Effects of chloramphenicol, florfenicol, and thiamphenicol on growth of algae

Chlorella pyrenoidosa, Isochrysis galbana, and Tetraselmis chui

Hong-Thih Lai *, Jung-Hsin Hou, Chyong-Ing Su, Chun-Lang Chen

Department of Aquatic Biosciences, National Chiayi University, 300 University Rd., Chiayi, 60004 Taiwan, Republic of China

ARTICLE INFO

Article history:
Received 8 November 2007
Received in revised form 27 February 2008
Accepted 2 March 2008
Available online 24 April 2008

Keywords:
Antibiotics
Growth inhibition
EC50
Toxicity
Algae

ABSTRACT

This study investigated the growth inhibition effects of three phenicol antibiotics on microalgae used in aquaculture. Different dose levels of chloramphenicol (CAP), florfenicol (FF), and thiamphenicol (TAP) were added to cultures of one freshwater green alga, Chlorella pyrenoidosa, and two marine algae, Isochrysis galbana and Tetraselmis chui. For the two marine algae, FF showed higher toxicity levels (EC50, 1.3–8 mg l−1) than CAP (4–41 mg l−1) and TAP (38–158 mg l−1). CAP was more toxic to the freshwater algae (EC50, 14 mg l−1) than FF (215 mg l−1) and TAP (1283 mg l−1). TAP was the least toxic to the three algae, but maintained the highest stability during the test period. Among the tested algae, T. chui was the species most sensitive to the three antibiotics. This study demonstrates that all three phenicol antibiotics can inhibit growth of the three microalgae and should be carefully used in aquaculture.

© 2008 Published by Elsevier Inc.

1. Introduction

Of the drugs approved for agriculture and aquaculture, antibiotics are among the most widely administered for animal health and management (Sarmah et al., 2006). Antibiotics have appeared on the market and are readily available for the following purposes: (1) to treat diseases in humans and in animals, (2) as growth promoters, and (3) to improve feeds’ nutritional efficiency (Sarmah et al., 2006). Today, antibiotics play a major role in the modern agriculture and aquaculture industries, and their use has been on the rise in many developed nations (Boxall et al., 2004; Cabello, 2006; Sarmah et al., 2006). These antibiotics are mainly administered through medicated feeds. This practice may result in the antibiotics entering the environment by leaching from uneaten feeds, unabsorbed parts in manure, or aquatic animals’ excrement (Robinson et al., 2007). For example, in an intensive fish farm, about 70–80% of applied antibiotics, administered to fish as food additives, end up in the aquaculture environment. This may result in adverse ecological effects, including the development of resistant bacterial populations, direct toxicity to microflora and microfauna, and/or possible risks in the transfer of antibiotics resistances to human pathogenic microbes (Cabello, 2006; Hektoen et al., 1995; Rigos et al., 2004). Therefore, the overuse and misuse of certain antibiotics have led to several adverse environmental effects and raised public concern (GESAMP, 1997; Heberer, 2002; Sarmah et al., 2006).

