Abbassa Int. J. Aqua., V0l.8 No.(1) 2015. *212-228*

EFFICIENCY OF FLUMEQUINE BIOENCAPSULATION IN *ARTEMIA* NAUPLII TO TREAT *OEROCHROMUS NILUTICUS* FRY INFECTED WITH *AEROMONUS HYDROPHILLA*

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Received 18/ 10/ 2015 *Accepted 24/ 11/ 2015*

Abstract

Bioencapsulated of flumequine by *Artemia* nauplii was evaluated as a function of its concentration in the enrichment medium and of the duration of the enrichment period. An emulsion containing 20, 30 and 40% (w/w) flumequine (30, 45 and 60 mg flumequine/ml of enrichment) was administered to nauplii for 4, 8, 12, 24 or 32 h. Increased uptake of flumequine (440.2 ±15.1 µg/g dry weight) and good survival rates of the nauplii were observed with the 30% emulsion and 24 h enrichment. Curve of the body tissue concentration of *Artemia* nauplii–time showed half-life were found to be 0.9 ± 0.10 h and the terminal phase elimination half-life were found to be 12.3 ± 6.90h. In the efficacy trial the cumulative mortalities in groups treated with bioencapsulation treatments appeared significantly reduced amounting to 20 ± 2.90%,19± 2.80%, and 22± 3.70% with groups taken 40%, 30% and 20% flumequine respectively. Mean cumulative mortality in unmedicated groups reached 91.80 ± 4.40% post challenge. Flumequine concentration was 65.40 ±6.80 µg/g in tilapia fry after a 5-day oral treatment with medicated nauplii. It could be concluded that flumequine appears effective in treating of *Aeromonus hydrophilla* in *Oerochromus niluticus* fry. The best level of flumequine concentration was 30 (w/w).

Key words: Flumequine, *Artemia*, *O. niluticus* fry.

INTRODUCTION

The importance of aquaculture has global implications as the world population continues to grow. The success of fish farming facilities can be jeopardized by the occurrence of infectious diseases that adversely affect production quotas. In order to manage aquatic animal health, there is an unavoidable need to develop and expand pharmacotherapy in this area of population medicine (Matthew *et al*., 2012). Stable productions of cod larvae

are limited by the outbreak of bacterial diseases during the early life stages. As the larvae possess no specific immune system when hatched, due to the immature status of the lymphoid tissues, vaccination is not an option at this stage. Hence, in order to treat an infection, antibacterial therapy is needed (Revertera *et al.*, 2014).

The development of microbial diseases in fish is of major importance especially when intensive culture practices are employed, during which large numbers of fish can be lost, resulting in adverse economic implications. These microbial diseases are mainly due to gram-negative bacteria and they are treated by the application of antimicrobials either in the culture water or in the fish feed (Samuelsen *et al*., 1997).

The delivery of drugs to aquatic animals is problematic. Delivery by medicated food leads to leaching of the drug from the pellets. In bath treatments, a substantial portion of the drug is not used and it can be difficult to dispose of the excess of drug safely. Recently, a new technique for the supplementation of the live fish feed artemia with nutrients, lipid soluble therapeutics or even with water soluble therapeutics became feasible (Touraki *et al*., 1999).

The nauplii of *Artemia* constitutes a food source that is very well accepted by both marine and freshwater fish fry while it can also be used as a carrier for compounds which are then easily introduced to the consumer organism. Such compounds are the highly unsaturated fatty acids and essential nutrients for the cultured species of fish, which have been incorporated to the fish larvae through the food chain (Touraki *et al*., 2010).

Quinolones are effective against all common bacterial infections and they have been used to treat aquaculture (Samuelsen, 2006).

Flumequine is a synthetic fluoroquinolone antibiotic used to treat bacterial infections. It is a first-generation fluoroquinolone broad spectrum antibacterial that has been killed bacteria by interfering with the enzymes that cause DNA to unwind and duplicate. Flumequine was used in veterinarian medicine to treat cattle, swine, chickens and fish (Geng *et al*., 2015).

