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EFFECTS OF ANTIBIOTICS ON THE CONCENTRATION OF BACTERIA IN BIOFILMS AND ON THE GROWTH OF *HALIOTIS RUFESCENS* POSTLARVAE

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ABSTRACT The effects of chloramphenicol and streptomycin/penicillin on the concentration of heterotrophic bacteria in biofilms associated with abalone postlarval culture and on the growth and survival of *Haliotis rufescens* postlarvae (3–5 days old) were studied under laboratory conditions. Two experiments were carried out in 12-well polystyrene tissue culture plates with 5 mL of 1-μm-filtered autoclaved seawater. Water was changed every other day and antibiotics were added during the water change. Experimental units were previously inoculated with the benthic diatom *Navicula incerta*. In the first experiment chloramphenicol at 0, 5, 10, and 20 mg L^{-1} was used and a mixture of streptomycin and penicillin at 0/0, 50/50, 100/100, and 150/150 mg L^{-1} was tested in the second experiment (in both cases with three replicates). Bacterial counts (Zobell plates) were performed for a period of 4 and 5 wk (experiments 1 and 2, respectively). Bacterial densities decreased 90% in 20 mg L^{-1} of chloramphenicol during the first 48 h; however, bacterial counts increased in all treatments thereafter and differences were not significant at the end of the experiment. The growth of abalone postlarvae was not significantly different among treatments. In the experiment with antibiotic mixtures, bacterial abundance was reduced 99% at the highest concentration (150/150) in the first 48 h, and remained significantly lower than the control for 2–3 wk. During this period, postlarval growth in this antibiotic treatment was also slower, as well as final survival, suggesting an important role of bacteria in the nutrition and/or digestion of abalone postlarvae. Results of this study also suggest that bacterial resistance to these antibiotics develops fast, discouraging their long-term use in abalone culture.

KEY WORDS: *Haliotis rufescens*, abalone postlarvae, antibiotics, bacteria

INTRODUCTION

Intensive rearing conditions allow the increase of bacteria caused by high levels of organic matter in culture systems. In many aquaculture facilities, bacteria are controlled using different antibiotics. (Li et al. 1999, Rombaud et al. 1999, Torkildsen et al. 2000). The triumph of antibiotics over disease-causing bacteria is one of modern medicine's greatest success stories. However over time, some bacteria have developed adaptations to minimize the effects of antibiotics (Fernández et al. 2000, Holström et al. 2003). The routine use of antibiotics in aquaculture may cause development of antibiotic resistance among pathogens infecting cultured animals and humans. Antibiotics may also cause negative effects on important ecosystem bacteria (Benbrook 2002, Holström et al. 2003, Costanzo et al. 2005).

Abalone (*Haliotis* spp.) postlarvae are cultured in tanks with surfaces covered with biofilms dominated by benthic diatoms that are grazed by postlarvae. Pathogenic bacteria in these biofilms can reach high concentrations and cause abalone mortality (Anguiano-Beltrán et al. 1998, Lizárraga-Partida et al. 1998).

Literature on the effects of antibiotics on bacteria associated with biofilms used in the culture of abalone is lacking, although in abalone postlarval research some antibiotics are used to control or prevent bacterial problems, including a mixture of penicillin G sodium/streptomycin sulphate (50–100 mgL^{-1}) and chloramphenicol (10–20 mg L^{-1}), (Searcy-Bernal et al. 1992, Roberts & Nicholson 1997, Roberts et al. 2001, Searcy-Bernal et al. 2001).

The main objective of this work is to study the effects of chloramphenicol and a mixture of penicillin/streptomycin on the bacterial concentrations in biofilms of the diatom *Navicula incerta*, which is widely used in abalone culture. Bacteria are also considered an important food source for early abalone postlarvae (Kawamura et al. 1998) and if antibiotics decreased their concentration substantially, an effect on early postlarval growth would be expected.

