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Bioencapsulation of Florfenicol in Brine Shrimp, Artemia Franciscana, Nauplii

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Abstract

The brine shrimp *Artemia franciscana* is one of the most common live-feed organisms for use in the larval culture of marine fish production. Bioencapsulation of florfenicol, an antibacterial agent, in *Artemia nauplii* was investigated as a potential carrier for this drug to marine larvae. Florfenicol was delivered directly to the organisms as particles, and the doses ranged from 100 to 2000 mg/l. Analysis of florfenicol concentrations in *Artemia* sp. nauplii were performed using high performance liquid chromatography (HPLC). The uptake of florfenicol in *Artemia nauplii* increased with particle size, dose, and exposure time, obtaining the highest concentration of 5.02 ng/nauplius, using a dose of 300 mg/l AQUAFLOR premix and 10 min exposure time. However, to obtain reproducible results, an enrichment time of at least 60 min is recommended.

Keywords: Antibiotics; Bacterial disease; Brine shrimp; Enrichment; Live feed; Treatment

Abbreviations: PF*: Precipitated Florfenicol; HPLC: High Performance Liquid Chromatography; OMP: Ormethoprim; OTC: Oxytetracycline; SDM: Sulphadimethoxine; SMX: Sulphamethoxazole; TMP: Trimethoprim; WSB: Wax Spray Beads

Introduction

Despite improved production of marine fish larvae the recent years, through better husbandry and increased knowledge of larval nutrient requirements, stable productions are, nevertheless, limited by the outbreak of bacterial infections during the early life stages. Due to the immature status of lymphoid tissues when hatched, the larvae possess no specific immune system, and vaccination is therefore not an alternative at this stage (Vadstein et al., 2004). Hence, in order to treat an infection, therapy using antibacterial agents is necessary.

At the earliest life stages, marine fish larvae are dependent on live feed organisms, and the two most important for use in aquaculture are rotifers (*Brachionus plicatilis*) and brine shrimps (*Artemia franciscana*) nauplii. Both are non-selective, continuous filter-feeding organism, grazing on particles from the surrounding water. For rotifers the optimal size of the particles was found to be in the range of 1.4 to 21 µm, with highest selectivity for particles of 4.5 µm (Vadstein et al., 1993; Hansen et al., 1997), whereas the preferred range for *Artemia* nauplii is approximately 7 to 28 µm (Fernandez, 2001; Dhont and Van Stappen, 2003). Prior to be offered to the larvae, the rotifers and *Artemia* nauplii are given nutrients, vitamins, and essential oils, during an enrichment process. The most common technique for enrichment is by adding lipid emulsions, containing the appropriate diet, to the water.

Live feed organisms are also used as carriers for the delivery of antibacterial agents to fish and shrimp larvae. Of the most studied antibacterials for the enrichment of *Artemia* nauplii are oxytetracycline (OTC) (Touraki et al., 1995; Gomez-Gil et al., 2001; Langdon et al., 2008), erythromycin (Majack et al., 2000; Cook and Rust, 2002; Cook et al., 2003) and the combinations of a sulphonamide and either trimethoprim (TMP) or ormethoprim (OMP) (Mohney et al., 1990; Nelis et al., 1991; Verpraet et al., 1992; Chair et al., 1996; Gapasin et al., 1996; Touraki et al., 1996; Touraki et al., 1999) whereas Dixon et al. (1995) studied the enrichment of the quinolones sarafloxacin and enrofloxacin. In the majority of these studies, the drugs were administered to *Artemia* nauplii via liposomes, or lipid emulsions, added to the water. An exception was the study by Mohney et al. (1990) where the antibacterials OMP and sulphadimethoxine (SDM) were added directly to the enrichment solution. The production of liposomes usually involves the use of expensive purified phospholipids and multiple steps in the preparation process, making these particle types both expensive and difficult to make on a large scale (Langdon et al., 2008). The enrichment time applied in most of these studies were 24h or more. In commercial hatcheries however, it is important to start medication as early as possible when an infection is revealed due to increased loss of appetite amongst the larva and subsequent mortality. Access to a rapid enrichment process is therefore of vital importance.

While little pharmacokinetic data is available for erythromycin and the potentiated sulphonamides in marine fish, oral administration of OTC is accomplished by a very low bioavailability (Elema et al., 1996; Rigos et al., 2003; Rigos et al., 2004) and must therefore be administered in rather high doses. Furthermore, erythromycin is an important antibiotic in human medicine and extensive use of this drug in aquaculture will not be approved in Norway.

