valenzuela et al. (2012) on the effect of the essential oregano oil (*Lippia berlandieri*) in pathogenic bacteria of shrimp *Penaeus vannamei* shows a viable or complementary alternative to control *Vibrio* spp. pathogens in penaeid shrimp. More recently, Stefanakis et al. (2014) shows the applicability of the essential oil of the species *Origanum vulgare* subsp. *hirtum*, *Origanum onites* and *Origanum marjorana* in rotifer's disinfection used in fish feeding, showing a significant decrease in the concentration of *Vibrio* spp. and higher rates of survival of rotifers applying 10 ppm of oregano oil.

In this study, we evaluated the effect of oregano essential oil (*Origanum vulgare*) compared to three antibiotics (enrofloxacin, florfenicol, and oxytetracycline) on embryogenesis and early larval stages for spat massive production at high and low initial densities of *N. subnudosus*.

2. Materials and methods

2.1. Obtaining gametes and general conditions

The broodstock of *N. subnodosus* was obtained by SCUBA in the coastal area surrounding of Salango Island, Manabí province, Ecuador (1° 35′28″S and 80° 52′13″N) and transported in isothermal containers with continuous aeration to CENAIM-ESPOL experimental mollusk hatchery. The reproduction conditioning to reach gonadal maturity was performed in three 400 L tanks, each with 10 organisms, fed with 300,000 cells mL^{-1} per day of *Tisochrysis lutea* (formely *Isochrysis* affinis *galbana* or T-Iso: Bendif et al., 2013), *Chaetoceros gracilis* and *Pavlova lutheri* (3:3:1 ratio), for one month: 2 initial weeks at 22–23 °C and 2 remaining weeks at 25–26 °C. The spawning was induced using thermal shock according to recommendations of Mazón-Suástegui et al. (2011). Fertilization, embryonic and initial larval development were conducted using microfiltered sea water (0.45 μm) irradiated with UV light.

2.2. Experimental development

After the appearance of the first polar corpuscle in the fertilized oocytes (> 95% after 15 min), they were transferred to 2.5-L plastic cylindrical containers (2 L of water effective volume). For this research, a combined factorial design of 2 densities of fertilized oocytes (low: 20 embryos mL^{-1} and high: 100 embryos mL^{-1}) was used in 4 therapeutic treatments: 1) florfenicol at 2 ppm, 2) oxytetracycline at 4 ppm (used in the production of scallops; Getchell et al., 2016), 3) enrofloxacin at 1 ppm (widely used in shrimp and fish production; WHO Press, 2006), and 4) extract of oregano oil (*Origanum vulgare*) at 17% in a concentration of 1 ppm. In addition, a control treatment without any therapeutic was tested.

The doses used of florfenicol and oxytetracycline were selected given the recommendations of Miranda et al. (2014), in scallop larvae. The doses used of enrofloxacin in aquatic organisms were determined based on considerations from Robinson et al. (2005), Chang et al. (2012) and Flores-Miranda et al. (2012), opting for a 1 ppm conservative concentration. The dose used of oregano oil was selected after a 24-h study of its lethal effect at concentrations 0 (control); 0.01; 0.1; 1; 10 and 50 ppm, contained in 20-mL bottles with 15 mL of sterilized seawater at an initial density of 20 fertilized oocytes mL^{-1} (5 replicates of each concentration). The 50% lethal dose (LD_{50}) of oregano oil was calculated by probit method (Finney, 1971) and the dose selected was based on the observation of the highest survival.