Moreover Gomes *et al.* (2007) reported the successful bioencapsulation of flumequine so, it has been reported that the nauplii of *Artemia* might well represent an experimental model for the evaluation of ecotoxicity of drugs possibly due to the increased sensitivity of the naupliar stages.

The aim of the present work; provide bioencapsulation technique to be applicable for the delivery of chemotherapeutics via live food in tilapia fry.

MATERIAL AND METHODS

Artemia Preparation:

Artemia cysts (E.G. grade, Great Salt Lake strain, Artemia International LLC center, USA) were regularly hatched in filtered artificial sea water (35 ppt), at 28 ± 1°C under continuous aeration and illumination (2000 lux). After 24 hr, instar I nauplii were separated from unhatched cysts, washed in clean artificial sea water and thereafter supplemented with the enrichment emulsion (Selco, INVE Aquaculture Artemia Systems, Belgium) with and without flumequine (Onwulata, 2012).

Enrichment of *Artemia* nauplii:

After rinsing of the nauplii was divided into four groups in three replicates in clean glass aquaria (30x40x40cm) with artificial seawater, 0.15 ml of an emulsion in which 20, 30 and 40 % (w/w) flumequine were administered (Photo. 1), at t0 + 2 h and t0 +8 h with t0 being the onset of their incubation. The flumequine concentrations (w/w) in the enrichment emulsion correspond to about 30, 45 and 60 mg flumequine/ml of enrichment emulsion. All experiments were performed in the dark, to avoid photodecomposition of the antibiotic.

The duration of the enrichment period was 0 to 4, 8, 12, 24 and 32 h for three separate replicates. Control *Artemia* samples were fed on non treated emulsion (Gomes *et al*., 2007).

Photo 1. *Artemia nauplii* feed with emulsion containing flumequine.

Duration Effect of Bioencapsulation on *Artemia* Survival:

Viability of *Artemia nauplii* was determined in experiment in random samples of 10 ml each from each one of the five experimental tanks, counting live and dead Artemia. Each tank was tested twice. The nauplii were collected at the end of the enrichment period, they were thoroughly washed and either used immediately or stored at −20 °C until used according to the procedure of Touraki *et al*., (2010).

Drug Leakage in Culture Emulsion Following Bioencapsulation Period:

Drug residue in culture medium following bioencapsulation period was determined according to the procedure of Samuelsen (2006).

Flumequine Concentration–Time Profile in nauplii following Enrichment Period:

Flumequine concentration–time profile in nauplii following enrichment period was determined according to the procedure of Samuelsen (2006).

Fish:

A total of 600 (2±0.3g) apparently healthy *Oerochromus niloticus* fry randomly collected from fish farm of Central Laboratory for Aquaculture Research, Abbassa (CLAR). Fish were acclimated to the laboratory conditions for 2 weeks supplied with dechlorinzed tap water at 25±1^{o}C and aeration in the wet laboratory of fish diseases department.

Twelve glass aquaria (77×37×48cm) were prepared for experiment using dechlorinated tap water; continuous aeration; maintained temperature at 25°C throughout the experiment. About half of the water was changed and fecal matters were siphoned out once daily during the experiment.

Fish was divided into four groups in three replicates, T1 fed nauplii free from flumequine (control), T2, T3 andT4 fed nauplii incubated with 30, 45 and 60 mg flumequine/ml of enrichment emulsion consecutively (correspond to 20, 30 and 40 % (w/w).

Challenge Test:

O. niluticus fry were challenged using a bath challenge model that has previously been described by Seljestokken *et al*., 2006. Experiments were performed after a conditioning period (fed nauplii containing flumequine) of 1 week and then they were challenged with the *Aeromonas hydrophila* which kindly obtained from Fish Health Department, CLAR. It was previously isolated from naturally diseased tilapia and identified according to standard bacteriological tests. It was used at density of 0·5 x 10^7 cell/ml for 4 h under continuous oxygenation at 25 °C, pH values ranged between 7.8 and 8·5, and the daily renewal of the water. The challenge was ended by changing the water twice. Survival percentage was measured for 8 days following the bacterial challenge.

