Bull Environ Contam Toxicol (2011) 87:621–625
DOI 10.1007/s00128-011-0415-8

Chronic Toxicity of Erythromycin Thiocyanate to *Daphnia magna* in a Flow-Through, Continuous Exposure Test System

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Received: 21 June 2011 / Accepted: 9 September 2011 / Published online: 9 October 2011

Abstract Approval of a new animal drug application for AQUAMYCIN 100® (erythromycin thiocyanate; ET) to treat freshwater salmonid species with bacterial kidney disease is being pursued in the US. As part of the approval process, ET's impact on an aquatic environment had to be described in an environmental assessment. The environmental assessment was lacking data to characterize the effect ET would have on a chronically exposed aquatic invertebrate organism. A major step to fulfilling the environmental assessment was completed after conducting a comprehensive study continuously exposing *Daphnia magna* to ET for 21 days. Results indicated that the no observable effect concentration for ET was 179 μg/L.

Keywords Toxicity of erythromycin thiocyanate to *Daphnia* · Assessing the environmental impact of an aquaculture drug

Erythromycin, a macrolide antibiotic, is a natural metabolic product of the fungus *Streptomyces erythreus* that has been approved by the US Food and Drug Administration (FDA) for the treatment and prevention of human and animal diseases. Erythromycin is not FDA approved for use in aquaculture with the exception it may be used under an investigational new animal drug permit as AQUAMYCIN 100®, a Type A medicated article containing 100 g erythromycin thiocyanate (ET)/lb (mention of trade or manufacturer name is solely for providing specific information and does not imply endorsement by the US Geological Survey). The Type A medicated article is added to feed to produce a Type C medicated feed that is used to control mortality associated with bacterial kidney disease in all freshwater salmonid species.

Approval of ET to control mortality associated with bacterial kidney disease in salmonid species is currently being sought. Approval only comes after the FDA has approved data submitted to the 5 major technical sections, including the Environmental Impact technical section. For aquaculture drugs, this technical section includes data that describes the potential impact a drug may have on an aquatic environment after the drug is released from an aquaculture facility. The impact is assessed through FDA's review of an environmental assessment (EA). An EA is required for any drug that is discharged directly to public water. In most cases where ET medicated feed will be used, uneaten feed has the potential to be directly discharged to public receiving waters, therefore an EA is required. The EA for ET has several components including an unfulfilled component describing the drug's toxicity to aquatic organisms. This study was conducted to fulfill ET's aquatic organism toxicity component by assessing the survival and reproduction of a representative, sensitive, aquatic invertebrate organism, *Daphnia magna*, after chronically exposing the organism to ET.

Materials and Methods

Erythromycin thiocyanate (CAS number, 7704-67-8; molecular weight, 793.02; potency = 791 μg/mg as is, 833 μg/mg anhydrous) was manufactured by Abbott Laboratories (Abbott Park, IL) and acquired from Bimeda, Inc. (La Seur, MN). Erythromycin (CAS number, 114-07-8;

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molecular weight, 733.94; purity, 98%) used as an analytical reference standard was acquired from Acros Organics (Fair Lawn, NJ). An erythromycin stock solution was prepared each day water samples were processed to determine ET concentrations. A stock solution was prepared by quantitatively transferring a known amount of erythromycin to a 250- or 500-mL volumetric flask with acetonitrile [high pressure liquid chromatography (LC) grade, Fisher Scientific, Pittsburg, PA]. The flask volume was adjusted to 250 or 500 mL with well water filtered through a type HA, 0.45 μm filter (Millipore Corp., Billerica, MA). Analytical standards were prepared by diluting the stock solution with well water. Solutions were used to generate LC-mass spectrometry (MS) calibration curves.

