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Final Report:
Pharmacokinetics and efficacy of oxytetracycline in RLP-infected abalone
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Abstract

Withering syndrome (WS) is a catastrophic disease of both wild and cultured abalone caused by "*Candidatus* Xenohaliotis californiensis", a Rickettsia-like procaryote (WS-RLP). Losses from this disease have severely hampered the abalone aquaculture industry and abalone restoration efforts in California. Oxytetracycline (OTC) was recently demonstrated to be an effective therapeutant for WS-RLP infections and associated losses due to WS. In addition, a long duration of protection (approximately one year) was noted anecdotally. Part one of this study examined the efficacy and pharmacokinetics of OTC treatment in red abalone, *Haliotis rufescens*, held at 13.4°C. Medication was administered orally in an artificial diet containing 1.85% active OTC at a rate of 103.4 mg/kg abalone for 10, 20 and 30 days. Control abalone were fed the same diet lacking OTC. Significant differences in prevalence and intensity of infection in both the post-esophagus and digestive gland were observed. Although the digestive gland from abalone medicated for 10 days and foot muscle from those medicated for 30 days was best described with a two-compartment model, pharmacokinetic trends were adequately described by a one-compartment model. Significant differences in OTC accumulation and depletion were observed among treatments. Animals medicated for 10 days accumulated significantly less than those medicated for 20 or 30 days. Despite this, terminal elimination rate constants were similar. Significantly less OTC was present in foot muscle samples relative to corresponding digestive gland samples. Estimated half-life values were similar among treatments for both foot muscle (10.7-13.4 d) and digestive gland (23.8-27.5 d) samples.

The second part of this study examined OTC pharmacokinetics and efficacy at a higher temperature. Therapeutic treatment of red abalone infected with WS-RLP was carried out at 17.3°C over 10, 20 and 30 days. After 3, 17, 23, 42, 63, 81, 102, 122, and 160 days, OTC-treated and untreated abalone were assessed for WS-RLP burden, WS-associated pathological changes and drug residue concentrations within their foot muscle and digestive gland. The highest concentrations of OTC were detected on the first sample day; digestive gland concentrations were higher than respective muscle concentrations at all time points. While drug depuration occurred rapidly from foot muscle, high concentrations and long depuration times were measured within the digestive gland, as observed in the first experiment. Digestive gland depuration conformed to a two-compartment model, with terminal phase elimination half lives of approximately 22 days with no significant differences between the 10, 20 and 30 day treatment durations. All three durations of drug treatment led to significant reductions in WS-RLP prevalence, decreased foot muscle atrophy, and fewer mortalities. Cohabitation trials with WS-RLP-infected abalone that were initiated 44, 88, and 122 days following the 10-day treatment showed reduced susceptibility to re-infection for up to 88 days after treatment, suggesting that digestive gland OTC residues confer long term resistance to pathogen. Atomic absorption spectroscopy conducted on both foot muscle and digestive gland found significantly higher concentrations of iron, zinc, and manganese in the digestive gland offering an explanation behind the underlying mechanism of OTC retention for extended periods within this tissue. The results from these studies will facilitate more effective use of OTC by providing insight as to what doses are effective and how often abalone need to be dosed to maintain WS-RLP resistance, in light of substantial OTC retention.

1. Introduction

Withering syndrome (WS) is a catastrophic disease of both wild and cultured abalone caused by "*Candidatus* Xenohaliotis californiensis", a Rickettsia-like procaryote (WS-RLP) (Haaker et al., 1992; Friedman et al., 2000; Moore et al., 2000, Moore et al. 2002). This intracellular pathogen infects cells of the digestive gland and postesophagus and is presumably directly transmitted through fecal-oral spread. Recent seasonal losses of cultured red abalone, *Haliotis rufescens*, in California combined with a newly established captive rearing program for the endangered white abalone, *H. sorenseni*, within the WS endemic zone have heightened concerns over the impacts of this disease. Oxytetracycline (OTC) is a broad-spectrum antibiotic with activity against a wide range of gram-positive and gram-negative bacteria, atypical procaryotes such as rickettsiae, chlamydiae and mycoplasmas, and some protistan parasites (See review by Chorpa and Roberts, 2001). This therapeutant is one of the few antimicrobial therapeutants approved for both therapeutic and sub-therapeutic (growth enhancer) use in terrestrial agriculture and for therapeutic use in selected aquaculture species in the U.S. and several other countries (Schnick, 1998; Chorpa and Roberts, 2001). Oral treatments of infected abalone with OTC have been shown to be effective in reducing WS-RLP infection prevalence, intensity, and mortality (Friedman et al., 2003). In the Friedman study, researchers found drug residues within foot muscle depleted to below the Federal tolerance level of 2 ppm between 15-22 days post medication. Interestingly, the efficacy of oxytetracycline appeared to increase with time; 5 days after the 14 day medication period over 50% of the abalone were infected, while less than 1% of the abalone were infected nearly one year later (Friedman et al., 2003). In addition, researchers have noted that the abalone digestive gland accumulated OTC at a much higher rate than the foot muscle (Friedman et al., 2003; Braid et al., 2005). It is possible that this accumulation may provide unprecedented long-term protection against rickettsial infection. These studies clearly indicate that further assessment of the efficacy and pharmacokinetics of this drug in both the foot muscle and digestive gland of abalone are needed.

In part one of this study we examined the efficacy and pharmacokinetics of three time-doses of oxytetracycline using a *per os* administration conducted at 13.4^{o}C, reflective of temperatures experienced in northern, central, and, seasonally, in southern California. The second portion of this study evaluated oxytetracycline pharmacokinetics and efficacy at 17.3^{o}C. Both field and laboratory studies with red abalone have shown a strong correlation between elevated Southern California seawater temperature and increased incidence and severity of WS, suggesting at least in the short term that WS-RLP infected red abalone may require a thermal stress to develop WS (Moore *et al.*, 2000; Braid *et al.,* 2005; Vilchis *et al.,* 2005). We hypothesized that given the poikilothermic nature of abalone, animals held at the higher water temperature would consume and accumulate more OTC than those held at the lower temperature. In addition, the OTC terminal depletion rate would be similarly enhanced in those held at higher water temperatures. To evaluate the relationship between OTC retention time in digestive gland and susceptibility to WS-RLP re-infection, we also re-exposed abalone to the pathogen at three time periods following OTC treatment. Finally, since chelation of ferric or magnesium deposits may influence OTC retention, we assessed, via atomic absorption/emission spectrometry, differential concentrations of Cu, Zn, Fe, and Mg in digestive gland and foot muscle.

2. Methods

2.1 Abalone and Oxytetracycline Administration- Experiment 1

On March 20, 2002 approximately 500 red abalone measuring 47.9 ± 1.1 mm in maximum shell dimension were donated by The Abalone Farm, Inc. (Cayucos, CA) and shipped overnight to the Pathogen Containment Facility at the UC Davis-Bodega Marine Laboratory where they were placed into triplicate 122 L bins receiving a constant supply of aerated, flow-through, ambient (~13°C) seawater. All effluent from this facility is disinfected with 10-15 ppm sodium hypochlorite for 2 hr and de-chlorinated prior to release. The abalone were exposed to the WS-RLP by cohabitation with WS-RLP-infected red abalone, a proven method of transmission (Moore et al. 2001, Friedman et al. 2002), for eight weeks and were subsequently transferred to six 11 L tanks (n=75 per tank), comprising triplicate control and triplicate experimental (OTC medicated) tanks. Cohabitation continued within each tank while the seawater temperature was slowly increased to 19°C and then held constant for three months to promote WS-RLP shedding. Animals were periodically sampled to ensure successful transmission of the WS-RLP and achievement of mean WS-RLP burdens of at least 1 on a scale of 0-3 as described below. Once infection intensities were verified through both histology and PCR, water temperatures were lowered to ambient conditions and held for 16 weeks prior to the initiation of the pharmacokinetic and efficacy study on April 16, 2003. During this same time period animals were acclimated to artificial unmedicated feed produced by The Abalone Farm, Inc. The experimental animals were held at 13.4±1.2°C throughout the remainder of the study.