Drug Accumulation in Fish:

Quantitative analysis of the fish body was carried out, after the fish were fed with nauplii enriched as described by Mnyone *et al.* (2009).

Statistical Analysis:

Data were statistically analyzed with one way ANOVA (Sokal and Rohlf, 1981). The analysis was performed with SPSS 16.0 and the statistical significance was set at $p > 0.05$.

Pharmacokinetic analysis of flumequine in *Artemia* nauplii and in fish larvae was carried out using model-independent standard methods. Calculations were carried out with the FreeKin Modeler (2014).

RESULTS

Enrichment of *Artemia* nauplii:

The concentrations of flumequine per g of dry weight of *Artemia* nauplii were determined in emulsions containing different amounts of flumequine (Table 1 and Fig. 1).

Table 1. Showed Duration Effect on Flumequine Concentrations in Bioencapsulated *Artemia* nauplii.

Time(hr) / Flumequine Conc.*	0	4	8	12	24	32
T1	---	----	------	------	------	------
T2	---	102.2^{C} ± 10.5	175.6^{C} ±11.3	234.7^{B} ±12.2	310.1^{B} ±14.6	209.4^{C} ±10.4
T3	---	165.4^{B} ±11.8	202.5^{B} ±12.4	353.6^{A} ±14.4	440.2^{A} ±15.1	304.2^{B} ±11.5
T4	---	174.2^{B} ±12.1	216.3^{A} ±12.5	371.2^{A} ±15.1	455.4^{A} ± 15.7	315.5^{B} ± 12.4

*Concentrations measured by µg/g.

Superscript letters explain degree of significantly at P <0.05. Means having the same superscript letters are no significantly different.

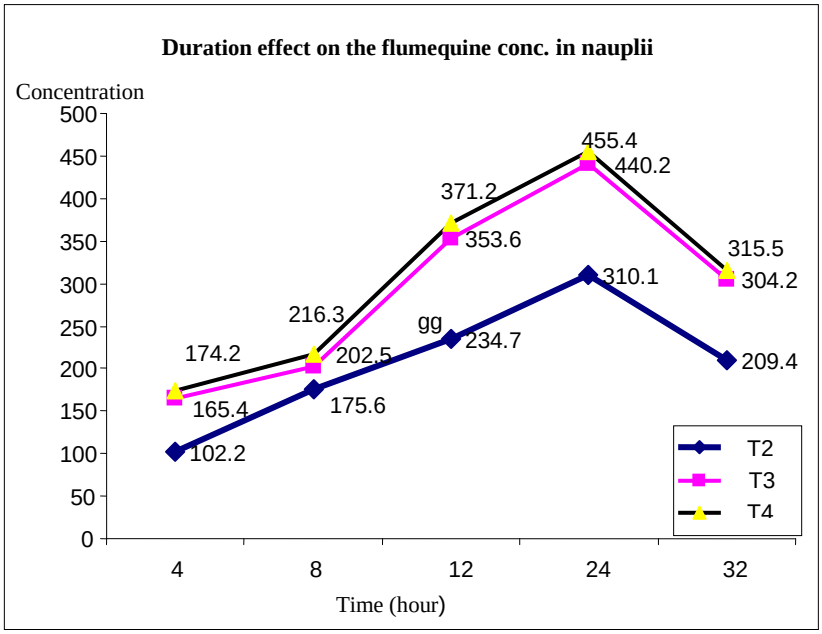

Fig. 1. Showed Duration Effect on Flumequine Concentrations in Bioencapsulated *Artemia* nauplii**.**

Survival of Treated *Artemia* in Relation to Time:

The survival data showed a decline in the survival of *Artemia* nauplii at 32h to 86.5 ± 2.6 when the 40% emulsion was used, which was significantly different from the control value of 92.6±3.4, as well as from the value of 90.5 ±3.2 corresponding to the 30% emulsion and 93.6 ±3.2 to the 20% emulsion (p b 0.05) (Table 2 and Fig.2).

Table 2. Showed Percentage of Survival of Treated *Artemia* in Relation to Time.