Identification and quantification of ET were performed using an Agilent 1100 LC system (Agilent Technologies, Inc., Santa Clara, CA) with a Waters XBridge™ C18, 3.5 μm, 2.1 × 150 mm analytical column (Waters Corporation, Milford, MA) and an Agilent model G1946D mass selective detector. The LC parameters included an isocratic mobile phase of 80% solvent A (74.9% water, 25% acetonitrile, and 0.1% acetic acid) and 20% solvent B (99.9% acetonitrile and 0.1% acetic acid), a flow rate of 0.3 mL/min, an injection volume of 100 μL, a column temperature of 30°C, and a run time of 8 min. Mass spectral detection was achieved using atmospheric pressure electrospray ionization in the positive mode. Mass spectrometer operating parameters included a capillary voltage of 3,250 V, a nebulizer pressure of 25 psig, a drying gas (nitrogen) flow rate of 10 mL/min, and a gas temperature of 350°C. The select ion monitoring parameters are presented in Table 1. The acceptable retention time difference between the quantitation and confirmation ions was ≤0.03 min. The ET concentrations were determined from ET's peak area and a quadratic regression equation developed from 5 analytical standard solutions with nominal ET concentrations ranging from 60 to 2,400 μg/L.

The *D. magna* mass culture was maintained in the following conditions: water temperature, 20°C; lighting, 16 h of incandescent light and 8 h of darkness with 20 min transition periods for lights to fade on and off. The exposure system was constructed with materials and operated with parameters described in the standard guide for conducting toxicity trials (ASTM 1997). The system was fully described in Meinertz et al. (2008). Test chemical working solutions were delivered to the stream of well water using MasterFlex® Digi-Staltic® model 77310-01 pump drives, MasterFlex® Easy-Load® II model 77202-60 heads, and a model 77310-02 controller (Cole-Parmer Instrument Co., Vernon Hills, IL). Working solutions were prepared every 3 or 4 days.

Table 1 Parameters for determining ET concentrations using the LC–MS system under select ion monitoring conditions (electrometer gain, 2 V; tolerance, 20%)

Product ion	Fragmentor voltage (V)	Ion assignments (m/z)	Confirmation/ quantitation ions ratio
ET quantitation	140	734.4[a]	8.9
ET confirmation	200	576.3	

[a] Mass + hydrogen

The experimental design included a randomized block design of 50 test chambers positioned in 10 blocks of 5 chambers per block and 5 treatment groups (target concentrations of 0, 200, 400, 800, and 1,600 μg/L) with 10 test chambers per treatment group. Each test chamber in a block was randomly assigned to 1 of 5 treatment groups so that each treatment group was represented only once in each block.

Flow of water and test chemical through the exposure system was initiated 3 days before stocking the test system with *D. magna*. On Day 0 (first day of exposure), one < 24-h old *D. magna* was randomly assigned to each test chamber. Chambers were inspected each day for live first generation *D. magna* and offspring production. Young were enumerated, if present, and discarded. A daily water sample (1 mL) was collected from each chamber before feeding and water from common treatment groups pooled in 20-mL glass vials. A portion (5 mL) of the pooled water sample was mixed with 5 mL of acetonitrile and filtered through a 0.2 μm GHP (polypropylene) membrane 13 mm Acrodisc® syringe filter (Pall Corporation, East Hills, NY) into an amber glass LC vial (Agilent Technologies Inc.) and stored on the laboratory bench top until analyzed to determine the erythromycin concentration.

D. magna food (100 μL) was dispensed into each test chamber, twice daily during weekdays, at an interval of more than 4 h, and once each weekend day. Daily dissolved oxygen concentrations ranged from 7.3 to 8.5 mg/L. Daily pH ranged from 7.3 to 7.7. Daily temperature ranged from 19.5 to 20.1°C. Flow rates through test chambers were about 2.6 mL/min producing about 19 volume exchanges per 24 h. Weekly measurements of alkalinity and water hardness ranged from 110 to 130 mg/L as $CaCO_3$ and 168 to 182 mg/L as $CaCO_3$, respectively. Weekly light intensity measurements directly over the chambers ranged from 80 to 260 lux during the 16 h periods of light.

First generation *D. magna* that survived through the 21-day trial were transferred to a 4% formalin–sucrose solution (Haney and Hall 1973). A wet mount of each preserved *D. magna* was used to determine length (mm) with a digital camera (model Coolpix 5000; Nikon Corporation, Japan) mounted on a microscope (model E600;

Nikon Corporation, Japan) and imaging software (Image Pro Express, version 4.5, Media Cybernetics, Silver Spring, MD). Length was measured from the top of the head to the base of the spine.