All therapeutics tested were added 2 h before incubation and vigorously aerated to allow their homogeneous dissolution. The combined treatments were conducted in triplicate, resulting in 30 incubation units, maintained with slight aeration and a temperature of 27 ± 0.5 °C. The trial lasted 24 h, enough time to reach the early veliger mixotrophic larval stage with calcified valves (Villavicencio-Peralta, 1997; Mazón-Suástegui et al., 2011). After this, the content of each incubation unit was collected with a 30 μm mesh and concentrated in 10-mL vials, with 2 mL of 4% formaldehyde, neutralized with seawater and 8 mL microfiltered seawater (0.45 μm). After homogenizing the contents, 3 samples of 1 mL were taken to quantify with optical microscopy (200×) the number of calcified, uncalcified, malformed and trochophore larvae. These last three conditions were considered as signs of poor or late larval development. Also, the length (anterior-posterior axis) of calcified larvae was measured in 30 larvae of each replica. The records for the analysis of the larvae were made through images, taken under the microscope with a digital camera (Lanoptik MDX 501) attached, for later measurement with the iWorks 2.0 program. For each replicate, the performance in larvae produced was estimated, which consisted in quantifying, at the end of the experiment, the production of normal mixotrophic larvae.

2.3. Statistical treatment

The percentage of D-larvae survival exposed to different concentrations of oregano oil was evaluated with a one-way ANOVA and significant differences between treatment means were determined using Duncan's *a posteriori* test. A two-way ANOVA was performed to evaluate the effect of the density of fertilized eggs, type of therapeutics and the interaction of these in the production of mixotrophic larvae (survival), percentage of normal larvae, as well as in the sizes of the larvae at 24 h. When significant differences were observed in some of the interactions, an orthogonal contrast analysis was developed following the recommendations of Yossa and Verdegem (2015). The significant differences among means (density and therapeutic) were evaluated by Duncan's test. All variables used satisfied the assumptions of normality (except survival) and homogeneity of variances, according to the Kolmogorov-Smirnov and Levene's tests, respectively. The data corresponding to survival were transformed using arcsine, to reach normalization. All tests were carried out according to the recommendations in Zar (2010) at a probability of $P = 0.05$.

3. Results

3.1. LD_{50} of oregano oil

The preliminary assay to establish the lethal dose of oil oregano showed that 50 ppm causes the total mortality of the embryos at 24 h ($P < 0.05$). The highest survival rate was recorded at the concentration of 1 ppm (> 90%). However, no significant differences were determined with the rest of the treatments ($P > 0.05$), except for the 10-ppm dose (Fig. 1). The concentration obtained for the LD_{50} was 16.5 ppm [95% CI (15.6, 17.7)]. A 1 ppm dose of oregano oil was chosen since it allowed greater survival of D larvae.

3.2. Survival

Significant differences of density (F = 49.18, df = 1, $P < 0.001$), therapeutics (F = 3.22, df = 4, $P < 0.05$) and their interaction (F = 5.82, df = 4, $P < 0.005$) on survival of the mixotrophic larvae were observed. The survival achieved in the D-larvae state was higher when fertilized eggs were incubated at low density (20 embryos mL^{-1}), which was practically observed in all treatments. When evaluating the combined effect of density and different therapeutics on survival, the significant differences for interaction cases are clearly

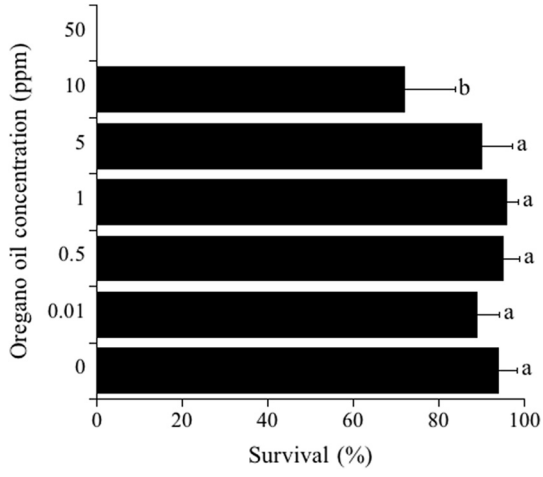

Fig. 1. Survival expressed in % D larvae produced in different concentrations of oregano oil, at an initial density of 20 fertilized oocytes mL^{-1}. Different letters among bars indicate significant differences (Duncan's test, $P < 0.05$). Each bar represents mean and standard deviation (n = 3).