Duration (hr) / Flumequine Conc.	4	8	12	24	32
T1	98.2 ^{A} ±3.6	98 ^{A} ±3.6	97.2 ^{A} ±3.5	95.6 ^{A} ±3.4	92.6 ^{A} ±3.3
T2	98.1 ^{A} ±3.6	97 ^{A} ±3.4	95.1 ^{A} ±3.4	93.6 ^{A} ±3.2	91.4 ^{A} ±3.1
T3	97.2 ^{A} ±3.5	96.3 ^{A} ±3.5	94.5 ^{B} ±3.4	92.1 ^{B} ±3.2	90.5 ^{B} ±3.0
T4	94.8 ^{B} ±3.3	91.2 ^{B} ±3.1	89.6 ^{C} ±2.2	88.1 ^{C} ±1.9	86.5 ^{C} ±2.6

Superscript letters explain degree of significantly at P <0.05. Means having the same superscript letters are not significantly different

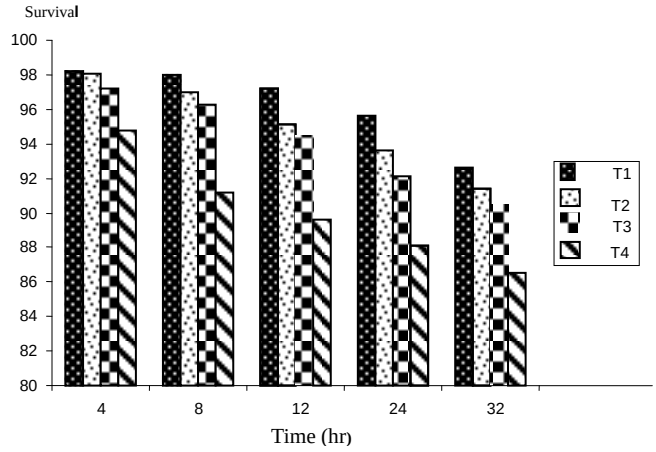

Fig. 2. Showed Relationship between Treated *Artemia* Survival and Time.

Drug Leakage in Culture Emulsion Following Bioencapsulation Period:

The concentration of flumequine in the culture emulsion following the bioencapsulation period increased reaching its highest value of 0.3 ±0.05 μg/ml medium when an emulsion containing T4 flumequine was used, and they were significantly different from the value of 0.26 ±0.05 μg/ml for the emulsion containing T3 flumequine, as well as from the value of 0.24 ±0.04 μg/ml corresponding to the T2 emulsion (Table 3 and Fig.3).

Table 3. Showed drug residue in culture medium following bioencapsulation period.

Time (hr) / Flumequine Conc. *	4	8	12	24	32
T1	-------	-------	-------	-------	-------
T2	0.09^{C} ±0.01	0.12^{C} ±0.02	0.15^{B} ±0.02	0.19^{C} ±0.03	0.24^{B} ±0.04
T3	0.11^{B} ±0.02	0.14^{B} ±0.02	0.16^{B} ±0.03	0.21^{B} ±0.04	0.26^{B} ±0.05
T4	0.13^{B} ±0.02	0.15^{A} ±0.02	0.18^{A} ±0.03	0.25^{A} ±0.04	0.3^{A} ±0.05

* Flumequine Concentrations measured by μg/ml
Superscript letters explain degree of significantly at P <0.05. Means having the same superscript letters are not significantly different