Concentration was considered to be a categorical variable rather than a continuous variable in data analyses because of the limited number of concentrations used. Statistical analyses were performed with SAS software (SAS 2004) and analyses considered to be significant if $p \leq 0.05$. A Kaplan–Meier test of homogeneity of survival time across concentration (Kaplan and Meier 1958) was used to assess differences in times to death. The 4 *D. magna* that died by Day 1 were assumed to have been damaged during transfer or were otherwise compromised individuals, therefore they were excluded from subsequent analyses. Pair-wise comparisons were conducted to identify differences in the survival function between ET levels using a log rank test with Šidák multiple-comparison adjustment. Comparisons were based on unadjusted *p*-values rather than adjusted *p*-values because this provided a more conservative estimate of the effect of concentration on survival (i.e., identification of the lowest concentration that significantly differed from the control).

The times to first brood could only be observed in *D. magna* that survived long enough to reproduce, therefore adult *D. magna* that died before having a brood were excluded from the analysis. A Kaplan–Meier test of homogeneity was used to analyze the effect of concentration on time to first brood.

The number of broods and total number of young are non-negative integer-valued counts where a Poisson distribution is assumed. A generalized linear model was developed to assess the effect of concentration on the number of broods and total number of young produced. The model parameters were estimated using maximum-likelihood methods and likelihood-ratio Chi-square tests were used to test hypotheses about the relation between the counts and concentration.

Body length data as a function of concentration were fit to a generalized linear model (McCullagh and Nelder 1989). Likelihood-ratio F-tests were used to test hypotheses about the relation between body length and concentration.

Results and Discussion

The selection of ET test concentrations for the chronic exposure study was based on the findings of Meinertz et al. (2010) where *D. magna* were chronically exposed to 2 pharmaceutical compounds, diphenhydramine hydrochloride and ET. The results indicated that *D. magna* survival, reproduction, and growth were not affected when *D. magna* were chronically exposed to ET concentrations <250 μg/L. However, survival, reproduction, and growth were affected when *D. magna* were chronically exposed to an ET concentration >250 μg/L. Therefore, the range of target exposure concentrations for the current study, 0, 200, 400, 800, and 1,600 μg/L were selected to include non-toxic and toxic concentrations.

Additional aquatic organism erythromycin toxicity data were primarily limited to acute studies. Isidori et al. (2005) reported exposing a variety of aquatic organisms to erythromycin. Effective concentrations (EC50; the concentration that evokes a response in 50% of the population) were based on different parameters for each organism. For *D. magna*, they reported a 24-h EC50 of 22.45 mg/L and for *Ceriodaphnia dubia*, a 48-h EC50 of 10.23 mg/L, each based on immobilization. They also report a 7-day EC50 for *C. dubia* of 0.22 mg/L based on population growth inhibition. Other aquatic organisms exposed included *Vibrio fischeri* (a luminescent bacterium; 24-h exposure, no effect at 100 mg/L based on light output), *Branchionus calyciflorus* (a rotifer; 24-h EC50 of 27.53 mg/L based on mortality and 48-h EC50 of 0.94 mg/L based on population growth inhibition), *Thamnocephalus platyurus* (a shrimp-like crustacean, 24-h EC50 of 17.68 mg/L based on mortality), *Danio rerio* (a tropical freshwater fish; 96-h exposure, no effect at 1,000 mg/L based on mortality), *Pseudokirchneriella subcapitata* (a green alga; 72-h EC50 of 0.02 mg/L based on growth inhibition). Other published erythromycin toxicity data included a 24- and 48-h EC50 of 388 mg/L and 211 mg/L, respectively, for *D. magna* based on mortality (Dojmi di Delupis et al. 1992).

In this study, exposure to ET had a significant negative impact on the time to death (degrees of freedom (DF) = 5; Log-Rank Chi-square = 12.3628, $p = 0.03$). The times to death in the 400 and 1,600-μg/L groups were significantly shorter than the time to death in the control group ($p = 0.03$ and $p < 0.01$, respectively). The time to death in the 1,600-μg/L group was significantly shorter than the times to death in the 200 and 800-μg/L groups ($p = 0.01$ and $p = 0.02$, respectively).