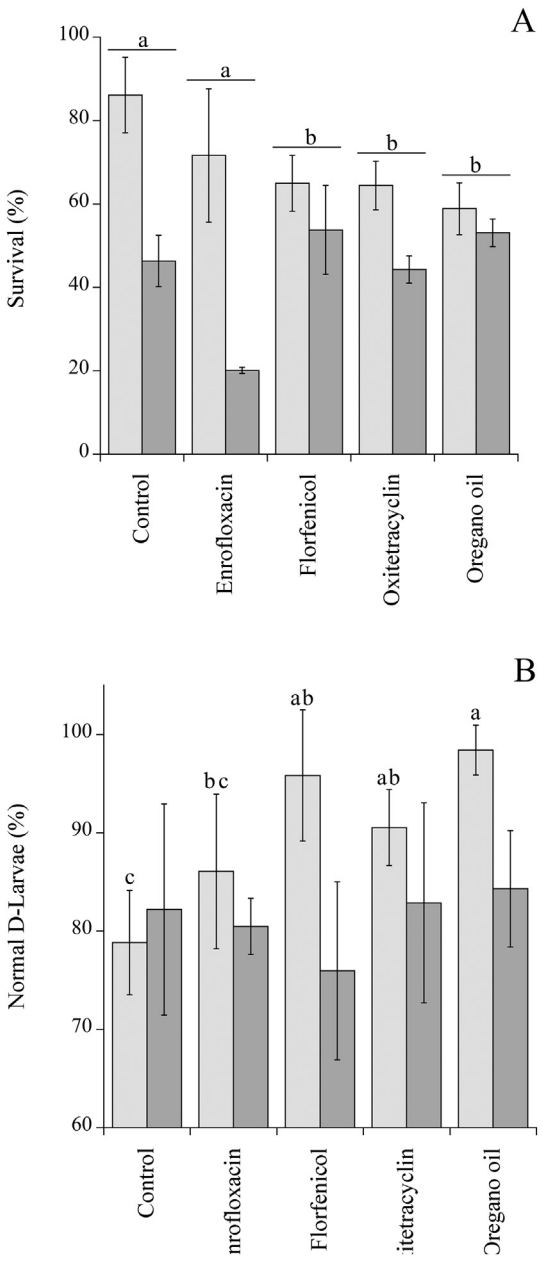

Fig. 2. Survival (A) and normal veliger D larvae (B) in the different treatments with therapeutics and control at initial densities of 20 (light gray bars) and 100 (dark gray bars) fertilized oocytes mL^{-1}. Different letters between bars indicate significant differences by orthogonal contrasts for survival and Duncan's test for normal larvae (both $P < 0.05$). Each bar represents mean and standard deviation (n = 3).

observed. No significant differences were observed ($P = 0.728$) in the interactions between density and therapeutics in the larvae survival when enrofloxacin and control were applied. However, each of the latter treatments showed significant difference with the other therapeutics used in the study (Fig. 2A). The interaction was present particularly by the significantly ($P < 0.05$) higher values of control treatments, enrofloxacin, and oxytetracycline at low density and the absence of significant differences ($P < 0.05$) in the rest of treatments between densities. At low density, the control treatment and enrofloxacin showed significantly higher survival values (86.1 ± 9.02% and 71.7 ± 16.00%) than the other treatments (58–65%). The treatment with enrofloxacin at high density, showed the lowest survival rate (20.1 ± 0.70%, Fig. 2A).