Beginning on April 16, 2003 animals (n=81) in the three 11 L OTC treatment tanks were fed a proprietary medicated diet that contained 1.85% active OTC at a daily dose of 103.4 mg/kg of abalone, while the other three tanks were maintained on control feed (identical but lacking OTC). After 10 days one third (n=27) of the animals from each of the six 11 L tanks were moved to six new tanks (6 x 4 L) and fed kelp, *Macrocystis pyrifera*, 2-3 times per week. This transfer of animals represented the 10-day medication and control treatments. The remaining animals were fed either the OTC or control diet for an additional ten days at which point, one half (n=27) of the abalone were removed into 6 x 4 L tanks and maintained on kelp (20 day medication and control treatments). The remaining animals stayed in the original "treatment" tanks for an additional 10 days of OTC treatment (30 day medication and control treatments). After the 30 day period, the latter abalone were also were transferred into 6 x 4 L tanks. Animals were maintained in the 4 L containers in all 18 tanks (3 replicates x 2 treatments x 3 medication duration levels). The day that animals were transferred out of the treatment tanks was designated as day 0. At selected time points (day 3, 17, 22, 42, 63, 81, 102, 122, and 161) three abalone per container were randomly selected and sacrificed; foot muscle and digestive gland samples were dissected and flash frozen in liquid nitrogen and stored at –80 ºC until analyzed. Selected tissues (foot, posterior esophagus (PE) and digestive gland (DG)) were excised and processed for histology (see below).

2.2 Abalone and Oxytetracycline Administration- Experiment 2

Red abalone (850; approx. 2.5 cm in length) were received on 10/23/03 from The Abalone Farm (Cayucos, CA) and placed into 122L bins at the Bodega Marine Laboratory Pathogen Containment Facility. Animals received aerated, flow-through,

ambient (approx. 13º C) seawater. After an initial two week acclimation period, the water temperature was slowly increased from 13°C to 17.3°C over a six-week period during which time approximately 70 visibly withered abalone were added as a source of WS-RLP. Acquisition of infections by the recipients was evaluated through periodic random selection of 10 animals and the presence of WS-RLP was determined using both histology and PCR (Friedman *et al.* 1997, Andree *et al*. 2000). Cohabitation continued for 18 weeks, at which point 80% infection prevalence was detected by both PCR and histology. WS-RLP donors were removed and the remaining abalone were equally divided among six 11L ("treatment") tanks providing three replicate containers for both OTC-treated and untreated animals. Animals were acclimated to the new tanks and weaned onto the artificial feed for two weeks prior to study initiation. During the treatment phase, animals in three of the tanks were maintained on medicated artificial feed, while the other three tanks were maintained on control feed. Ten, 20 and 30 day treatments were conducted exactly as for Experiment 1. At selected time points similar to those for Experiment 1 (days 3, 17, 23, 42, 61, 81, 102, 122, and 160) three abalone from each container were randomly selected and sacrificed. Wet weights and shell weights and lengths were recorded and digestive gland and foot muscle were excised and stored at -80°C for OTC residue analysis. Additional digestive gland and foot muscle samples were collected from each individual for histology. On sample days 3, 81, and 102 an additional piece of digestive gland was excised from each sampled abalone and stored in 95% ethanol at -20°C for PCR analysis (below).

2.3 Resistance to Re-infection (Experiment 2)

To understand the relationship between digestive gland OTC concentrations and conferred resistance to the pathogen, OTC-treated animals in the second experiment were re-challenged with the pathogen at three time points following drug treatment. At 42, 81, and 122 days after the 10-day dosing, four animals were removed from each of the three replicate OTC-treatment tanks and transferred into three 4 L cohabitation tanks receiving effluent from a header tank that contained 40 red abalone with clinical WS (Braid *et al.,* 2005). Three additional cohabitation tanks each contained four control abalone that had never been exposed to the WS-RLP or OTC. These uninfected and previously untreated animals served as positive indicators that the cohabitation system would result in infections among recipients. PCR and histological examination demonstrated that the control animals were WS-RLP free prior to each cohabitation trial. Cohabitation tanks received contaminated effluent for 30 days, at which point they were disconnected and received a constant supply of aerated, flow-through, sand-filtered seawater (approx. 17.3 º C) for an additional 30 days. They were then sacrificed and the presence of WS-RLP was evaluated using histology and PCR (below). Each of the six cohabitation tanks was scored by the percent of WS-RLP positive animals detected by each method. Two-way ANOVAs evaluating the prevalence of infection in control and treated abalone at 42, 81, and 122 days post 10-day OTC treatment were conducted on PCR and histology results separately.

2.4 Histology

Selected tissues were placed in Invertebrate Davidson's solution (Shaw and Battle, 1957) (Experiment 1) or 3.5% paraformaldehyde in phosphate-buffered saline

(Experiment 2) for 24 h and processed for routine paraffin histology. Deparaffinized 5µm sections were stained with hematoxylin and eosin (Luna 1968) and viewed by light microscopy. The intensity of WS-RLP infection in the postesophagus and digestive gland was quantified at 200x using the logarithmic scale of Friedman et al. (1997): (0): no bacterial foci, (1): up to10 foci per field of view, (2): 11-100 foci per field of view, and (3): >100 foci per field of view. Condition of the digestive gland and foot muscle were assessed using a modification of the condition scales of Friedman et al. (2003) in which (0) represented normal, (1) represented <10% alteration from normal**,** (2) represented moderate (up to 25 %) alteration from normal, and tissue that was severely (> 25%) altered was scored as (3). Morphological alterations in digestive gland architecture involved two specific tissue changes including degeneration (characterized by an increase in connective tissue between digestive tubules, the primary tissues responsible for secretion of digestive enzymes and nutrient absorption in abalone (Voltzow, 1994), and transport duct metaplasia.

2.5. Polymerase Chain Reaction detection of WS-RLP (Experiment 2)

The presence of the WS-RLP in tissue or fecal samples was assessed during the second experiment using a modification of the PCR test of Andree *et al.* (2000). Briefly, DNA was purified from either abalone digestive glands or feces collected from a tank using DNeasy spin columns (Qiagen Inc.). For every eight DNA extractions conducted on unknown abalone samples, DNA from known WS-RLP infected and uninfected tissues were extracted, along with one blank (no-tissue), as positive and negative controls, respectively. A 160 bp segment of the 16s rDNA gene was amplified from WS-RLP infected tissues or feces using the primers of Andree et al. (2000). All amplifications were performed in standard 50 µL reactions containing 100ng of template DNA, 2U Sigma JumpStarTAq, 1x JumpStarTAq buffer, 3mM MgCl2, 400ng/ul BSA, 200uM dNTPs, and 0.5uM of each primer. The rickettsial DNA was amplified with an initial denaturation step of 95ºC for 3 minutes; followed by 40 cycles of 95ºC for 1min, 62ºC for 30 seconds, 72ºC for 30 seconds; and a final extension of 72ºC for 10min using an Eppendorf Master Cycler Gradient thermal cycler. After amplification, DNA was separated and visualized on 1.5% agarose gels impregnated with ethidium bromide.