It is well known that prolonged use of a single antibacterial agent, or one combination, will eventually expedite the development of resistant bacteria. Therefore, in order to reduce resistance development, alteration between antibacterial agents with different sites of microbial action is an important reaction. Thus, it is necessary to have a selection of different antibacterials available for treatment.

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Florfenicol is a fluorinated derivative of chloramphenicol with similar chemical structure and antibacterial activity (Fukui et al., 1987). Florfenicol is bacteriostatic and inhibits protein synthesis by binding to the 50S ribosomal subunits of susceptible bacteria. Commercially, florfenicol is available as AQUAFLOR premix and NUFLOR VET., both from Schering-Plough (Union, NJ, USA). AQUAFLOR premix consists of 50% inactive compounds (mainly lactose monohydrate) and 50% florfenicol particles. In Norway, AQUAFLOR premix is approved for the treatment of furunculosis and cold-water vibriosis in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) and is at present the second most used antibacterial agent (Grave et al., 2008; Grave and Litleskare, 2008). Orally administered florfenicol, given as medicated pellets, is well absorbed in fish, obtaining bioavailabilities of 91 and 99%, respectively, in cod (*Gadus morhua*) and Atlantic salmon held in seawater (Horsberg et al., 1996; Samuelsen et al., 2003). NUFLOR VET. is an injection fluid that is approved for treatment of bacterial infections in mammals. A solubility test revealed precipitation of florfenicol particles at a concentration of approximate 10 mg/l when dissolved in methanol and added to seawater. Hence, in seawater much of the drug will be present as particles.

In an unpublished study, florfenicol was used successfully in the enrichment of rotifers (Roiha et al., submitted). Highest concentrations of the drug were achieved using a modified form of AQUAFLOR premix where the lactose monohydrate had been removed and micro-particles of florfenicol were liberated to the enrichment solution. Blended AQUAFLOR premix and NUFLOR injection fluid added directly to the enrichment solution gave lower concentrations. The enrichment was, however fast for all the three forms of florfenicol and within 2 hours a steady state condition was reached, i.e. no further increase in concentration over time was detected.

Using a similar experimental set-up as described in Roiha et al. (submitted) a study was initiated to examine to what extent particles of florfenicol was ingested by the *Artemia* nauplii, what concentrations of the drug could be obtained and how fast did the enrichment process proceed.

Materials and Methods

Chemicals and reagents

AQUAFLOR®, 50% w/w premix, and NUFLOR® VET., 300 mg/ml injection solution, were supplied by Schering-Plough (Union, NJ, USA). The chloramphenicol standard was purchased from Sigma-Aldrich, Norway AS (Oslo, Norway). 1-Heptane sulphonic acid was purchased from Fluka Chemie (Buchs, Switzerland). Methanol (HPLC-grade), acetone, methylene chloride, phosphoric acid (H_3PO_4), disodium hydrogen phosphate (Na_2HPO_4), trisodium phosphate (Na_3PO_4), hydrochloric acid (HCl), triethylamine and n-hexane (all p.a. grades) were from Merck (Darmstadt, Germany). Stock solutions of florfenicol and chloramphenicol were prepared in a concentration of 1 mg/ml in methanol and stored at –20°C. Working standards were prepared by dilution from the stock solutions with methanol.

Experimental set-up

Cysts of the brine shrimp *Artemia franciscana* were purchased from INVE Aquaculture, Inc. (Salt Lake City, Utah, USA). The cysts were hatched in a commercial hatchery, SagaFjord Sea Farm AS (Stord, Norway), using seawater with salinity of 34-35%, continuous illumination of 1000 ± 100 lx, and a temperature of 25 ± 1°C. Twenty-four hours following hatching, nauplii were harvested, separated from hatching debris, thoroughly rinsed, and were ready for use.

The enrichment studies were performed in 10 L containers, with continuous aeration, and a density of *Artemia* nauplii of approximately 600 individuals per ml. The seawater had a salinity of 35% and a temperature of 20°C. In Experiment 1, florfenicol was applied to the *Artemia* nauplii in a modified form of AQUAFLOR premix. The modified form termed PF* was prepared by adding AQUAFLOR premix to distilled water of approximately 60°C in order to dissolve lactose monohydrate and thereby liberate the micro-particles of florfenicol. According to the producer, the size of these particles will be in the range of 4-10 µm. The enrichment solutions were prepared at concentrations from 400 to 2000 mg/l and with an enrichment time of 120 min. Three parallel tanks were used for each dose and samples were collected after 10, 20, 40, 60, and 120 min of enrichment.