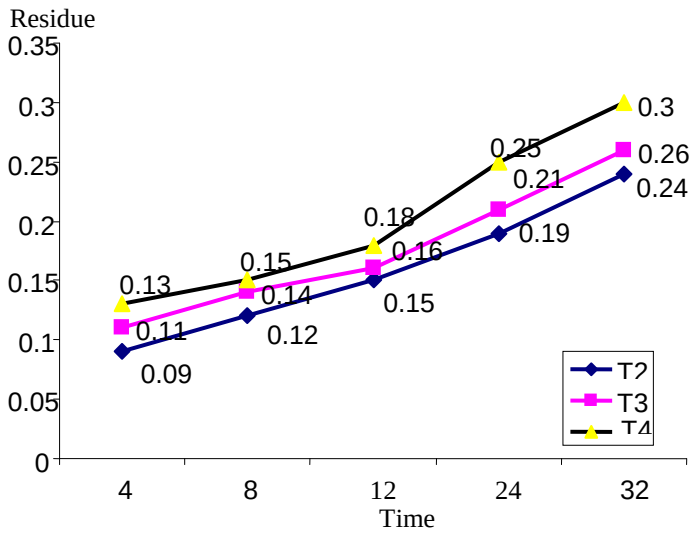

Fig. 3. Showed Drug Residue in Culture Medium Following Bioencapsulation Period.

Flumequine Concentration–Time Profile in Nauplii Following Enrichment Period:

Curve of the body tissue concentration of *Artemia* nauplii–time showed half-life were found to be 0.9 ± 0.1 h and the terminal phase elimination half-life were found to be 12.3 ± 6.9 h. The body tissue concentration at time zero after the end of the 24 h enrichment period (Table 4 and Fig 5). There weren't significant differences between experiment groups.

Table 4. Showed Flumequine Concentration–Time Profile in Nauplii Following Enrichment Period.

Time after 24 h / Flumequine Conc	0	4	8	12	24	32
T1	-----	-----	-----	-----	-----	-----
T2	$310.1^{B} \pm 12.4$	$280.4^{B} \pm 10.8$	$115.2^{B} \pm 8.2$	$75.8^{B} \pm 6.1$	$18.0^{B} \pm 4.1$	$6.4^{C} \pm 2.1$
T3	$440.2^{A} \pm 14.2$	$295^{B} \pm 11.2$	$119.4^{B} \pm 8.2$	$74.2^{B} \pm 6.1$	$16.8^{C} \pm 3.8$	$8.1^{A} \pm 2.6$
T4	$455.4^{A} \pm 14.7$	$300.4^{B} \pm 11.6$	$128.5^{B} \pm 9.1$	$80.6^{A} \pm 7.2$	$20.4^{B} \pm 5.2$	$8.6^{A} \pm 2.6$

Superscript letters explain degree of significantly at $P < 0.05$. Means having the same superscript letters are not significantly different.