2.6 Analysis of oxytetracycline residues in abalone tissues

OTC levels in the tissue samples were quantified using a modified FDA-approved method for measuring drug residues (AOAC, 1990) as modified from Friedman et al. (2003) and outlined here. Frozen foot muscle and digestive gland samples were allowed to slightly thaw on ice, diced with a razor blade, and briefly macerated. A 0.25 g piece of macerated foot muscle or digestive gland was weighed and homogenized in 4 ml of 0.1 M phosphate (K_2HPO_4) buffer (pH 4.5) in a 7 mL glass homogenizer on ice. The homogenate was centrifuged at 800 x *g* for 10-12 minutes at 4-8°C and the resultant supernatant was collected and stored at -80°C until assayed. Previous studies revealed that short duration frozen storage did not affect OTC concentrations (Friedman et al. 2003). If the sample extracts were expected to be out of the range of the standard curve because of previous results, further dilutions were made with phosphate buffer.

A stock solution of 0.5 mg/mL oxytetracycline dihydrate (Sigma #O-5750) in 0.1 N HCl was used to prepare a working dilution of 5ppm. Standards were prepared daily in

supernatant from homogenates of control tissue type (no OTC exposure) at the following concentrations for the first experiment: 0.8, 0.6, 0.3, 0.2, 0.1, and 0 ppm, and for the second experiment: 1.0, 0.9, 0.7, 0.5, 0.3, 0.1 and 0 ppm.

Bacillus cereus (ATCC 11778) was cultured overnight in Tripticase Soy Broth (TSB, Difco; on a shaker set at 120 rpm) at room temperature (~21°C). Slightly different methods were then followed for the two experiments. For the first experiment, a 100 mL aliquot of molten (48 ^{o}C) Mueller Hinton II agar (BBL, #11438) was inoculated with 0.5 mL of the *B. cereus* suspension. Aliquots (11 mL) of the inoculated agar were added to each petri plate and allowed to solidify. Four 8 mm x 10 mm stainless steel penicylinders (Fisherbrand) were added on top of the agar, equidistant from one another. For each standard or unknown (abalone tissue homogenate), triplicate 200 µl aliquots were added to each of three cylinders per plate; a 0.2 ppm standard was added to the fourth cylinder to control for interplate variation. For the second experiment, a volume of 4.5 mL of the *B. cereus* suspension was used to inoculate a 450 mL aliquot of molten (48°C) Mueller Hinton II agar (BBL, #11438). A 3.5mL aliquot of the inoculated agar was added to each partition of a tri-sectioned petri plate and allowed to solidify. A 250 µL aliquot of either standard or unknown (tissue homogenate) was added to penicylinders in triplicate. For both experiments, the plates were then incubated at room temperature (~21°C) for 20 hours after which the diameter of the zone of inhibition was measured to the nearest 0.1 mm using vernier calipers. Unknown concentrations were calculated by comparison to a standard curve. Zones on each plate were corrected by the proportion of the internal 0.2 ppm standard and the grand mean of 0.2 ppm controls across all plates. The mean of three zones for each sample was used to calculate the concentration of OTC residues in the abalone tissue via the standard curve.

2.7 Pharmacokinetic data analyses

Excluding sample points below the limit of detection (0.1 ppm) from foot muscle and DG tissue residue, raw data were analyzed as a function of time with one- and two-compartment models using the naïve-pooled data approach (Ette and Williams, 2004) and WinNonlin® pharmacokinetic data fitting software (Pharsight Corp., Mountain View, CA, USA). The equations for the one- and two-compartment models used are :

1) One-compartment model: $C_{(t)} = C_{zero}e^{-\beta t}$
2) Two-compartment model: $C_{(t)} = Ae^{-\alpha t} + Be^{-\beta t}$

where C is the OTC concentration in the tissue at time (t), C_{zero} is the intercept of the one-compartment model elimination rate constant, A is the t_0 intercept of the distribution phase, α is the distribution rate constant, B is the t_0 intercept of the elimination phase and β is the terminal elimination rate constant. Selection of the best fit model was based on the Akiake information criteria (AIC; Akaike 1974), and visual inspection of both the residual plots and curve fit to the data. The elimination half-life ($t_{1/2}$) of OTC from the foot muscle and digestive gland was calculated as $t_{1/2}$=0.693/β.

2.8. Metals Analysis (Experiment 2)

Metals analyses for both treated and untreated foot and digestive gland tissues were conducted on samples taken 42 and 81 days post 20-day OTC treatment durations.

A 0.5 g dry weight sample of tissue was digested with 1 mL conc. HNO_3 and 2 mL 30% hydrogen peroxide. The concentrations of Zn, Mn, Fe and Cu were determined using microwave digestion and atomic absorption spectrometry (AAS). Metal concentrations were determined via seven-point standard calibration curves with detection limits of 1 ppm. Results here are generally reproducible within 8% for all analytes (University of California, Davis, Division of Agriculture and Natural Resources analytical lab SOP 590).

2.9 Statistical analyses

ANOVAs followed by Tukey-Kramer post hoc analysis were used in the first experiment to examine differences in prevalence and infection intensity among replicates of each treatment (SigmaStat 2.03, Systat Software, Inc., Point Richmond, CA). If no significant differences were observed the data from this balanced design, they were pooled for further analysis. Drug residue values from treated foot and DG were log transformed to achieve normality and having met the assumptions of normal distribution and homogeneity of residuals, ANCOVAs and Tukey's pairwise comparison were used to compare the terminal (linear) portion of the regression lines from different treatments in both digestive gland and pedal tissues using withdrawal day and treatment as factors (SAS version 9.1). The Spearman Rank Order Correlation was used to examine if a linear relationship existed between OTC levels in the foot and digestive gland and the withdrawal time (SigmaStat 2.03).

In the second experiment, ANOVAs were conducted using Number Cruncher Statistical Systems (2001 Edition; NCSS Statistical Software, Kaysville, UT). Data sets found to have non-normal distributions were re-evaluated after square root or log transformation. If normality was achieved through transformation further analyses were conducted using one and two-way ANOVAs followed by Tukey-Kramer post hoc analysis. If data continued to display non-normal distributions after transformation, analyses were conducted using Kruskal-Wallis one-way ANOVAs tests. Drug residue values were analyzed as for Experiment 1.

3. Results

3.1 Mortalities and OTC efficacy (Experiment 1)

Only three medicated and six control abalone died during the 161day study. Losses were observed in two of the medicated and three of the control tanks. Significant differences in rickettsial prevalence ($p<0.0001$, Table 1) and intensity of infection (Table 1) were observed between medicated and control animals in both the post-esophagus ($p<0.0001$) and DG ($p<0.05$). While control (non-medicated) abalone maintained high prevalences of WS-RLP infection throughout the experiment, medicated abalone were nearly free of rickettsial infections (Table 1). There were two exceptions: Two abalone in the 10 day treatment (one abalone with one WS-RLP inclusion 3 days post medication and one with a light infection, scale = 1, at 161 days post medication; Table 1, Figure 1). Thus, overall 99.2 % of the medicated abalone were free of the WS-RLP relative to only 21% of the non-medicated control animals. On a per treatment basis the relative percent clearance (1 – [No. Infected/(Total No. Abalone – No. uninfected] x 100) was 97% for the 10 day treatment and 100% for both the 20 and 30 day treatments.