The results obtained in experiment 1 were however disappointing and showed significant lower concentrations in *Artemia* nauplii, compared to rotifers using the same experimental set-up. Therefore, an additional experiment was performed (Experiment 2) studying the enrichment of florfenicol in *Artemia* nauplii using blended AQUAFLOR premix and NUFLOR VET. in the enrichment solutions.

For AQUAFLOR premix the correct quantity of florfenicol was mixed with 500 ml tap water using a blender for 3 min, followed by addition of 2 L of seawater, and filtering through a 150 µm mesh filter. For NUFLOR the determined quantity of the drug was added directly to the enrichment containers. Three parallel tanks were used for each dose, and samples were collected after 10, 20, 40, 60, and 120 min of enrichment. The doses used were 100 and 300 mg/l for AQUAFLOR premix and 400 mg/l for NUFLOR VET.

Sampling and sample preparation

At each sampling, 500 ml was removed from the containers, and the *Artemia* nauplii were filtered off, using a 150 µm sieve, and washed thoroughly with 5 L seawater at 25°C. From the filtrate, a 2 ml sample was stored in an Eppendorf tube at - 20°C until analysed. The samples were prepared and analysed, following a modified procedure of the method described by Samuelsen et al. (2003). The 2 ml sample was transferred to a 10 ml tube (Sarstedt, Numbrecht, Germany) and sonicated using an ultrasonic bath for 10 min. Prior to sonication 100 µl of a solution of 1 mg/ml of chloramphenicol in methanol was added as internal standard. Following sonication, 2.5 ml acetone was added to the homogenate, mixed vigorously by vortexing, and centrifuged at 2500 g (5000 rpm) for 10 min, using a Jouan centrifuge (Thermo Scientific, Waltham, MA, USA). The supernatant was extracted with 5 ml methylene chloride, and a 2.2 ml fraction of the methylene chloride extract was evaporated to dryness using nitrogen and a water-bath set at 30°C. The residue was dissolved in 500 µl of a solution of 0.01 M Na_2HPO_4 (pH 2.8): methanol (80:20%), washed with 1 ml n-hexane, and filtered through a Spin-X Micro Centrifuge Filter (0.2 µm) from Corning (NY, USA). Twenty µl of the filtrate was used for the HPLC analysis. Samples were taken prior to initiation of the study and analysed to confirm the absence of florfenicol and chloramphenicol in *Artemia* nauplii. Standard curves for florfenicol, in the range of 10 to 1200 µg per sample, were prepared in triplicates.

Since the florfenicol concentrations were calculated for individual organisms in this investigation, the numbers of *Artemia* nauplii in ten 2 ml samples were determined following each enrichment study. The 2 ml samples were dissolved in 2 L seawater and the nauplii were

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evenly distributed by aeration. Five parallels of 200 µl were collected and counted, using a magnifying loupe, and the number of nauplii per 2 ml sample could be calculated. At the same time mortality was recorded and the presence of particles in the stomach of the nauplii were inspected visually.

Analytical procedure

The HPLC system used consisted of an SP 8800 ternary HPLC-pump (Spectra-Physics, San José, CA, USA) connected to a Gilson 234 Auto-injector (Gilson, Middleton, WI, USA) and a Spectra-Physics SP-8480 UV-detector operating at a wavelength of 225 nm. The detector output was coupled to a computerised data system consisting of a Dionex UCI-50 Universal Chromatography Interface, the program Dionex Chromeleon Version 6.80 (Dionex Softron, GmbH, Germering, Germany), and a PP04X Dell computer for storage and integration of the chromatograms. The analytical column was a 150 x 4.6 mm Zorbax SB-C-18, 3.5 µm (Agilent Technologies, Karlsruhe, Germany) connected to a short C-18 pre-column (10 x 4.6 mm). The column was operated at room temperature. The mobile phase was a mixture of two solutions, A and B, at a ratio of 60:40%. Solution A was made by dissolving 0.02 M heptansulphonate and 0.025 M Na_3PO_4 in water and adjusting the pH of the solution to 3.85 using phosphoric acid 1 and 5 M. Solution B was methanol containing 0.1% triethylamine. The mobile phase was filtered through a 0.2 µm Millipore filter and degassed using helium and sonication (5 min). The flow rate was 1 ml/min, giving elution times of 5.6 min (florfenicol) and 7.8 min (chloramphenicol).