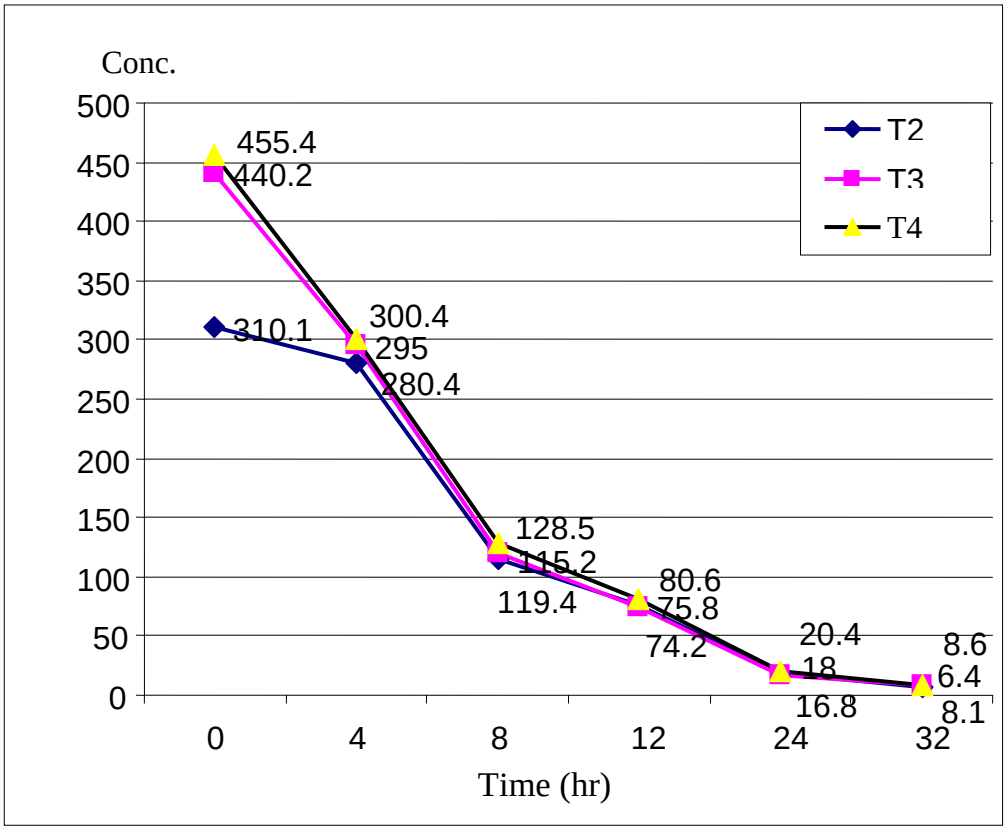

Fig. 5. Showed Flumequine Concentration–Time Profile in nauplii following Enrichment Period**.**

Challenge Test:

Data collected after challenge experiment showed high mortality rates 2 days post the challenge, indicating the potency of the used strain. There was no significant difference was observed between groups treated with 30% and 40% flumequine concentration. However, there were marked significant differences between the untreated and treated challenged fish. The mortality percentages were 91.8, 22, 19 and 20 % in 0, 20, 30 and 40% treated groups (Table 5 and Fig.4).

Table 5. Showed Cumulative Mortalities after Challenge Test.

Group	T1	T2	T3	T4
Cumulative mortality	91.8 ±4.4%	22 ±3.7%	19 ±2.8%	20 ±2.9%

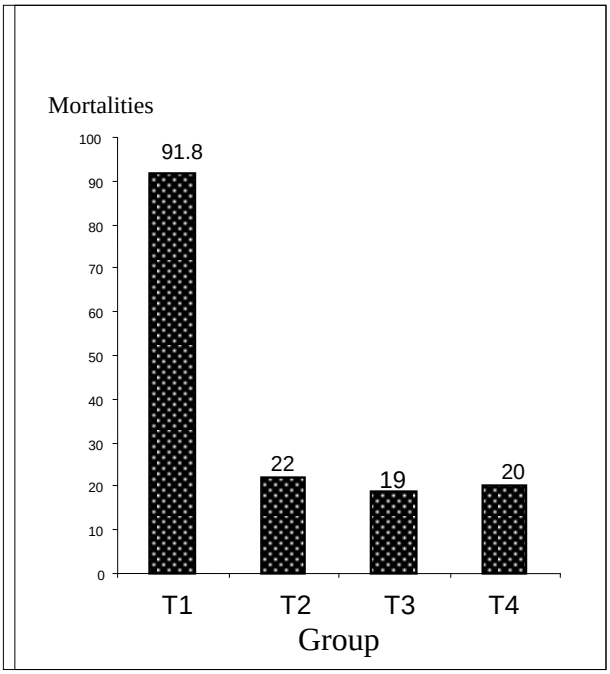

Fig. 4. Showed Cumulative Mortalities after Challenge Test**.**

Drug Accumulation in Fish:

Table 6 and Fig. 5 illustrates tilapia which were performed on treatment with flumequine bioencapsulated in *Artemia* nauplii.

Table 6. Showed Drug Accumulation in Fish.

Conc. in fish \ Time /days	1	3	5	7	9	10
T1	---	---	---	---	---	---
T2	---	$28.9^{C} \pm 3.6$	$59.5^{B} \pm 6.6$	$30.0^{C} \pm 4.1$	$9.5^{B} \pm 2.1$	---
T3	---	$32.2^{B} \pm 4.3$	$65.4^{A} \pm 6.8$	$40.4^{B} \pm 4.5$	$11.2^{A} \pm 2.9$	---
T4	---	$36.4^{A} \pm 4.1$	$70.2^{A} \pm 7.1$	$49.4^{A} \pm 5.4$	$13.5^{A} \pm 3.1$	--

*Superscript letters explain degree of significantly at P <0.05. Means having the same superscript letters are not significantly different.