No differences in prevalence or intensity of rickettsial infections were observed among replicate control tanks (p>0.05) and among treatments tanks (p>0.05). When rickettsial prevalence and intensity was examined over the entire experiment duration, no differences were observed between the 20 and 30 day control treatments (p>0.05). However, within the 10 day control treatment, animals examined at withdrawal day 122 had significantly lower rickettsial prevalences than all other sample points (22% versus 56-100%, p<0.0001). Similarly, the postesphagus of abalone examined at 122 days had lower infection intensities (p<0.05). Differences in abalone tissue condition were non-significant among all treatments (p>0.05) except for the foot muscle of animals in the 10 day treatment (p<0.05); mean foot muscle atrophy ratings of 0.02 and 0.43 were observed in medicated and control animals, respectively.

3.2 Drug depletion kinetics (Experiment 1)

Our limit of detection was 0.1 ppm (data not shown). Individual animal data points and the predicted concentrations using a one-compartment model for foot muscle and digestive gland after 10, 20 or 30 days of per os medication with 103.4 mg/kg OTC are illustrated in Figures 2 and 3. Three days after completion of each treatment (duration of medication), mean digestive gland OTC residues of 1734 ± 287 ppm (10 day), 2171 ± 257 ppm (20 day) and 2622 ± 708 ppm (30 day) were observed in the DG of medicated animals, while foot muscles accumulated only 8.54 ± 0.34 ppm (10 day), 10.9 ± 0.52 ppm (20 day) and 9.73 ± 0.71 ppm (30 day) of OTC. Significantly less OTC accumulated in foot muscle relative to digestive gland tissue in all experimental treatments (p<0.001). ANCOVA using the terminal (linear) portion of the depletion curves for all three treatments revealed that medication duration had a significant influence on DG (p<0.0001) but not on pedal (p>0.05) OTC accumulation. Abalone medicated for 10 days accumulated significantly less drug in the digestive gland than those medicated for 20 or 30 days (p<0.001); no differences in OTC levels were observed between those medicated for 20 or 30 days (p>0.05). No differences in the slope of the depletion curves were observed among the three treatments for both digestive gland and foot muscle (p>0.05).

While the elimination curves for the DG in the 10 day medication and foot muscle in the 30 day treatment are best described with a two-compartment model, data from remaining samples were well fit with a one-compartment model (Figures 2, 3) and thus it was chosen for all the treatments. The rate constants of elimination, the corresponding half-life of elimination and the predicted concentration at time=0 (C_{zero}) are shown in Table 2 for both foot muscle and digestive gland. The DG terminal phase half-life for the 10 day treatment calculated using a two-compartment model is shown in parentheses under the one-compartment parameter. Despite differences in medication exposure, both C_{zero} and the elimination half-life for OTC were similar for the 10, 20 and 30 day treatments (Table 2). The DG of animals medicated for 10 days fell below the FDA tolerance level for OTC of 2 ppm after 161 days of withdrawal, while those medicated for 20 or 30 days still retained a mean of 4.53 and 6.98 ppm of OTC, respectively. OTC residues in the foot muscle of all treatment groups fell below 2 ppm by withdrawal day 42 with no detectable OTC in the 10 day treatment after 42 days and in the 20 and 30 day treatments after 63 days (Figure 2). Spearman rank order correlations revealed a significant linear relationship between foot muscle and DG OTC levels and withdrawal

time ($P<0.0001$). Correlation coefficients were high and direct for DG and foot muscle ($C=0.800$ to 0.847), while high, inverse correlation coefficients were observed for withdrawal time and OTC levels in both tissues examined ($C=-0.847$ to -0.975).

3.3 Morphologic condition of OTC treated abalone (Experiment 2)

No significant differences in wet weight or shell length and weight were detected between 10-, 20-, and 30-day OTC-treated animals; the same was observed for the three untreated groups (one-way ANOVAs; $p<0.05$). Without significant differences, the three treated groups were pooled, and the untreated groups were separately pooled to focus morphologic analysis on differences between treated and untreated animals. Whole animal wet weights increased significantly over time in both treated and untreated groups ($p<0.05$; one-way ANOVAs), however treated animals had higher mean wet weight values than in untreated abalone ($p\leq0.0001$; Kruskal-Wallis one-way ANOVAs). Tukey-Kramer post hoc analysis revealed that this increase over time in treated and untreated wet weights was observed in animals sampled only after 160 days (Figure 4). While treated animals had significantly longer mean shell lengths, no increases over the time course were observed in either treated or untreated animals ($p\leq0.01$; two-way ANOVAs). In addition no significant differences due to OTC treatment or post-treatment sampling time were detected in shell weight. OTC treatment led to significantly lower foot muscle atrophy ratings ($p\leq0.000$; Kruskal-Wallis one-way ANOVAs)

3.4 Efficacy of OTC treatment (Experiment 2)

No mortalities were detected in any of the tanks treated with OTC while 5.0% (17/339) of the animals in the untreated tanks died (Figure 5). The first mortalities were observed in the 10-day non-medicated tanks 240 days after initial exposure to WS-RLP, while the 30-day non-medicated tanks suffered from the highest overall number of mortalities. All dead animals were severely shrunken and appeared to have died from WS. While histological examination of some individuals was not possible due to tissue necrosis, 91% of those with samples available had advanced WS-RLP infections.

Histological observations over the entire time course showed a 99.2% clearance of the WS-RLP in animals treated with OTC (2 infected out of 243 animals). One of the two WS-RLP positive animals found within the OTC treated tanks was detected on day 102 post-treatment in a 20-day tank and one was found on day 160 in a 10-day tank. In both cases very low levels of WS-RLP were present. In untreated animals 86% (213 out of 243 animals) were infected with the WS-RLP (Figure 6). In untreated animals, WS-RLP infection intensity increased significantly over the time course (one-way ANOVA; $p<0.0001$) with mean WS-RLP ratings increasing from 0.74 three days post-treatment to 1.4 at the last time point.

PCR analysis showed 100% (81 of 81) of the untreated animals tested positive for WS-RLP, while only 7.4% (6 out of 81) of those treated were PCR-positive. Four of these six PCR-positives were found 3 days post-treatment in the 10-day OTC-treated tanks, one was detected 3 days post-treatment in the 20-day OTC-treated tanks; and one was found 122 days post-treatment in the 20-day OTC-treated tanks.

3.5 OTC accumulation and depletion kinetics (Experiment 2)

During the treatment phase abalone did not readily consume either the medicated or non-medicated food; however, measurable concentrations of OTC were found in the tissues of all animals treated for 10, 20 and 30 days with medicated feed, while sham treated abalone lacked evidence of OTC in either foot muscle or digestive gland. Within treated animals, significantly higher concentrations of OTC were measured in the digestive gland relative to the foot muscle ($p \leq 0.0001$; Kruskal-Wallis one-way ANOVAs). Within digestive glands maxima of 831 $\pm$ 104 ppm (10 day), 928 $\pm$ 165 ppm (20 day) and 1216 $\pm$ 223 ppm (30 day) of OTC were detected, while corresponding foot muscle concentrations were 14.2 $\pm$ 7.1 ppm (10 day), 11.7 $\pm$ 2.6 ppm (20 day) and 6.8 $\pm$ 4.0 ppm (30 day) of OTC (Table 3). In both tissues the highest concentrations of drug were detected 3 days post-treatment. Digestive gland OTC concentrations 3 days post-treatment were significantly higher in animals exposed for 30 days than in the 10-or 20-day treated animals (one-way ANOVAs; $p < 0.001$). This same test for foot muscle at three days post OTC treatment, found 30 day treatment animals had significantly lower OTC concentrations than those observed in 10 day exposures (one-way ANOVAs; $p < 0.01$). Foot muscle OTC levels in the 20 day treated animals measured 3 days post-treatment was not significantly different than levels measured 3 days post-treatment in either the 10-or 30-day treated animals ($p > 0.05$).