Chloramphenicol (CAP), florfenicol (FF), and thiamphenicol (TAP) are broad-spectrum antibiotics commonly used, in veterinary or aquacultural practice, as chemotherapy agents to control diseases (Campa-Córdova et al., 2006; Nagata and Oka, 1996; Uriarte et al., 2001). CAP is highly effective when used in veterinary practice because it inhibits a variety of aerobic and anaerobic microorganisms (Huang et al., 2006); however, the toxicity of CAP to human bone marrow has been linked to blood disorders such as aplastic anemia (Anadon et al., 1994; Cravedi et al., 1987; Fraunfelder et al., 1982). Consequently, consumption of CAP by farm animals, intended for human consumption, is restricted by most countries (Munns et al., 1994; Sarmah et al., 2006). Nevertheless, CAP’s broad spectrum of activity, its ready availability, and its low cost continue to attract some farmers and aquaculturists despite its illegality (Huang et al., 2006; Takino et al., 2003). Both TAP and FF are part of the CAP family of drugs and are found to be effective antibacterials (van de Riet et al., 2003). In some countries, TAP has restricted use in aquaculture because its possible side effects on consumers (van de Riet et al., 2003). Even when they are restricted, the illegal use of these drug to treat fish and seafood products remains a possibility (Pfenning et al., 2000). FF, in spite of being a structural analog of TAP, has a superior spectrum of activity and greater potency, but without the toxic effect on human beings (Pfenning et al., 2000; Samuelsen and Bergh, 2004; Xu et al., 2005). Therefore, FF is widely employed in aquaculture for treating diseases in cultured animals (Ferreira et al., 2007; Samuelsen and Bergh, 2004; van de Riet...
Microalgae are the primary producers, and undoubtedly the food source, of a large quantity of aquatic biomass. Additionally, they also have fundamental importance in the production and balance of dissolved oxygen in an aquatic environment. Microalgae also play a key role in aquaculture development (Riquelme and Avendaño-Herrera, 2003). They are widely used in aquaculture as food in the early larval stages of mussels, fish, and crustaceans. Besides being grown as food for commercially valuable organisms, microalgae also optimize the water quality of an aquaculture's ecosystem (Riquelme and Avendaño-Herrera, 2003; Utting, 1985).

On the other hand, some microalgae are highly sensitive to the contamination of aquatic environments (Eguchi et al., 2004). They are considered indicators of bioactivity, such as industrial waste, and vary in response to a variety of toxicants (Ma et al., 2006). Some algae are more sensitive to antibiotics than higher trophic organisms in the same aquatic environments. For example, both *Chlorella* sp. and *Selenastrum capricornutum* have much higher sensitivities when compared with *Acartia tonsa* (crustacean) and *Brachyhydrio rario* (zebrafish) (Lanzky and Halting-Sorensen, 1997). *T. chui* (algae) is more sensitive than *Artemia parthenogenetica* (crustacean) to FF and oxytetracycline (Ferreira et al., 2007).

Only very limited information has been collected about the toxic effects of phenicol antibiotics on microalgae used in aquaculture (Campa-Córdova et al., 2006; Eguchi et al., 2004). This study collects growth inhibition data for CAP, FF, and TAP on three microalgae, *Chlorella pyrenoidosa*, *Isochrysis galbana*, and *Tetraselmis chui*. The objective of this paper is to evaluate the inhibition effects of the three phenicol drugs on the trophic levels of producers. The results may be referred to for further properly managed manipulation research consisting of phenicol drugs applied to aquaculture.

2. Materials and methods

2.1. Algal cultures

The freshwater alga, *C. pyrenoidosa*, was kindly provided by Dr. Todd Hsu (Institute of Marine Biology, National Taiwan Ocean University, Taiwan). *C. pyrenoidosa* was cultivated in a liquid medium, which was prepared as described in Hsu et al. (2000). Cultures of marine algae, *I. galbana* and *T. chui*, were donated by Dr. Lee An-Chin (Department of Aquatic Biosciences, National Chiayi University, Taiwan). The two marine algae were cultivated in Walne medium (Walne, 1974) with 20 psi brackish water, which is commonly used in the culture of mussels, fish, and crustaceans in brackish areas of southeastern Asia (Chien, 1992). The brackish water was prepared by adding synthetic sea salt (TAAM Inc., CA, USA) in RO-deionized water and was adjusted to the nominal salinity. Then both freshwater and brackish water, used for the preparation of cultivation media, were further UV-irradiated and 0.2 μm-filtered before use.

Cultures of algae were grown at 25 °C under constant illumination (6 klx) with gentle shaking. Cells of the three microalgae were collected in 5-mL glass tubes and observed under a phase-contrast microscope (Nikon E600, Japan). The number of algae was counted by image analysis software (Image-Pro Plus, Media Cybernetics, Inc., MD, USA) and further confirmed with a hemacytometer. Growth curves, of number over time, were set up to determine the increase rates for each of the three algae during cultivation for 7 days.