3.3. Calcified D-larvae

Significant differences in density were observed in the percent of normal calcified D-larvae (F = 9.85, df = 1, $P = 0.0052$). The therapeutic treatments and the interaction between this factor and larvae density was non-significant ($P > 0.05$). At low density, the percentage of trochophores, amorphous and uncalcified larvae led to a smaller number of normal calcified D-larvae in the enrofloxacin and control treatments (86.1 ± 7.86% and 78.2 ± 5.29%, respectively, Fig. 2B), despite control and enrofloxacin showing significantly higher survival values ($P < 0.05$). The rest of the treatments showed values > 90% of normal larvae. At high density, there were no significant differences among treatments ($P > 0.05$), and their values were between 82 and 85% of normal D-larvae.

3.4. Calcified larval length

Significant differences in antero-posterior length of the normal D-larvae were observed with density (F = 5.03, df = 1, $P = 0.036$) and therapeutic treatments (F = 4.85, df = 4, $P = 0.006$); interaction between these factors was non-significant ($P > 0.05$). The length of the calcified D-larvae was larger when the fertilized eggs were incubated at low density than at high density, which was an effect particularly observed when florfenicol was used (Fig. 3). Under this condition, the anteroposterior length reached 90.3 ± 2.16 µm of at low density, and 84.1 ± 1.67 µm at high density. In the other treatments, the difference in length of the D-larvae was not significantly different between densities, despite recording the highest length values in the larvae treated with the oregano essential oil (91.6 ± 1.91 µm). The length of D-larvae, when treated with enrofloxacin, was significantly shorter than the other treatments (85–86 µm, Fig. 3).

4. Discussion

Considering the larvae survival at 24 h, it could be inferred that there was a better production in treatments with lower density (20 embryos mL^{-1}) without therapeutics, given the higher survival values in the control. This could suggest the non-use of therapeutics in embryonic development at low density, especially knowing the sensitivity of the embryos. However, in the control treatment at both densities, the results presented around 20% of deformed larvae, without calcification or with delayed development, which suggests the existence of a possible bacterial interaction during embryonic development.

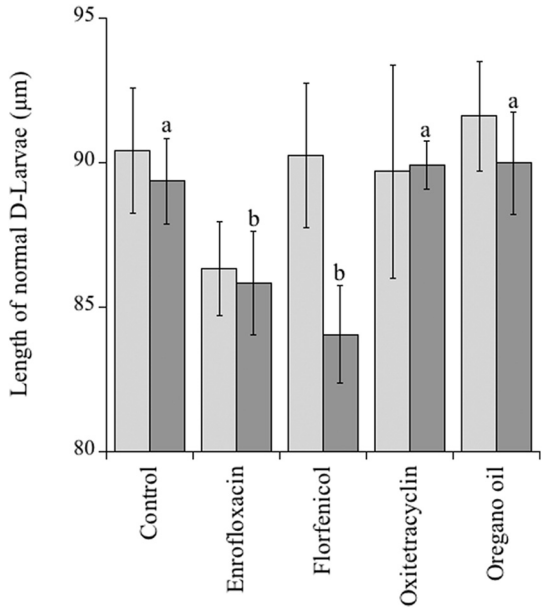

Fig. 3. Antero-posterior length of the normal D larvae in the different treatments with therapeutics and control at initial densities of 20 (light gray bars) and 100 (dark gray bars) fertilized oocytes mL^{-1}. Different letters among bars indicate significant differences (Duncan's test, $P < 0.05$). Each bar represents mean and standard deviation (n = 3).

At high densities, the normal D-larvae produced increases above 300%, demonstrating the higher harvest of mixotrophic larvae for the subsequent larval culture to spat production. Given this performance, our results show that it is indifferent to use oregano oil or florfenicol; however, the greater length of D-larvae achieved with the treatment with oregano oil, the antibiotic oxytetracycline and without any treatment, show a better condition than D-larvae produced with florfenicol and enrofloxacin treatments. This suggests that the treatment with antibiotics could be discarded. This worse condition of D-larvae could be associated with some toxicity to embryos and early larvae in *N. subnodosus*, as has been reported for clams and oyster larvae (Fitt et al., 1992; Doroudi and Southgate, 2002; Wassnig and Southgate, 2011), and shrimp larvae (Soto-Rodríguez et al., 2006).