Before reaching a reproductive age, 1 *D. magna* died in the control, 3 in the 200–μg/L group, and 1 in the 400, 800, and 1,600-μg/L groups. After reaching a reproductive age, 1 death occurred in the control group, 0 in the 200-μg/L group, 5 in the 400-μg/L group, 3 in the 800-μg/L group, and 8 in the 1,600-μg/L group.

Production data from only those *D. magna* that reached a reproductive age (>9 day old) were used in assessments of production. For comparison purposes, in the cases where a *D. magna* reached a reproductive age and did not produce a brood, the time to first brood was denoted as the last day it was observed to have no brood, i.e. its day of death or day 21.

Table 2 Production data from *Daphnia magna* reaching a reproductive age (>9 day old) during exposure to ET for 21 days

Treatment group (μg/L)	Reproductive age adults	Mean and range of days to first brood	Mean broods per adult	Total broods	Mean young per adult	Total young
0.0	9	11 (10–20)[a]	4	32[a]	99	895[a]
200	7	11 (10–14)[a]	4	27[a]	100	703[a]
400	9	10 (10–11)[a]	2	21[b]	21	191[b]
800	9	11 (11–21)[a]	2	19[b]	15	131[b]
1,600	9	15 (13–21)[b]	1	5[c]	3	23[c]

Data denoted with a common letter were not significantly different ($p > 0.05$)

Table 3 Body length data from *Daphnia magna* exposed to ET for 21 days

Treatment group (μg/L)	Adults surviving for 21 days	Mean length (mm)	Range (mm)
0.0	8	4.02[a]	3.66–4.22
200	7	3.89[a]	3.50–4.25
400	4	3.17[b]	2.90–3.38
800	6	3.79[a]	3.15–4.17
1,600	1	4.04[a]	–

Data denoted with a common letter were not significantly different ($p > 0.05$)

Table 4 Erythromycin thiocyanate concentrations in pooled water samples from all test chambers within a treatment group

	Treatment group (target concentration)			
	200 (μg/L)	400 (μg/L)	800 (μg/L)	1,600 (μg/L)
Mean	179	414	789	1,594
sd	9.4	20	37	120
% rsd	5.2	4.9	4.6	7.7

Exposure to ET had a significant negative impact on the time to first brood (DF = 5; Log-Rank Chi-square = 17.5523, $p < 0.01$). The times to first brood in the control, 200, 400, and 800-μg/L groups were significantly less than the time to first brood in the 1,600-μg/L group ($p < 0.02$; Table 2).

Exposure to ET had a significant negative impact on the total number of broods (DF = 5, Log-Rank Chi-Square = 58.58, $p < 0.01$). The total numbers of broods produced in the control and 200-μg/L group were significantly greater than the total numbers of broods produced in the 400, 800, and 1,600-μg/L groups ($p < 0.05$). Likewise the total numbers of broods produced in the 400 and 800-μg/L groups were significantly greater than the total number of broods produced in the 1,600-μg/L group ($p < 0.04$).

Exposure to ET had a significant negative impact on the total number of young produced (DF = 5, Log-Rank Chi-Square = 242.01, $p < 0.01$). The total numbers of young produced in the control and 200-μg/L groups were significantly greater than the total numbers of young produced in the 400, 800, and 1,600-μg/L groups (all $p < 0.01$). The total numbers of young produced in the 400 and 800-μg/L groups were significantly greater than the total number of young produced in the 1,600-μg/L groups (all $p < 0.01$).

Although exposure to ET statistically impacted growth (DF = 5, F value = 10.39, $p < 0.001$), the effect was not dose-dependent. Lengths in the control and 200-μg/L groups were not significantly different from lengths in the 800 and 1,600-μg/L groups (Table 3).

Mean ET concentrations in pooled water from test chambers of each treatment group were within 11% of the target concentrations (Table 4). ET was not present in water samples from the control group.

In summary, relative to the control treatment group, *D. magna* survival and production, specifically the number of broods and total number of young produced, were significantly impacted during 21 days of continuous exposure to ET concentrations >179 μg/L.

Acknowledgments We thank Mark Gaikowski of the USGS Upper Midwest Environmental Sciences Center for statistical support.

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