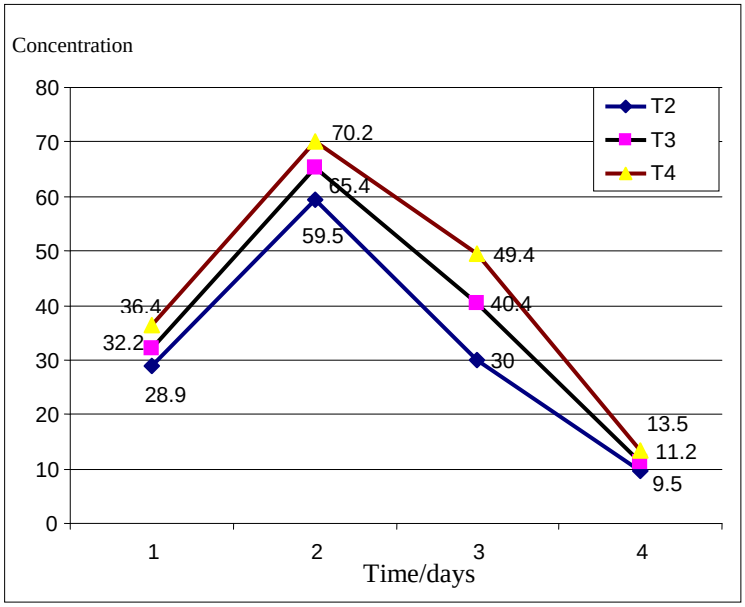

Fig. 5. Drug Accumulation in Fish.

DISCUSSION

Using of live food as a carrier of substances to the organism that feed on it has certain benefits as to present high uptake, low discharge of the substance to be delivered and satisfactory survival rates under the experimental conditions used. Artemia nauplii have been extensively used for the delivery of nutrients and chemotherapeutics to fish larvae (Gomes *et al*., 2007).

The uptake of flumequine by *Artemia* nauplii in the presented study reaching 455.4 μg/g dry weight with the 40% enrichment emulsion, 440.2 μg/g for the 30% emulsion and 310.1 μg/g for 20% emulsion, appeared quite lower than the 256 mg/g previously reported (Gomes *et al*., 2007). This difference might be due to the different methods used for the determination of flumequine in *Artemia* and the different developmental stages of *Artemia* that were employed in the two studies. While, it was near to 520.4 μg/g that reported by Touraki *et al*., 2010 but at 50% enrichment flumequine emulsion.

The survival data showed a decrease of survival as the duration of the bioencapsulation period increased. Decreased survival of nauplii to 88.1%, 89.5 % and 90.6% was observed in present study with the emulsion containing 40%, 30% and 20% flumequine. The result was supported with (86.49% at emulsion containing 40% flumequine) that recorded by Ferreira *et al*., 2007 who explained the reason to the toxicity of several antibiotics including flumequine has been reported for various aquatic organisms including *Artemia*.

The widespread use of antibiotics in aquaculture implicates the possibility of an emergence of bacteria resistant to antibiotics including quinolones. Although flumequine is subject to photodegradation in the upper levels of the water column it is not degraded in sediment (Samuelsen *et al*., 1997). Therefore the minimization of the leakage of antibiotics from the fish feed appears of great importance. The results showed that the leakage of flumequine from the enriched nauplii was evident after 4 h following the enrichment period slower than 2h that recorded by Touraki *et al*., 2010. The rapid levels reported in the culture medium might be due to photodecomposition of the drug (Lai and Lin, 2009), metabolic conversion or Artemia take full dose and then excreted some

drugs. At any time, it would be preferable for the enriched nauplii to be used immediately after the end of their enrichment, avoiding excess of medicated feed that might contribute to environmental pollution.

The data of the body tissue concentration of *Artemia* nauplii–time showed half-life were found to be 0.9 ± 0.1 h and the terminal phase elimination half-life were found to be 12.3 ± 6.9 h similar results were recorded by Touraki *et al*., 1999 who reported results in the same average.