Foot muscle OTC concentrations were plotted against time in Figure 7. However, due to the rapid elimination of OTC from foot muscle and the lack of sampling during the first 17 days, we were unable to calculate elimination rate constants (β) and half-lives ($t_{1/2}$) for this tissue. For all three treatment durations the number of animals with detectable concentrations of foot muscle OTC dropped rapidly between the first and second time points. Conservative withdrawal estimates based on the mean values obtained from only animals with detectable foot muscle OTC showed concentrations first dropping below 2 ppm on sample days 17, 23, and 17 for the 10- 20- and 30-day treatment durations, respectively.

In this experiment OTC depuration from the digestive gland best fitted a two compartment model (Figure 8). The pharmacokinetic parameters calculated from the three depuration models are shown in Table 4. The elimination half life ($t_{½\beta}$) of the terminal part of the elimination phase was estimated to be 22.7, 22.4, and 22.4 days for the 10-, 20-, and 30-day treatments, respectively. Intercepts of the extrapolated terminal (linear) portion of the curve to the Y-axis for the three OTC treatments were significantly different (ANCOVA, F=35.52, $p < 0.001$, df=2), confirming that the three treatment durations led to significantly different mean accumulated digestive gland OTC concentrations. Tukey-Kramer post hoc tests indicate that longer OTC treatment periods resulted in significantly higher drug concentrations in the digestive gland. However the interaction between post-treatment sample time and durations of treatment were not significant, indicating that the slopes of the elimination phases from the three OTC treatments did not significantly differ.

3.6 Susceptibility to re-infection after 10-day OTC treatment (Experiment 2)

Within the experiment designed to investigate the long term resistance conferred by OTC treatments, PCR analysis found 100% of the control animals (animals never previously exposed to pathogen or OTC) to be WS-RLP positive in the first and third

trials (42 and 122 days post 10-day OTC treatment respectively) and 83.3% were positive after the second (88 days post 10-day OTC treatment) cohabitation experiment (Figure 9). Histological examination indicated the presence of WS-RLP inclusions in 58%, 25%, and 50% of the control abalone used in the first, second and third cohabitation trials, respectively. Thus, both methods show lower transfers of WS-RLP occurring during the second cohabitation trial. PCR and histological measurements were made after 30 days of exposure to effluent containing pathogen and an additional 30 days of grow-out.

Histology and PCR conducted on 10-day OTC-treated animals taken from the drug depuration study at 42, 81, and 122 days post-treatment and before WS-RLP re-exposure, showed no evidence of WS-RLP infection. PCR conducted on 10-day OTC-treated animals after re-exposure to pathogen indicated the presence of WS-RLP DNA in 50% of the animals re-exposed 42 days after treatment and in 16.7%, and 72% on 81, and 122 days post-treatment, respectively. Histological examination of these same animals detected WS-RLP inclusions after cohabitation in 16.7%, 8.3%, and 50% of the animals 42, 81, and 122 days post 10-day OTC-treatment, respectively.

PCR analyses suggested that animals never exposed to OTC were significantly more susceptible to infection by cohabitation than those previously medicated with OTC (two-way ANOVAs; $p \leq 0.05$). Tukey-Kramer post hoc tests indicated that this difference exists at both the 42 and 81 days cohabitation initiation time points, while similar rates of infection occurred in both control and OTC-treated animals exposed 122 days after treatment. Histological examination, while showing the same trend as that observed with PCR (decreased presence of WS-RLP in abalone previously treated with OTC during the first two cohabitation trials), did not indicate significant differences between control and OTC-treated animals (ANOVAs; $p > 0.05$).

Use of the predicted elimination model for 10-day OTC-treatment indicates that digestive gland OTC concentrations over the 30 day exposure to WS-RLP effluent were in the range of 59-23, 14.9-5.8, and 5.15-1.8 ppm for the 42, 81, and 122 days post-treatment cohabitation trials, respectively, while OTC residues measured directly from 10-day treated tanks found OTC concentrations of 60 ± 39, 9 ± 4.5, and 3.4 ±3.9 ppm at sample times of 42, 81, and 122 days post-treatment. Predicted OTC concentrations during the subsequent 30-day grow-out period ranged from 23-9, 5.8-2.3, and 1.8-0.79 ppm for each of the three cohabitation trials, respectively.

3.7 Metal concentrations in the foot and digestive gland (Experiment 2)

The mean concentrations of Zn, Mn, and Fe were significantly lower in foot muscle relative to the digestive gland (one-way ANOVA; $p \leq 0.001$,), while those of Cu were similar (Table 5). Two-way ANOVAs, after log transformation of the data, were used to evaluate the factors of OTC treatment and post-treatment sample time (42 and 81 days post 20-day treatment) on both foot muscle and digestive gland metal concentrations. Within foot muscle significant increases occurred in the concentrations of all four metals between the two time points (two-way ANOVAs; $p < 0.05$); however, similar concentrations existed in animals regardless of OTC treatment. Within the digestive gland, significant increases in the concentrations of Zn and Fe occurred between the two time points, and a significant effect was observed in the interaction of post-treatment time and OTC exposure for both metals (two-way ANOVAs; $p < 0.05$). Tukey-Kramer post hoc testing revealed that this was due to significant increases over

time occurring only in the untreated animals, not in the OTC-treated animals, leading to a disordinal change in which the relative concentrations of Zn and Fe between OTC-exposed and unexposed animals changed over time. No significant effects of OTC treatment or post-treatment sample time were observed in digestive gland Mn and Cu concentrations.

4. Discussion

4.1 OTC efficacy and WS-RLP infections

These studies confirmed the efficacy of *per os* OTC medication to effectively treat rickettsial infections in abalone (Friedman et al., 2003). In the first experiment with ~13°C water temperatures, no significant differences in abalone survival or DG condition were observed. We attribute this observation to the lack of clinical WS observed during the trial. As OTC is bacteriostatic and not bacteriocidal (Martindale, 1996; Chopra and Roberts, 2001), a medicated individual requires a period of time to rid themselves of the bacterial infection. Thus, the observation of a single infected individual 3 days following the 10 day treatment (measured DG OTC residue in this animal was 2,706 ppm) suggests that this individual was in the process of purging the bacterium from its system and had received an adequate OTC medication. Whether the second infected abalone observed from the 10 day medication on withdrawal day 161 represents either incomplete treatment or reinfection through cross contamination from an unmedicated control tank is less clear. Since the DG of this animal contained 1.4 ppm of active OTC on day 161 when it was sampled, it is likely that this individual received a high therapeutic dose during the medication period (e.g. ~2000 ppm, Table 1) and that, perhaps, the infection was recent and due to cross-tank contamination. However, Braid et al. (2005) illustrated that the WS-RLP is not effectively transmitted at low ambient water temperatures similar to those in this study (12.3°C versus 13.4°C); only 1.7% of abalone held at a mean temperature of 12.3°C became infected relative to 72% infection prevalence in abalone held at 18.7 °C. Despite the presence of these two lightly infected individuals, the medication was 97% effective in clearing WS-RLP infections in the 10 day medication and 100% in the 20 and 30 day treatments.