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MATERIALS AND METHODS

Two assays were performed to evaluate the effects of different concentrations of the antibiotics: chloramphenicol (experiment 1) and a mixture of penicillin G sodium/dihydrostreptomycin (experiment 2), on bacterial counts in biofilms associated with *Haliotis rufescens* culture and on the growth of postlarvae.

The Microalgae Laboratory of the Instituto de Investigaciones Oceanológicas provided the diatom *Navicula incerta* used to form the biofilm to feed abalone postlarvae. The farm "Abulones Cultivados S. A. de C. V." (Ejido Eréndira, B.C., México) donated the veliger larvae of *Haliotis rufescens*. Larvae were reared in 1-μm filtered, UV treated seawater changed every day. Competent larvae (7–8 days old) were induced to metamorphose in rectangular plastic vessels (1 L) with gamma-aminobutyric acid (GABA) at 1.5-μM (Searcy-Bernal & Anguiano-Beltrán 1998).

Sterile 12-well tissue culture plates (Corning, 3.8 cm^2 bottom area, and 6 mL volume) were used as experimental units (EU). Diatoms were inoculated at 250-cell mm^{-2} one day before the beginning of the trials and 4–5 postlarvae (5-d old in experiment 1 and 3-d old in experiment 2) were transferred to each EU the next day. All antibiotics were tested at four concentrations with three replicates per treatment following a completely randomized design. Stock solutions of antibiotics were prepared with sterile distilled water and work solutions were prepared with sterile seawater both of them at the moment of use.

Before adding the antibiotics to the EU samples (1-mL) from the biofilm were collected with an automatic sterilized pipette from a previously standardized area, to estimate the initial number of bacteria. These samples were suspended in a test tube with 9 mL of saline solution (0.9% NaCl), and immersed in an ultrasound bath (Fisher Scientific) for 3 min to disaggregate diatoms and to detach bacteria from diatoms (Lizárraga et al. 1998, Gorrostieta-Hurtado & Searcy-Bernal 2004). Second and third dilutions of these sonicated test tubes were prepared and samples of these (100 µL) were plated in triplicate onto Zobell agar media and incubated at 24°C to 25°C for 5 days. Bacterial counts at 5 days after incubation were made to determine CFU mL^{-1}. Experiments were conducted at 17 ± 1°C under constant fluorescent illumination (ca. 50 µE s m^{-2}).

For experiment 1, concentrations of chloramphenicol were 0, 5, 10, and 20 mg L^{-1} and biofilm samples were taken at 1, 2, 7, 15, and 22 days to evaluate bacterial concentrations. In experiment 2, 0/0, 50/50, 100/100, and 150/150 mg L^{-1} of penicillin G sodium/dihydrostreptomycin were used and biofilm samples were taken at days 1, 2, 7, 14, 21, 28, and 35. Antibiotics were replaced every other day during the water changes.

To measure shell length, video-images of 2–4 postlarvae from each EU were recorded with a Sony SSC-C374 high-resolution camera on an inverted microscope (Meiji Techno) and analyzed digitally (NIH image 1.59 Power PC). Survival was evaluated counting live and dead postlarvae under an inverted microscope only in the experiment 2. The statistical significance of the differences between antibiotic concentrations was determined by one-way analyses of variance. Percent survival data were subjected to the arcsine square-root transformation before the analyses (Zar 1999).

RESULTS

The bacterial growth in the biofilm of *Navicula incerta* associated with abalone postlarvae under different chloramphenicol concentrations after 22 days of experimentation is presented in Figure 1. Only at the highest antibiotic concentration, bacterial concentrations decreased to 2.2 × 10^3 CFU mL^{-1} in the first 24 h of treatment, but increased in all treatments the next day reaching concentrations of 10^6 at the end of the experiment. Differences among treatments were significant at day 1 ($F = 4.12$, $P = 0.05$) and day 11 ($F = 5.01$, $P = 0.03$).