2.2. Phenicol antibiotics

The CAP, FF, and TAP (purity > 98%) used were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of each antibiotic were prepared by dissolving the selected antibiotic with RO-deionized water or brackish water as in microalgae cultures. The concentrations of each antibiotic in the inhibition test were then adjusted from each stock solution.

2.3. Procedure for the growth inhibitory test

Growth inhibitory tests were performed following the guidelines of the Organization for Economic Cooperation and Development (OECD) for testing chemicals no. 201 with modifications (OECD, 1984): In brief, conical flasks (250 mL) containing 100 mL of freshwater or brackish water, as described in Algal Culture, were autoclaved at 121 °C for 20 min. All glassware used in the tests was rinsed with 1 M HCl and then RO-deionized water for at least 1 h before use. Condensed medium solutions sterilized by filtration (pore size 0.2 μm) were added and precultivated algae cells were inoculated. The algal inocula were taken from exponentially growing precultures and added to 100 mL of test solutions to obtain initial cell densities on the order of 10^5 cells per milliliter. The flasks were covered with perforated polyethylene film to avoid contamination and evaporation, but also to allow gas exchange. The polyethylene film was changed every day after a subsample was taken for measurement and no contamination in test solutions was observed during the test period. Algal preculture, as well as test solutions, was grown and counted under the same conditions as described in the section Algal Culture. The flasks were shaken and changed in position relative to each other four times per day to equalize irradiating light intensity (6 klx). Cell growth lasted for 4 days and was measured at 1-day intervals. The pH increase during the tests did not exceed 1.5 pH units (MP 2000 pH Meter, Mettler, Toledo, Switzerland).

Two-step experiments, range-finding and determination, were conducted and modified from Isidori et al. (2005). The range-finding test, which consisted of six concentrations of test substances, 2000, 1000, 500, 100, 50, and 10 mg L^-1^, was carried out to estimate the approximate EC50. The main test was carried out with a negative control and 5–6 concentrations of test substances, in geometric series (common ratio = 2), determined for the estimated EC50 value located in the center of the concentration's distribution. However, as necessary, some test concentrations were modified for more precise EC50 ranges. The concentration setups of the determination experiment were shown in Figs. 1–3 for each of the phenicol antibiotics. Each treatment combination and negative control had three replicates.

2.4. Antibiotics analysis procedures

A high-performance liquid chromatography (HPLC) apparatus was applied to determine concentrations of the three antibiotics in each test solution of algae. This consisted of a Hitachi L-7100 intelligent pump, an L-7200 autosampler, and a Hewlett Packard Model 1050 absorbance detector and D-2500 chromato-integrator. CAP, FF, and TAP determination was separated and determined with a LiChrosorb 10 ODS column (3.2 mm i.d. × 250 mm L, Phenomenex Co., Torrance, CA, USA). The mobile phase consisted of methanol and water, whose ratios were 3:2 for CAP, 1:1 for FF, and 3:7 for TAP. The flow rate was 1.0 mL min^-1^ with ambient temperature. All detections were performed by UV absorption, in which the wavelength was 275 nm for both FF and TAP. Quantitation was performed using external standards and was based on peak areas.

2.5. Statistical methods

The data on growth inhibition were analyzed with SAS software (SAS Institute, 2001). The EC50 values were calculated with linear regression analysis of antibiotics' concentrations versus percentage inhibition (Ma et al., 2006). Significant differences between treatments and controls were measured with the Tukey test. Values were considered significantly different at P < 0.05.

3. Results

3.1. Toxicity of three antibiotics to microalgal growth

All three of the phenicol antibiotics showed inhibition effects on the three algae. CAP inhibited the growth of *C. pyrenoidosa* at concentrations of 20 and 40 mg L^-1^ (Fig. 1a). However, CAP did not affect the growth of *C. pyrenoidosa* at concentrations of 2.5, 5, and 10 mg L^-1^, CAP inhibited the growth of *I. galbana* at concentrations of 20 mg L^-1^, including 50 and 80 mg L^-1^, and did not affect the growth of *I. galbana* below concentration of 5 mg L^-1^, including 2.5 mg L^-1^ (Fig. 1b). CAP significantly inhibited the growth of *T. chui* at all treatment concentrations, 2.5, 5, 10, 20, and 50 mg L^-1^ (Fig. 1c).