Statistical analysis

Data from the experiments were statistically analysed using a Student's t-test with a probability of $P \leq 0.05$.

Results

The standard curves for florfenicol in *Artemia* nauplii were linear over the range studied ($r^2 = 0.97$-0.99) and based on these curves a limit of quantitation of 2 µg/sample could be determined. Examining 10 parallels, only a minor variance in the number of *Artemia* nauplii in the 2 ml samples was found (61000 ± 2000). When applying blended AQUAFLOR, particles were observed in the stomach of the *Artemia* nauplii inspected under the microscope. No sign of mortality was registered as a result of the enrichment procedures.

In experiment 1, using the modified form of florfenicol (PF*), the accumulation was both dose and time dependent and no significant increase ($P \geq 0.01$) in concentrations were seen after 40 min of enrichment for any of the doses. Following 120 min of enrichment, the concentrations were 0.13, 0.39, 0.78 and 1.55 ng/nauplii using doses of 400, 800, 1000 and 2000 mg/l, respectively (Figure 1).

In experiment 2 using NUFLOR injection fluid, accumulation of the drug followed a similar pathway as was seen for PF* and following 120 min of enrichment the concentration obtained was 0.14 ng/nauplius using a dose of 400 mg/l. When florfenicol was administered as AQUAFLOR premix the uptake was very rapid and peak concentrations of 1.71 and 5.20 ng/nauplius for the 100 and 300 mg/l dose, respectively, were seen after 10 min of enrichment. The rapid increase was followed by a substantial decrease in the concentrations for the next 30 min. After 40 minutes, however, no significant change ($P \geq 0.01$) in concentrations over time were seen and following 120 minutes of enrichment, the concentrations were 0.49 and 2.49 ng/nauplius for the 100 and 300 mg/l doses, respectively (Figure 2).

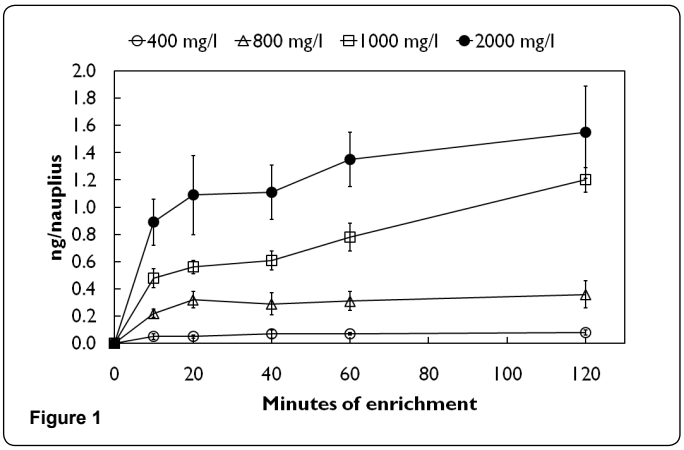

Figure 1

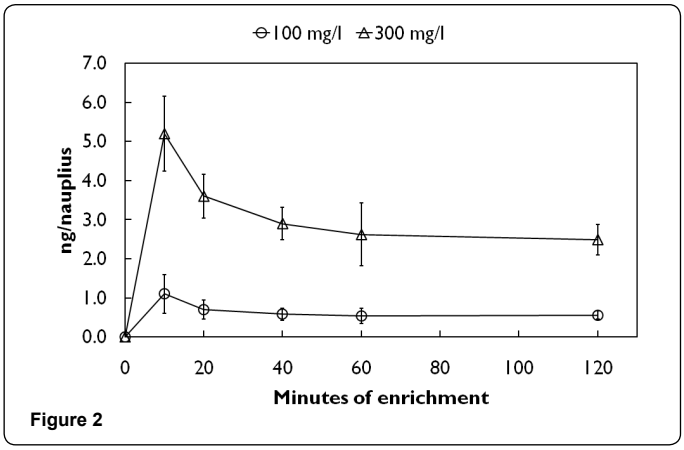

Figure 2

Discussion

There are different approaches to present the amount of a drug in live feed. The concentrations of drugs in *Artemia* nauplii are often presented as either mg drug/mg protein or mg drug/mg dry weight (Verpraet et al., 1992; Touraki et al., 1995; Touraki et al., 1996; Robles et al., 1998; Touraki et al., 1999; Mejia et al., 2007). However, this is rather impractical for a fish farmer and in order to ease the calculation of correct doses of a drug to fish, prawn and shellfish larvae the use of ng or µg per nauplius is recommended (Mohney et al., 1990; Gomez-Gil et al., 2001). Hence, in this study we have presented the concentration of florfenicol in *Artemia* nauplii as ng/nauplius. To make use of this information, however it is necessary to know the average weight of the larvae to be treated and the approximate number of nauplii ingested by an individual larva per feeding.