There was not a clear relationship between postlarval growth and chloramphenicol concentration (Fig. 2). At the end of the experiment the largest average shell length (± s. error.) was 557.3 ± 19.1 µm at 20 mg L^{-1} and the smallest (472.8 ± 18.8 µm) at 5 mg L^{-1} and this difference was significant ($F = 5.70$, $P = 0.02$).

Bacterial counts in the experiment with the mixture of penicillin G sodium/dihydrostreptomycin are presented in Figure 3. The highest antibiotic concentration (150/150 mg L^{-1}) depleted the bacterial population to 0.2 ± 0.1 × 10^3 CFU mL^{-1} after 48 h of treatment although differences among treatments were not significant ($F = 1.0$, $P = 0.5$). During the next three weeks there were important differences (of ca. one order of magnitude) between the control and the highest concentration, which were significant at day 7 ($F = 8.05$, $P = 0.007$) and day 14 ($F = 4.4$, $P = 0.04$). At the end of the experiment (day 35) biofilms were severely grazed by postlarvae and, probably as a consequence, bacterial concentrations decreased in all treatments (Fig. 3). The highest value (2 × 10^5 CFU mL^{-1}) was still in the control and the lowest in the 100/100 mg L^{-1} mixture (6 × 10^3 CFU mL^{-1}). This difference was significant ($F = 4.2$, $P = 0.05$).

During the first three weeks, postlarval growth in the highest concentration of the antibiotic mixture (150/150 mg L^{-1}) was slower than in the other treatments (Fig. 4), and this difference was significant at day 21 ($F = 5.4$, $P = 0.03$). At the end of the experiment, postlarval survival was also lower (31%) in the highest antibiotic concentration (Fig. 5), and a significant difference among treatments was detected ($F = 7.77$, $P = 0.009$).

DISCUSSION

Bacteria are some of the most important agents that cause mass mortalities of animals. The use of antibiotics to control diseases in aquaculture promotes the development of resistant strains of both harmful and harmless bacteria that are replacing

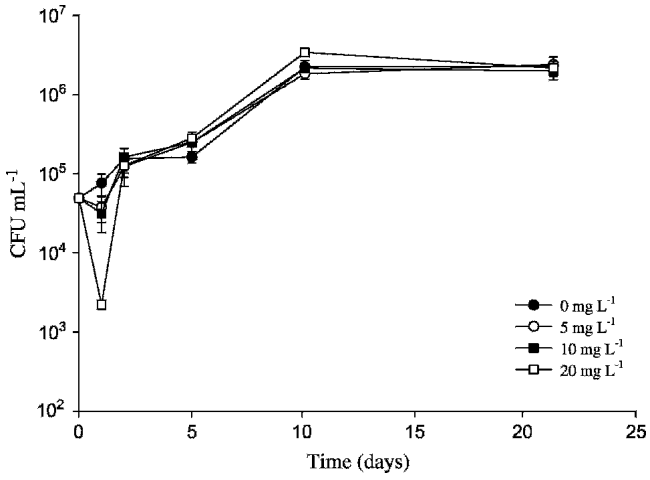

Figure 1. Bacterial growth in a culture of abalone postlarvae under different chloramphenicol concentrations. Vertical bars are s.e. ($n = 3$).

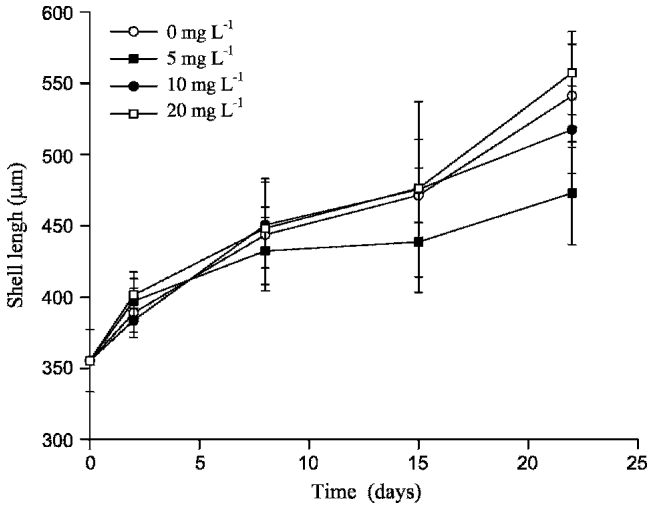

Figure 2. Abalone postlarvae shell length under different concentrations of chloramphenicol. Data are means of three replicates and bars are standard errors.