In the second experiment, conducted at 17.3 °C, unmedicated abalone suffered higher losses and developed clinical WS at a higher rate than did medicated animals. These findings support earlier observations in which 14-day oral administration of OTC to WS-RLP infected abalone led to significantly reduced mortalities in a farm setting (Friedman *et al.,* 2003). Detection of WS-RLP in the first sampling post-OTC treatment in both the 10-and 20-day treated tanks, and in the Friedman study (2003), suggests that clearance of pathogen occurs over a multi-week time course, while infections found on days 102, 122 and 160 in the 10-and 20-day treated tanks suggests incomplete treatment of pathogen. It is possible that treatments were not 100% effective as some animals did not eat the food and/or were less exposed to the drug. The absence of WS-RLP detection by both histology and PCR between sample days 17-81 however, does not support this and it is more likely that WS-RLP was reintroduced through contamination from the adjacent tanks, equipment, water source, or food supply, or transferred during the post-treatment period due to poor handling. It is also possible however that infection persisted through treatment, and the sampling of only three abalone per time point would allow a very low level infection, to go undetected. Regardless, these results demonstrate the

possibility of re-infection relatively soon after application of the drug is ceased can occur at temperatures known to favor the spread of WS-RLP pathogen.

4.3 OTC depletion kinetics

Observed OTC levels and predicted C_{zero} values combined with different elimination rate constants (β), clearly illustrate differences in OTC pharmacokinetics among treatments in Experiment 1. The significantly higher DG therapeutant levels in abalone medicated for 20 or 30 days relative to those medicated for 10 days suggests a lack of DG saturation during the 10 day medication. As the metabolism of abalone is directly temperature dependent, we hypothesized that abalone medicated at a higher temperature would consume more feed and attain higher initial OTC tissue levels, but that depletion would occur more quickly than in abalone held at lower water temperatures. Contrary to expectations, the abalone medicated at 17.3 ^{o}C only accumulated half as much OTC (~1000-1300 ppm) as those medicated at 13.4 ^{o}C (~1700-2600 ppm). Except for the 10 day medication in Experiment 1, DG β values were similar, suggesting similar OTC depuration rates at the two experimental temperatures despite differing OTC maxima. However, if the terminal elimination rate constant from the two-compartment model for the 10 day expsoure is considered, then all three treatments eliminated the drug from the DG at similar rates. In additional studies on OTC kinetics in marine invertebrate species, the terminal elimination rate constants are typically ~0.03, similar to that observed in all treatments of both of our experiments (β=0.025-0.031). Reed et al. (2004) observed that β =0.036 in penaeid shrimp hemolymph and Campbell et al. (2001) found β =0.028 in sea urchin gonad. This latter species also accumulated a relatively high level of OTC (mean of 69.5 ppm after a 12 day medication at 29 mg g^{-1} feed) and a long $t_{1/2}$ of 24.6 days. Only in the urchin study and that of Rosenblum (2006) did animals in any other trials mirror the long OTC half-life observed herein ($t_{1/2}$ = 22.4-25.7 days vs our 23.8-27.5 days). Collectively, these data suggest that OTC saturation in the DG may be influenced by duration of medication as well as temperature; perhaps OTC saturation may occur more readily at elevated water temperatures.

4.4 Influence of di- and trivalent cations on OTC pharmacokinetics

Pedal OTC levels found in abalone were similar to levels observed in other species (especially fish liver). However, OTC levels in abalone DG far exceeded those reported in the literature for all other aquatic species examined to date (Bayer and Daniel, 1987; Malvisi et al., 1996; Mahoney et al., 1997; Chen et al., 2004; Reed et al., 2004; Ueno et al., 2004). Predicted zero-time drug concentrations in both experiments were nearly 2,000 ppm or higher. As suggested in previous studies, high di- and trivalent metal cations in abalone digestive gland (Bryan et al., 1977; Hyne et al., 1992) combined with a low digestive tract pH (Kusamoto et al., 2000; Edwards and Condon, 2001; Friedman et al., 2003) favor OTC retention (Doi and Stoskopf, 2000). However, the cytoplasmic pH of abalone cells is likely to be neutral or slightly basic. Due to higher pH and divalent metal cation content, the thus negatively charged cytoplasmic OTC is likely to be chelated. Metal-bridging has been proposed as a mechanism of metal ion-OTC complex formation in soils containing high amounts of divalent metal ions and organic material, and is dependent upon the ability of the drug to complex metal ions in solution

(MacKay and Canterbury, 2005). Such a mechanism may play a role in the long term OTC retention in abalone DG and not in the foot muscle; we found that the DG contains roughly 10 fold more divalent metal cations than the foot muscle. It should also be noted that OTC is highly lipid soluble, and has been shown to accumulate in lipid rich tissues (Jobling, 1994). The digestive gland of abalone may contain relatively high lipid concentrations, potentially facilitating the accumulation of high concentrations of OTC within this tissue. Thus the disproportionately high metal concentrations, high lipid content and low pH may potentially all contribute to the high concentrations of OTC, and long depuration periods recorded for digestive gland. Further analysis on differential uptake and retention of OTC relative to DG cation and lipid content is needed to better understand the dynamics of this drug in abalone.

4.5 Abalone growth post-treatment (Experiment 2)

While OTC reduced WS-RLP loads and associated tissue atrophy, little change was observed in both shell length and wet weights over the full time course. Trevelyan et al., (1998) found typical linear growth rates of H. rufescens in commercial production range from 33 to 50 μm day−1 when animals were fed on M. pyrifera at temperatures between 12 and 16 °C. In black foot abalone, *H. iris*, Stuart and Brown (1994), found growth rates range between 17-29 mm/year and 5.4 mg/day and concluded that during short-term studies these rates were largely dependent upon both the nutritional value of the food and the feeding rate. It is possible that the lack of significant shell growth and the low wet weights increases over this 5 ½ month study were due to WS-RLP effects on digestive capabilities. Several researchers (Moore et al., 2000; Friedman et al., 2002; Braid et al., 2005) have shown that WS-RLP infection results in a loss of tissue involved in both digestive gland enzyme production and nutrient uptake. The lack of mortalities in the OTC-treated tanks implies the treatment occurred prior to the terminal stages of WS, however, it is possible that recovery of a fully functional digestive gland post WS-RLP infection occurs over a longer period of time than that followed in the current study.

It is also anticipated that OTC treatment may impact gut microflora preventing efficient digestion and indirectly reducing overall growth, as found in sea urchins (Campbell et al., 2001). In addition, slow growth in this study may be explained by changes in the ability to utilize the nutritional content of the diet. It has also been shown that endogenous digestive gland enzymes varied with diet and that kelp-fed abalone appear to adjust their polysaccharide-digesting enzymes to utilize this different substrate (Cook and Coyne, 1997). Thus, in our study any effect that either the WS-RLP had on digestive gland architecture or antibiotic had on gut microflora, may also have been compounded by the change in diet that occurred after OTC treatment, when all abalone were switched from artificial food to kelp. The inclusion of a second control group (uninfected and maintained on a non-medicated artificial feed) is needed to definitively ascertain the effects of OTC therapy and WS-RLP infection on abalone growth.