No mortality of *Artemia* nauplii was recorded in this study, showing that no acute lethal effect was caused by the enrichment procedure or the drug used. This is in contrast to the study by Cook and Rust (2002) who found that three out of five forms of erythromycin gave significant lower survival of adult *Artemia*. Figueiredo et al. (2009) found increasing mortality with prolonged enrichment time in *Artemia* nauplii enriched with fatty acids and where the maximum enrichment time was 24h.

All three administered forms of florfenicol were ingested by *Artemia* nauplii, but blended AQUAFLOR premix gave much higher concentrations than both the modified form PF* and NUFLOR (Figure 1 and Figure 2). When comparing doses of 400 mg/l (NUFLOR), 400

mg/l (PF®) and 300 mg/l (AQUAFLOR premix), respectively, steady state concentrations of 0.14, 0.13 and 2.49 ng/nauplius, were found. The reason for this difference may be due to the difference in particle size and how the drug is presented in the enrichment solution. NUFLOR is a liquid containing emulgators (n-methyl-2-pyrrolidone, propylene glycol, polyethylene glycol qs) and the florfenicol is associated to micelles when added to seawater whereas PF® consists of florfenicol particles in the range of 4 to 10 μm, which is in the lower range for *Artemia* nauplii that normally graze on particles between 7 and 28 μm, with an optimum of about 16 μm (Fernandez, 2001). Furthermore, since *Artemia* nauplii have the ability to ingest particles as large as 70 μm, it is obvious that blended AQUAFLOR premix gave a range in particle sizes with a higher preference as food particles for the nauplii than both PF® and NUFLOR. In rotifers which have preference for particles in the range of 1.4 to 21 μm, with highest selectivity for particles of 4.5 μm (Vadstein et al., 1993; Hansen et al., 1997), the concentrations achieved using the same doses of PF® were approximately twice the concentrations obtained in *Artemia* nauplii. The results therefore illustrate the importance of presenting food particles of optimal size to the organism that is to be enriched.

The rapid ingestion of florfenicol followed by a fast elimination as seen in Figure 2, is from a pharmacokinetic perspective rather atypical. However, Reeve (1963a; 1963b) showed that *Artemia* nauplii ingested indigestible sand particles ten times faster than algae of the same size that may explain the fast uptake of the florfenicol particles. Reeve (1963b; 1963c) also showed that the production of faecal pellets increased when the amount of sand particles increased in the diet whereas Evjemo et al. (2000) found that the assimilation efficacy decreased with increasing food concentrations, and observed considerable amounts of undigested food in the faecal particles when food was presented in a high amount. Increased faecal pellet production and florfenicol particles forced through the gut undigested may explain the decrease in the drug concentrations that occurred between 10 and 40 min in this study.

In the literature the concentrations obtained from enrichment of antibacterial agents using *Artemia* nauplii varies considerably and is mainly due to variations in doses, enrichment time and in what form the drug was administered. While some authors report drug concentrations slightly lower or similar to those obtained in this study, others report much higher concentrations. In the current study a maximum florfenicol concentration of 5.20 ng/nauplius was detected after 10 minutes enrichment and 2.49 ng/nauplius after 120 minutes using 300 mg/l AQUAFLOR premix (Figure 2). These values are in the same range as those found by Langdon et al. (2008). They investigated the enrichment of OTC in *Artemia* nauplii using OTC-containing wax spray beads (WSB) or an aqueous solution of OTC. The enrichment time was 22h and the enrichment media were added a total of 206 mg/l OTC using WSB and 214 mg/l OTC in the aqueous solution. The concentrations of OTC in *Artemia* nauplii were measured to 2.85 and 11.47 ng/nauplius using the aqueous solution and WSB, respectively (Langdon et al., 2008). Gomez-Gil et al. (2001) studied the bioencapsulation of OTC and the quinolone enrofloxacin in *Artemia* nauplii. The density of the nauplii was 2130 nauplii/ml and the antibacterials were delivered as percentage of a lipid emulsion, where the highest concentrations were equivalent to 160 mg/l enrofloxacin and 320 and 640 mg/l of OTC. Samples were taken successively for 24 h. Maximum concentrations of 1.07 ng/nauplius of enrofloxacin and 9.32 and 9.37 ng/nauplius, respectively, for the two doses of OTC were obtained after 4h. Worth noticing is the minor difference in the concentration between the two OTC doses indicating a maximum enrichment dose of 320 mg/l or less for OTC. Based on their results (Gomez-Gil et al., 2001) recommended a minimum of 4h for complete enrichment. In comparison, Touraki et al. (1995), applying OTC in concentrations of 4000, 8000 and 12000 mg/l administered via liposomes and applying enrichment times of 8, 24 and 32h, found peak concentrations of 23.18, 43.03, and 61.98 ng/nauplius following enrichment for 24h. These results indicate that a maximum enrichment dose for OTC was not reached.