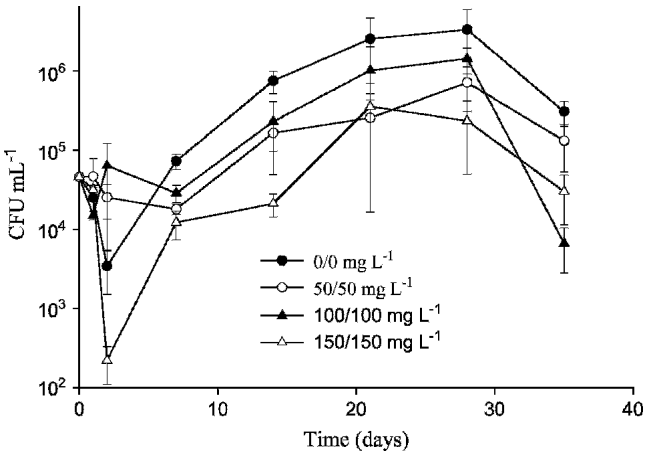

Figure 3. Bacterial growth in a culture of abalone postlarvae under different concentrations of penicillin G sodium/dihydrostreptomycin. Vertical bars are s.e. (n = 3).

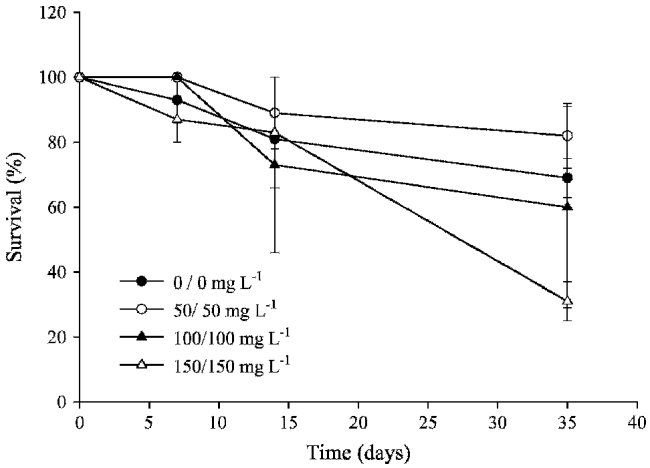

Figure 5. Abalone survival postlarvae under different concentrations of penicillin G sodium/dihydrostreptomycin.

antibiotic susceptible bacteria (Son et al. 1997, Li et al. 1999). Fitt et al. (1992) mention that some antibiotics can control bacterial growth if added daily to culture; but in this study chloramphenicol and penicillin/dihydrostreptomycin were effective only during the first 48 h, despite continuous replacement with fresh antibiotics. These findings suggest the development of antibiotic resistance by bacteria because of the frequent use of antibiotics, in agreement with other reports (Son et al. 1997, Bruun et al. 2000, Chelossi et al. 2003). Bacteria were probably sensitive to antibiotics during the first two days, because diatoms and postlarvae never received an antibiotic treatment before.

On other hand, because diatoms and bacteria are forming a biofilm, it is possible that its complex structure, based on exopolysaccharides, provides protection to bacterial populations against antibiotics as suggested by Nickel et al. (1985), Nichols et al. (1988), and Davey and Otoole (2000).

Abalones require gut bacteria to digest algae (Erasmus et al. 1997, Sawabe et al. 1998). The digestive system of the early postlarvae is not yet well developed, thus they need to acquire the bacterial flora from the environment to feed efficiently on microalgae (Tanaka et al. 2003). Erasmus et al. (1997) found that chloramphenicol and cefotaxime at 20 and 150-µg mL^{-1} respectively inhibited the growth of abalone enteric bacteria.