Treatment of experimentally challenged with *A. hydrophilla* of *O. niluticus* fry with flumequine performed was successful for oral bioencapsulation treatments, as mortalities were significantly reduced in treated groups compared to the challenged untreated group. Mean cumulative mortality in unmedicated groups reached 91.8 ± 4.4% post challenge which is similar to the value of 90.5% reported for juvenile cod (Samuelsen *et al.*, 1997).The cumulative mortalities in groups treated with bath or bioencapsulation treatments appeared significantly reduced amounting to 20 ± 2.9%, 19± 2.8%, and 22± 3.7% with groups taken 40%, 30% and 20% flumequine respectively. There was no significant difference was observed between groups taken 30% and 40% flumequine concentration.

The elimination of flumequine after the last dose was 12.3 ± 6.9 h for oral treatment. These values were higher than the value of 10.7 h reported for large sea bass after intraperitoneal injection and lower than the corresponding value of 22 to 30 h reported for seabream (Tyrpenou *et al*., 2003). Elimination of flumequine was slower in Atlantic large turbot compared to small turbot (Samuelsen *et al.*, 1997) with the elimination half-life amounting to 43 h and 10 h respectively. Although the oral route and multiple dosing should be preferred for the treatment of bacterial infections with flumequine at which bath treatments do not depend on actively feeding fish, while oral treatments control the level of infection in the group rather than in the individual (Samuelsen, 2006).

It could be concluded that flumequine appears effective in treating of *A. hydrophilla* in *O. niluticus*. The bioencapsulation treatment, if used under conditions that minimize the leakage of the drugs to the environment, could be

an environmentally friendly method and a suitable alternative to bath treatments against *A. hydrophilla* of small fish. The best level flumequine concentration in which 30 (w/w) flumequine was administered.

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كفاءة الفلموكوين المغلف حيويا بالأرتيميا فى علاج الإيروموناس هيدروفيلا فى زريعة البلطى النيلى

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الملخص العربى

تم دراسة التغليف الحيوى للفلموكوين داخل الأرتيميا وذلك لمعرفة قدرة هذه الصورة فى علاج الأمراض البكتيرية لزريعة البلطى. ولهذا الغرض تم عمل مجموعات من الأرتيميا تم تغذيتها على ثلاث مركزات من الفلومكوين ٢٠، ٣٠، ٤٠% (٣٠، ٤٥، ٦٠ مج فلومكوين لكل مل من مغذات الأرتيميا) ثم أخذت عينات من الأرتيميا بعد ٤ ، ٨ ،١٢ ،٢٤ ،٣٢ ساعة. وقد لوحظ أن أفضل تركيز للفلومكوين كان ٤٤٠.٢ ميكروجرام من الوزن الجاف للأرتيميا عند تركيز ٣٠% بعد ٢٤ ساعة من تحضين الأرتيميا مع مخلوط الدواء.

أظهر المنحنى المعبر عن تركيز الدواء فى أنسجة الأرتيميا أن فترة عمر النصف له كانت بعد ٠.٩ ساعة و فترة الإختفاء من الأنسجة هى ١٢.٣ ساعة. فى محاولات لإختبار كفاءة التغليف من خلال العدوى الإصطناعية لاصبعيات البلطى وجد أن نسبة النفوق التراكمى كانت ٢٠، ١٩، ٢٢ % للمجموعات التى أخذت جرعات من الفلموكوين ٤٠ ، ٣٠ ، ٢٠% على التوالى بينما كانت نسبة النفوق التراكمى فى المجوعة الضابطة هى ٩١.٨%. وكان تركيز الفلومكوين هو ٦٥.٤ ميكروجرام فى أنسجة زريعة البلطى بعد ٥ أيام من العلاج بإستخدام الأرتيميا.

ومن هنا وجد أنه من الممكن إستخدام الفلومكوين المغلف داخل الأرتيميا فى علاج زريعة البلطى من الإصابة ببكتيريا الإيروموناس بتركيز ٣٠% من المستحلب المقدم لتغدية الأرتيميا.