In a review by Robles et al. (1998), a study of the bioencapsulation of florfenicol in *Brachionus* and *Artemia* nauplii was described. Florfenicol was administered as a lipid emulsion containing 20 and 50% florfenicol and applying an enrichment time of 24h the concentrations achieved in *Artemia* nauplii were 119 and 265 μg/g dry weight, respectively (Robles et al., 1998). According to Dhont and Van Stappen (2003) the dry weight of a Great Salt Lake *Artemia* nauplius is approximately 2.42 μg, hence there should be approximately 413.000 nauplii per gram dry weight. The findings described in Robles et al. (1998) therefore refer to concentrations of approximately 0.29 and 0.64 ng/nauplius, which is in the same range as our findings. However, since the doses used for enrichment were not reported in the study, a direct comparison with our results is difficult to make. Verpraet et al. (1992) found concentrations of 0.19 ng/nauplius of TMP and 0.51 ng/nauplius of sulphamethoxazole (SMX), using 300 mg/l of an emulsion containing 10% TMP and SMX in the ratio 1:5 and an enrichment time of 24h. Using the same drugs, ratio between the drugs and similar enrichment conditions comparable results to those described by Verpraet et al. (1992) were found in two studies by Touraki et al. (1996; 1999).

The recommended dosage of florfenicol to treat bacterial infections in larger marine fish is 10 mg florfenicol per kg fish daily for 10 consecutive days (Samuelsen and Bergh, 2004). At day 35 post-hatch, the average length of a cod larva is approximately 14 mm and the wet weight approximately 24 mg/larva (Otterlei et al., 1999). Hence, in order to achieve the daily dosage, larvae of 24 mg (wet weight) must consume approximately 100 nauplii with a concentration of 2.50 ng florfenicol/nauplii and approximately 1000 nauplii with a concentration of 0.25 ng florfenicol/nauplii. From a daily requirement of approximately 300 *Artemia* nauplii for a 30 days old cod larva, the requirement increases successively to 3850 when the larva has reached an age of 50 days (Van der Meeren, et al., 2005). Similarly, a halibut (*Hippoglossus hippoglossus*) larva with a length of 17 mm require daily approximately 500 *Artemia* nauplii and increasing to over 2000 when the larva has reached a length of 22 mm (van der Meeren, 1995; Gara et al., 1998; Mangor-Jensen, 2004). Furthermore, giant freshwater prawn larvae (*Macrobrachium rosenbergii*) were reared at a feeding rate of 200 *Artemia* nauplii twice a day (Devresse et al., 1990). Therefore, based on the results presented in this study it is possible to design suitable feeding regimes for the administration of a daily dose of the drug using any of the forms of florfenicol (AQUAFLOR premix, NUFLOR, PF®) presented in this study. It should however be noted that these investigations were performed using *Artemia* nauplii hatched 24h prior to the enrichment and at a density of approximately 600 individuals/ml in the enrichment tank. This study does not address any effect the density of nauplii in the enrichment tank may have on the feeding activity or any variation in appetite that might occur due to differences in time from last feeding, or hatching, to enrichment. It is therefore important to adapt the enrichment conditions specified in this study to obtain similar results.

As a conclusion, the present study shows that florfenicol is ingested by *Artemia* nauplii, and that this organism can be used as a carrier of the drug. Highest concentrations were achieved using AQUAFLOR premix, giving concentrations similar or higher to those

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described for several other antibacterials. The method is rapid, but an enrichment time of at least 60 min is recommended to obtain reproducible results.

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