Therefore, since in our study it was found that high levels of chloramphenicol and penicillin G sodium/dihydrostreptomycin decreased bacterial numbers during the first days of experimentation, it is possible that in these treatments postlarvae did not develop a normal gut microflora, either because of a low availability in the biofilm or the potential inhibition of bacterial growth in the digestive system. In the case of chloramphenicol, this was probably not an important issue, because the concentrations of bacteria increased quickly to control levels (10^6 CFU mL^{-1}) and postlarval growth was similar to that in the control (Figs. 1, 2). However, in the highest penicillin/streptomycin (150/150 mg L^{-1}) treatment, bacteria remained lower than in the control during all the experimental period and postlarval growth was also slower during the first 3–4 wk (Figs. 3, 4). The lowest survival in this high antibiotic treatment (Fig. 5) might also be related to a digestive deficiency caused by the lack of appropriate microflora. On the other hand, these results might be also partially explained by the potential role of bacteria as a nutritional source for abalone postlarvae (Kawamura et al. 1998). The potential toxic effect of high doses of antibiotics on abalone postlarvae cannot be ruled out, although literature on this issue is lacking.

In abalone research, antibiotics have been used in an effort to control bacterial interference during the chemical induction of larval metamorphosis, as well as mortality during postlarval development. These include the mixture of penicillin/streptomycin at 150/150 mg L^{-1} each (Searcy-Bernal et al. 1992, Roberts et al. 2001, Takami et al. 2002) and chloramphenicol at 10 mg L^{-1} (Martínez-Ponce & Searcy-Bernal 1998, Searcy-Bernal et al. 2001). Results from this study suggest that these antibiotics may be helpful in short-term studies such as those dealing with metamorphosis induction of abalone, because this process takes only a few days (Roberts & Nicholson 1997, Searcy-Bernal & Anguiano-Beltrán 1998, Roberts 2001). However, this may not be the case in long-term postlarval studies, because bacterial concentrations recover in a few days, unless antibiotics affect pathogenic bacteria more efficiently and these are reduced in the final microbial composition. Gapasin &

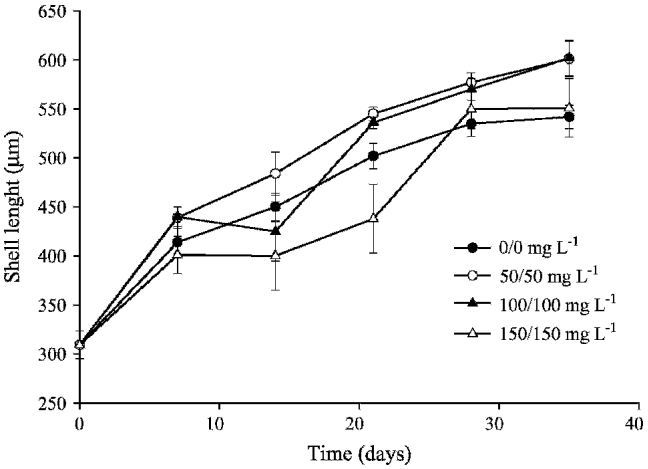

Figure 4. Abalone postlarvae shell length under different concentrations of penicillin G sodium/dihydrostreptomycin. Data are means of three replicates and bars are standard errors.

Polohan (2004) used streptomycin sulfate at 10 mg L^{-1} in their metamorphosis induction experiments, but it is doubtful whether this low concentration had any effect in the control of potential bacterial infection and larval mortality as intended.

This study suggests that the long-term prophylactic use of antibiotics in abalone culture systems may not be the best health practice and it is necessary to find some other alternatives, such as the use of probiotics (Douillet & Langdon 1993, Avendaño & Riquelme 1999, Makridis et al. 2000, Bachère 2003). Further research is required on the effect of antibiotics on the species composition in the microbial community of biofilms.

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