FF inhibited the growth of *C. pyrenoidosa* at all treatment concentrations, including 100, 200, 400, 600, 800, and 1000 mg L^-1^ (Fig. 2a). Exposure of *I. galbana* to FF at 2.5 and 5 mg L^-1^ did not
significantly affect growth. However, FF significantly affected the growth of *I. galbana* at doses of 10 mg l\(^{-1}\) and higher concentrations, including 20, 50, and 80 mg l\(^{-1}\) (Fig. 2 b). FF inhibited the growth of *T. chui* at all treatment concentrations, including 2.5, 5, 10, 20, and 50 mg l\(^{-1}\) (Fig. 2 c).

TAP inhibited the growth of *C. pyrenoidosa* at doses of 1400 mg l\(^{-1}\) and higher concentrations, including 1600, 1800, and 2000 mg l\(^{-1}\) (Fig. 3 a). Exposure of *C. pyrenoidosa* to TAP at 1200 mg l\(^{-1}\) did not affect growth. TAP inhibited the growth of *I. galbana* at doses of 400 mg l\(^{-1}\) and higher concentrations, including 600, 800, and 1000 mg l\(^{-1}\) (Fig. 3 b). Exposure of *I. galbana* to TAP at 50, 100, and 200 mg l\(^{-1}\) did not significantly affect growth. TAP inhibited the growth of *T. chui* at all treatment concentrations, including 10, 20, 50, 80, and 100 mg l\(^{-1}\) (Fig. 3 c).

### 3.2. Sensitivity of the three algae to the antibiotics

Levels of sensitivity of the three microalgae to the antibiotic treatments were evaluated. In general, both *I. galbana* and *T. chui* are more sensitive than *C. pyrenoidosa* (Table 1). In the inhibition of the growth of *C. pyrenoidosa*, EC\(_{50}\)'s range from 14 to 1283 mg l\(^{-1}\). The sensitivities to the antibiotics rank in increasing order as follows: TAP < FF < CAP. In the inhibition of the growth of *I. galbana*, EC\(_{50}\)'s ranges from 8 to 158 mg l\(^{-1}\). The sensitivities to the antibiotics rank in increasing order as follows: TAP < CAP < FF. Similarly to the results of *C. pyrenoidosa*, the sensitivities of *I. galbana* to both CAP and FF are significantly higher than that to TAP. In the inhibition of the growth of *T. chui*, EC\(_{50}\)'s ranges from 1.3 to 38 mg l\(^{-1}\). The sensitivities to the antibiotics rank in increasing order as follows: TAP < CAP < FF.

### 3.3. Stabilities of the three antibiotics in tests

The daily residues left by antibiotics during the test period (96 h) were examined by HPLC. The results can be seen in Fig. 4. At the end of the test, the residual ratios for *C. pyrenoidosa* were 76.5% for CAP, 95.4% for FF, and 90.2% for TAP. In test of *I. galbana*,...
4. Discussion

The antibiotics commonly used to avoid the adverse effect of pathogens in aquaculture are TAP, CA, streptomycin, FF, kanamycin, oxytetracycline, neomycin, and oxolinic acid (Benbrook, 2002). Despite the potential risks of selecting for antibiotic-resistant strains and possible toxicity to aquatic organisms, antibiotics are still used in some countries without appropriate regulations (Report of a Joint FAO/OIE/WHO Expert Consultation on Antimicrobial Use in Aquaculture and Antimicrobial Resistance, 2006). Studies using test systems indicated that various antibiotics in waste water remain active against different groups of bacteria, microflora and microfauna (Christensen et al., 2006; Kummerer, 2001, 2003; Quinn et al., 2008). In addition, growth inhibition effects of several antibiotics against algae and daphnids have been reported at surprisingly low concentrations of 5–100 μg l−1 (Holten Lützhøft et al., 1999; Macri et al., 1988; Wollenberger et al., 2000). Halling-Sørensen (2000) also recounted that several antibiotics used in intensive fish farming have various growth inhibition (EC50 0.0037–3108 mg l−1) effects on algae depending on the antibiotics and algae used.