4.6 Relationship between Resistance to Re-Infection and OTC Presence (Experiment 2)

Horizontal transmission of WS-RLP among previously treated and untreated abalone was demonstrated within each of the three cohabitation experiments. In addition, evidence of WS-RLP infection was detected by histology 102 days following the 20-day treatment in an animal that contained 58 ppm of OTC. These observations clearly show

that re-infection with detectable concentrations of digestive gland OTC is possible. Nevertheless, it is also clear that treatment provides a reduction in susceptibility to re-infection supporting earlier observations of long-term resistance to re-infection in OTC-treated red abalone (Friedman et al., 2003; Braid et al., 2005). These results may prove useful in plans to restore the endangered white abalone populations; animals may be 'loaded' with OTC prior to outplanting providing resistance to infection during the outplanting process when animals may be exposed to the pathogen. WS is endemic in southern California and, thus, its waters are likely to contain the WS-RLP thereby exposing outplanted abalone. In addition, as susceptibility to pathogens is typically enhanced in stressed organisms, OTC-loading may also reduce infection during the initial stages of settlement into the animals' natural environment.

Within the cohabitation studies, the efficiency of pathogen transfer to both control (previously untreated and uninfected) and OTC-treated animals appeared to vary between the three trials. This variation may be due to fluctuations in the bacterial loads that the infected animals were shedding. WS-RLP infected animals inhabiting the header tank were obtained shortly before the first cohabitation experiment and were not replaced during the three cohabitation trials. While PCR analysis of feces from the header tank confirmed the presence of WS-RLP prior to the onset of each cohabitation trial, conventional end-point PCR is not quantitative.

PCR data indicated higher infection prevalence than histology. While much more sensitive than histology, PCR detection is unable to differentiate between WS-RLP inclusions and DNA from OTC-killed bacteria or potential contamination during DNA purification and analysis. Conversely while infection detected by histology unequivocally shows the presence of WS-RLP it is dependent upon collection of sufficient amount of appropriate tissue and is apt to miss detection of low-level infections. However, neither method provides information on viability which would require a re-infection bioassay.

5. Conclusion

OTC is clearly an effective therapeutant for the treatment of rickettsial infections and to reduce losses associated with abalone withering syndrome. The judicious application of this drug is imperative to avoid the development of resistance to OTC in target and other bacteria (Chopra and Roberts, 2001). Rapid accumulation and long retention of OTC in abalone digestive tissues, the target organ of etiological agent of withering syndrome ("*Candidatus* Xenohaliotis californiensis", Friedman et al. 2000), has been observed. Even under conditions known to favor WS progression, as little as 10 day per os delivery of OTC therapy is capable in reducing WS-RLP loads and associated mortalities associated in California red abalone. It is also clear that the long residence time of OTC within the digestive gland confers a resistance to re-infection, and thus reduces the need for multiple dosing. Further examination of the application of shorter durations of medication, lower OTC levels in the feed to maximize palatability and examination of the influence of temperature and divlaent cations on OTC kinetics are needed to optimize application of this important antimicrobial therapeutant in abalone aquaculture.

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Table 1. Efficacy of three levels (medication durations) on the prevalence and intensity of WS-RLP infection in control relative to medicated abalone, Experiment 1.

Treatment	Factor	Control	Medicated	Significance (p value)
10 days	Prevalence (%)	80.95	1.25	p<0.0001
	PE RLP Intensity	1.05	1.23	p<0.0001
	DG RLP Intensity	0.66	1.25	p<0.05
20 days	Prevalence (%)	78.97	0.00	p<0.0001
	PE RLP Intensity	0.67	0.00	p<0.0001
	DG RLP Intensity	0.33	0.00	p<0.05
30 days	Prevalence (%)	76.39	0.00	p<0.0001
	PE RLP Intensity	0.93	0.00	p<0.0001
	DG RLP Intensity	0.33	0.00	p<0.05

Table 2. Pharmacokinetic parameters for foot muscle and DG as determined by a one-compartment model, Experiment 1. Results from the two-compartment model analysis of the 10 d exposure are in parenthesis below the one-compartment values. [1] Terminal elimination rate constant.

Treatment	Tissue	C_{zero} (ppm)	Equation $C_{(t)}$ =	β^1	$t_{1/2}$ (days)	r^2
10 days	DG	1996	$1996e^{-0.0582t}$	0.0582	11.9	0.90
			$(1500e^{-0.199t} + 1000e^{-0.0250t})$	(0.0250)	(23.8)	
	Foot	10.4	$10.4e^{-0.0647t}$	0.0647	10.7	0.82
20 days	DG	2710	$2710e^{-0.0274\ t}$	0.0274	25.3	0.83
	Foot	13.1	$13.1e^{-0.0604t}$	0.0604	11.5	0.80
30 days	DG	2833	$2833e^{-0.0252t}$	0.0252	27.5	0.86
	Foot	11.0	$11.0e^{-0.0355t}$	0.0355	19.5	0.51
			$(6.14e^{-0.052t}+5.7e^{-0.052t})$	(0.052)	(13.4)	(0.75)

Table 3. OTC concentrations (mean± s.d.) in red abalone after oral administration of medicated feed (100 mg/kg biomass) for 10, 20 and 30 days, Experiment 2. All means calculated from positive OTC values only.

Digestive gland

Post-treatment (days)	10-day OTC Exposure Concentration (ppm) mean±std (n)	20-day OTC Exposure Concentration (ppm) mean±std (n)	30-day OTC Exposure Concentration (ppm) mean±std (n)
3	831± 104 (9)	928 ± 165 (9)	1216 ± 223 (9)
17	176 ± 53 (9)	393 ± 193 (9)	569 ± 160 (9)
23	120 ± 32 (9)	147 ± 60 (9)	634 ± 103 (9)
42	60 ± 39 (9)	103.5 ± 21.6 (9)	368 ± 175 (9)
61	37± 22 (6)	105.1 ± 32.4 (9)	218 ± 169 (9)
80	9 ± 4.5 (9)	48.3 ± 40.4 (9)	134 ± 74 (9)
102	10.5 ± 5.8 (8)	30.6 ± 18.9 (9)	51.5 ± 38.6 (9)
122	3.4 ± 3.9 (7)	18.0 ± 13.5 (9)	37.3 ± 18.8 (9)
160	3.1 ± 1.1 (4)	5.8 ± 1.8 (8)	7.4 ± 2.9 (9)
200	Not done	5.3 ± 1.6 (3)	13.7 ± 6.6 (9)
250			7.5 ± 3.2 (7)

Foot Muscle

Post-treatment (days)	10-day OTC Exposure Concentration (ppm) mean± std (n)	20-day OTC Exposure Concentration (ppm) mean± std (n)	30-day OTC Exposure Concentration (ppm) mean± std (n)
3	14.2 ± 7.1 (9)	11.7 ± 2.6 (9)	6.81 ± 4.0 (9)
17	1.05 ± 1.4 (6)	2.5 (1)	1.6 ± 1.7 (4)
23	3.4 ± 2.2 (2)	ND	2.3 ± 1.0 (4)
42	ND	0.88 ± 0.8 (2)	2.4 ± 0.4 (2)
61	ND	2.5 ± 2.9 (2)	0.15
80		ND	ND

Table 4. Digestive gland pharmacokinetic values for OTC after oral dosing with 100 mg/kg biomass for 10, 20, and 30 days, Experiment 2. A ,B, zero-time tissue drug concentration intercepts of biphasic depuration curve; α, β values related to the slopes of distribution and terminal phases respectively; $t_{1/2\alpha}$, $t_{1/2\beta}$ distribution half life and elimination half life. Terminal elimination rate constants (day $^{-1}$) and half lives of OTC in the digestive gland of red abalone at 17.2°C water temperature. Theoretical withdrawal periods required to reach OTC levels of 2 ppm

	OTC Treatment Duration		
Parameter	10 days	20 days	30 days
A (ppm)	1133.5	991	1333
α (days^{-1})	0.2	0.165	0.067
B (ppm)	232.9	321	197
β (days^{-1})	0.027	0.031	0.031
$t_{1/2\beta}$ (days)	22.4	22.4	25.7
Theoretical withdrawal periods to 2 ppm	152	163	173

Table 5. Metal concentrations (ppm) in red abalone foot muscle and digestive gland, Experiment 2. Measurements were made on both treated and untreated animals 44 and 88 days after 20-day OTC treatment. Values are means ± standard deviations (n=3), and are on a dry tissue weight basis.