Studies on embryo culture of the mussel *Perna perna*, considering the density in relation to area, found that 20, 50 and 100 embryos cm^{-2} (densities considered low by these authors) did not show differences with respect to the number of normal D-larvae obtained (Aarab et al., 2013). In the case of the pectinid *P. maximus*, the consequences of incubation density in larval production are evident, obtaining a lower number of abnormal larvae when they were planted at 7 eggs cm^{-2}, compared to 700 eggs cm^{-2} (Gruffydd and Beaumont, 1970) However, these authors maintain that the high percentages of normal larvae obtained at high densities represent an advantage for hatcheries. This implies the management of a greater number of abnormal larvae. Similar results were obtained in the present study when analyzing the number of normal larvae of *N. subnodosus* at high density, especially with the use of oregano oil, oxytetracycline and without therapeutics (control).

The exposure to high doses of natural compounds can have detrimental effects on survival (including oregano oil at concentrations > 5 ppm as shown in this study), for this reason they have been considered effective antifouling (Qian et al., 2010). This is the case of neem oil (*Azadirachta indica*), which has been shown to be lethal in larvae of *Limnoperna fortunei* at a concentration of 8 ppm (Pereyra et al., 2012). The results obtained for LD_{50} in this study could suggest a lower toxicity of oregano with respect to neem oil. Oregano essential oil is rich in C10 monoterpenes, containing considerable amounts of carvacrol and phenols such as thymol (Burt, 2004; Teixeira et al., 2014; Baruah et al., 2017), that could be the source of its antimicrobial properties (Lambert et al., 2001; Karakaya et al., 2011; Alvarez et al., 2014; Diler et al., 2017; Mabrok and Wahdan, 2018). These compounds may have acted in the control of bacteria associated with embryos and early larval stages of *N. subnodosus*. Studies on the usefulness of this compound, in terms of dose and its bactericidal capacity in both embryogenesis and larval development, are recommended for both *N. subnodosus* and for other species of bivalve molluscs and aquatic organisms under culture. Since the production obtained of normal mixotrophic D veliger larvae represent > 80% of the oocytes fertilized and incubated initially at a density of 100 embryos mL^{-1}, future studies will be carried out at higher densities to obtain larger yields in the production of mixotrophic larvae.

It is expected that embryonic cultures at higher densities will produce a greater number of dead larvae as a consequence of a greater presence of organic and bacterial matter introduced with the gametes. However, during some infectious processes in bivalve larvae, such as vibriosis, the larvae go through an asymptomatic stage during the first 24 h after the infection started (Dubert et al., 2016a). The clinical signs show the onset of the disease with the colonization of the digestive gland that is absent or partially developed during this phase of the assay. Quantifying and characterizing the bacterial load at the

beginning of the culture, as well as recognizing the host-pathogen interactions in real time (Westermann et al., 2012), would provide very useful information to understand the mortalities that occur during the ontogenetic phase. There is a possibility that the doses used have been insufficient to reduce the bacterial load in embryos at high density, especially in the treatments of 1 ppm of enrofloxacin (survival < 20%), and 2 ppm of florfenicol (normal D-larvae < 80%, small size). In this sense, Wassnig and Southgate (2011) during the incubation of *P. penguin* embryos at densities of 100 eggs mL^{-1}, observed similar results with the use of 5 ppm of two antibiotics (streptomycin-sulfate and tetracycline-erythromycin).

The results suggest the use of oregano essential oil as a therapeutic treatment in the embryonic development of *N. subnodosus* for massive production of normal mixotrophic larvae. This reinforces the hypothesis that natural products are presented as a viable alternative to the use of antibiotics in the production of spat of bivalve molluscs.

Conflicts of interest

All authors declare no conflict of interest.

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