Results show that growth of the three algae is inhibited by the three phenicol antibiotics. T. chui is the most sensitive to all three antibiotics. The EC50 values indicate that T. chui is about 4–10 times more sensitive than I. galbana and 3–110 times more than C. pyrenoidosa (Table 1). Both of the two marine algae, T. chui and I. galbana, are more sensitive to TAP and FF than the freshwater alga, C. pyrenoidosa. However, to CAP, C. pyrenoidosa is more sensitive than I. galbana. The two marine algae are widely used in early larval culture of mollusks, fish, and crustaceans as food sources (Campana-Cordova et al., 2006). If these antibiotics were also to be used in larval culture systems (Gaunt et al., 2006; Uriarte et al., 2001), the feeding and growth of larvae could be adversely affected owing to the inhibition of algal populations by these antibiotics. Furthermore, if released with effluent or overflow, these antibiotics could potentially upset an aquatic environment.

The three phenicol antibiotics are recorded with different toxicities toward the algae in this study. FF is more toxic to the two marine algae than the other two antibiotics. FF has been used in the aquaculture of Japan, since the early 1990s (Xu et al., 2005) and is still popular in aquaculture and veterinary practice at present (Ferreira et al., 2007). In this study, the 96-h EC50 of FF to the marine alga T. chui is 1.3 mg l−1. Ferreira et al. (2007) also found that the 96-h EC50 of FF to T. chui is about 6 mg l−1. Both of these results indicate that an algal population can be affected by FF at low concentrations. It is suggested that use of this antibiotic in animal culture should be more carefully monitored to reduce the potential risk of contamination of algae in receiving waters. On the other hand, some reports indicate that, though exerting potential inhibitory effects on sensitive algae, Skeletonema costatum and Pasteurella multocida, FF has no significant impact on the environment as was expected; this is owing to dilution effects (NADA, 2007). However, besides the possible selection of resistant microbes, recent research suggests that water-borne pharmaceutical mixtures as low as ng l−1 levels may still have potential risks to aquatic life (Cabello, 2006; Pomati et al., 2006).

CAP is also toxic to the three algae in this experiment. The results show that CAP is the most toxic to the algae, T. chui, and less so to C. pyrenoidosa, and I. galbana being the last (Table 1). Though treatments with CAP in animal cultures are strictly prohibited, illegal uses are still being reported (Huang et al., 2006; Takino et al., 2003). The residues of CAP from animal culture and/or human medication may result in the contamination of receiving waters. For example, residues of CAP were detected in one sewage treatment plant effluent and one small river in southern Germany.

### Table 1

Average and standard deviation (in parentheses) of half-inhibition concentrations (EC50, in mg l−1) of three antibiotics, thiamphenicol (TAP), florfenicol (FF), and chloramphenicol (CAP) on three algae, Chlorella pyrenoidosa (CHL), Isochrysis galbana (ISO) and Tetraselmis chui (TET).

<table>
<thead>
<tr>
<th>Algae</th>
<th>Antibiotics</th>
<th>CAP</th>
<th>FF</th>
<th>TAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISO</td>
<td>CAP</td>
<td>41 (35–48)</td>
<td>8 (5–11)</td>
<td>158 (120–196)</td>
</tr>
<tr>
<td>TET</td>
<td>CAP</td>
<td>4 (2.7–4.3)</td>
<td>1.3 (0.2–2.4)</td>
<td>38 (33–43)</td>
</tr>
</tbody>
</table>

Note: Numbers in brackets are 95% confidence limits.

residual ratios were 93.2% for CAP, 81.9% for FF, and 97.7% for TAP.

In test of T. chui, residual ratios were 78.4% for CAP, 78.2% for FF, and 97.9% for TAP.
at concentrations of 0.56 and 0.06 μg l⁻¹, respectively (Hirsch et al., 1999). Though lower than the concentrations of acute toxicities in this study, the effect of chronic toxicity needs to be elucidated in the future. Furthermore, this study found that I. galbana is less sensitive to CAP than the other two algae. CAP inhibits the growth of I. galbana at concentrations above 20 mg l⁻¹; the 96-h EC₅₀ was 41 mg l⁻¹. Campa-Córdova et al. (2006) also reported no significant effect on growth of I. galbana with CAP doses at 12 mg l⁻¹ and below. It is suggested that discrepancies in the inhibition results of these two studies may be due partly to the various salinities used.

TAP shows the least amount of toxic effects to the three algae in this study. The toxicities (EC₅₀’s) of TAP to C. pyrenoidosa and I. galbana are all above 100 mg l⁻¹, which is not possible to exist in the natural environment. However, a significant inhibition of T. chui by TAP at 10 mg l⁻¹ is observed in this study. Therefore, the use of TAP in marine aquaculture also needs to be monitored for possible adverse effects on algal populations. Otherwise, TAP was found to be very stable during the test periods. The residues of TAP were all above 90% and even more than 97%. Therefore, the potential risk of chronic toxicity of TAP on algae requires further investigation.

According to environmental hazard assessments and classifications by the European Community (Directive 67/548/EEC), the acute aquatic toxicity of FF and CAP for algae can be classified in criterion R51 (Toxic to aquatic organisms); acute toxicity (EC₅₀) was above 1 mg l⁻¹ and below 10 mg l⁻¹. TAP can be classified in R52 and 53 (Harmful to aquatic organisms and may cause long-term adverse effects in the aquatic environment); acute toxicity on algae is between 10 and 100 mg l⁻¹ (Carlsson et al., 2006).

Antibiotics are considered only to be potential micropollutants since they are usually present at low concentrations (ppb range or less) in the environment (Ferreira et al., 2007; Le Bris and Pouliquin, 2004). The concentrations required to induce growth inhibition effects on the algae used in this study may occur only in exceptional conditions in the environment. For example, the antibiotics may arrive in aquatic environments of higher concentrations due to extraneous circumstances (e.g., runoff of contaminated soils, overflow of medicated aquaculture ponds, and domestic, industrial, or hospital effluents) (Ferreira et al., 2007; Sarmah et al., 2006). Consequently, the application of these antibiotics in aquaculture or veterinary use is a potential source of pollution in aquatic environments. Further investigations are suggested for the environmental fate and distribution of these antibiotics and their chronic and synergistic effects on wild organisms.

5. Conclusion

This study demonstrates the adverse effect of phenicol antibiotics, CAP, FF and TAM, on algae C. pyrenoidosa, I. galbana and T. chui. All three antibiotics can inhibit growth of the three algae at different dose levels. FF shows higher toxicities to the two marine algae than CAP and TAP. Though least toxic to the three algae, TAP has the highest stability during the test period, and chronic toxicity effects are suggested to be further elucidated. The marine algae, T. chui, is more sensitive to all three antibiotics amended than the other two algae. I. galbana and C. pyrenoidosa are more sensitive to FF and CAP than TAP, respectively. It is suggested that the possible exposure and effect of these antibiotics on aquatic environments should be carefully monitored.

Acknowledgments

This study was supported by the National Science Council, Taiwan, under Project NSC96-2313-B-415-003. Special thanks are due to Nick Howard for his editorial assistance.

References


NADA, 2007. Finding of no significant impact for Aquaflor Type A medicated article for control of mortality in freshwater-reared salmonids due to coldwater disease associated with Flavobacterium psychrophilum. Schering-Plough Animal Health, Summit, NJ, USA.