		Foot Muscle				Digestive Gland			
		44 days post OTC treatment		88 days post OTC treatment		44 days post OTC treatment		88 days post OTC treatment	
	(n)	untreated	treated	untreated	treated	untreated	treated	untreated	treated
Zn (ppm)	3	36±0	38±1.5	47±11	45±3	168±27	237±43	321±64	232±41
Mn (ppm)	3	2±0.7	1±0	3±1.2	2±0.6	12±2	15±2	17±7	14±2.6
Fe (ppm)	3	184±4.4	209±20	331±139	258±28	2638±217	2969±512	5481±1499	3413±688
Cu (ppm)	3	7±1	7±2.5	12±5	13±1.8	8±0.3	9±3.5	11±1.6	9±0.6

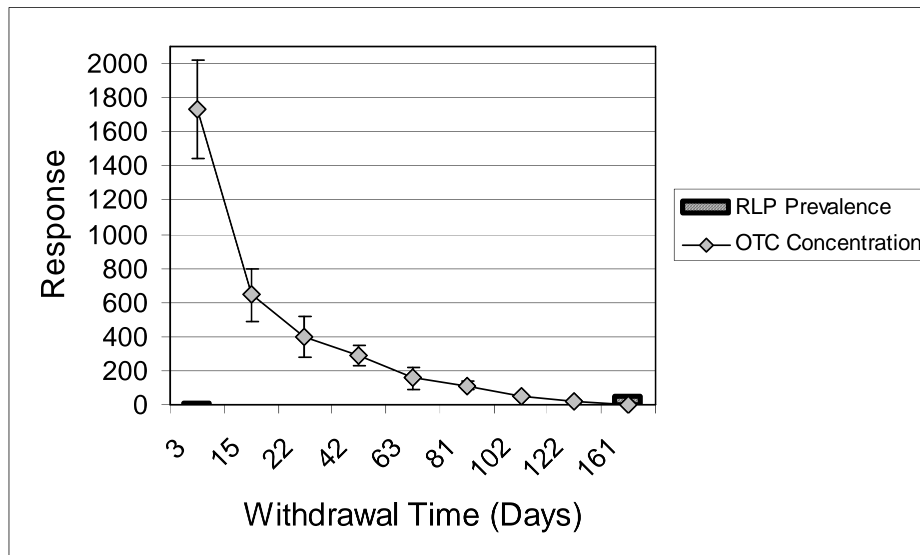

Figure 1. Response of medicated abalone in the 10 day, Experiment 1 treatment: WS-RLP prevalence (%) (bars) and oxytetracycline (OTC) concentration in the digestive gland (ppm or µg/mL; line). These data illustrate a low WS-RLP prevalence during the first and last sampling periods when OTC levels were high (1734 ppm) versus low (<2 ppm), respectively.

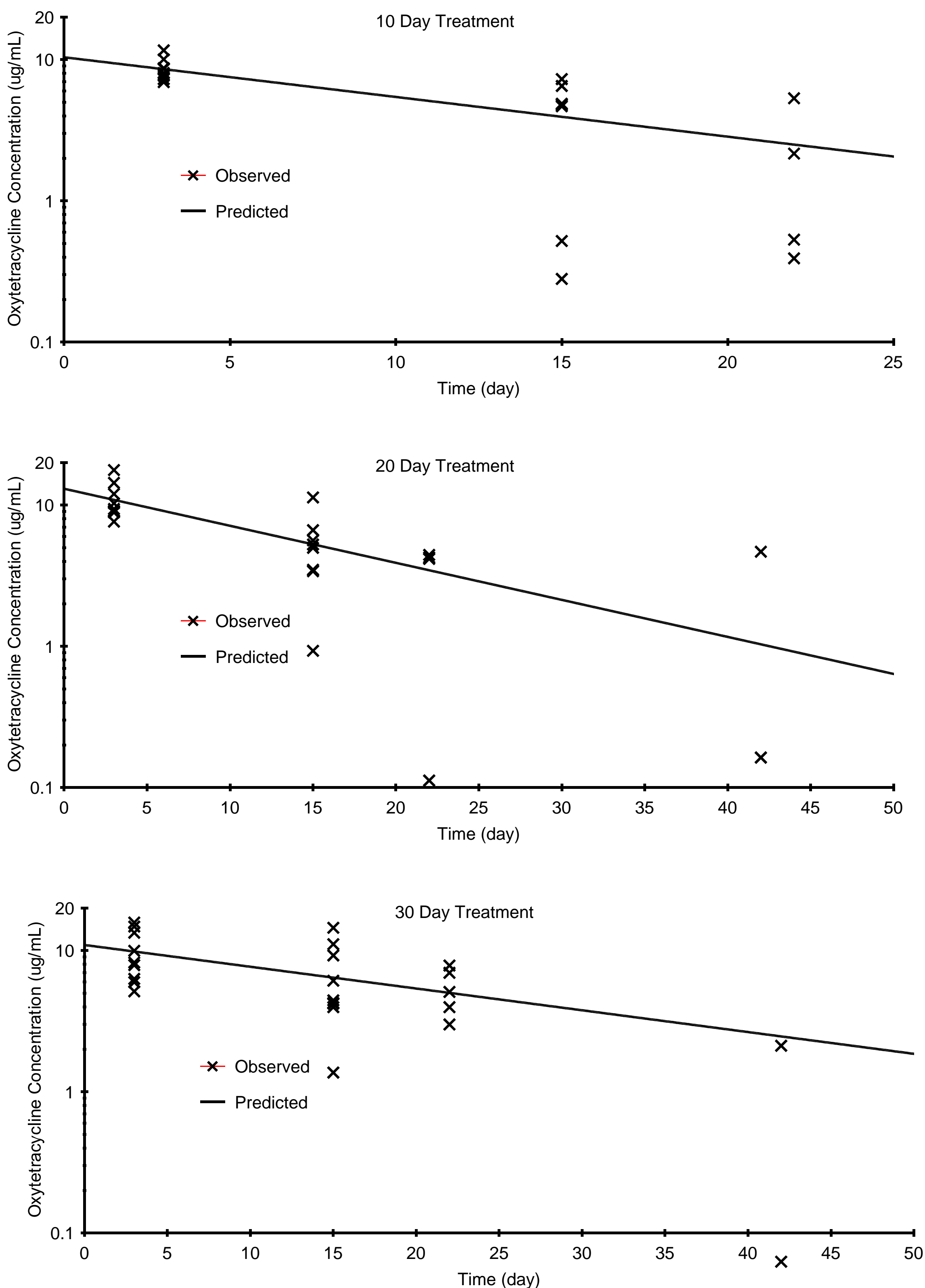

Figure 2. Oxytetracycline concentrations in the foot muscle of abalone medicated for 10 days, 20 days, and 30 days, Experiment 1. The predicted values are based on one-compartment pharmacokinetic model parameters.

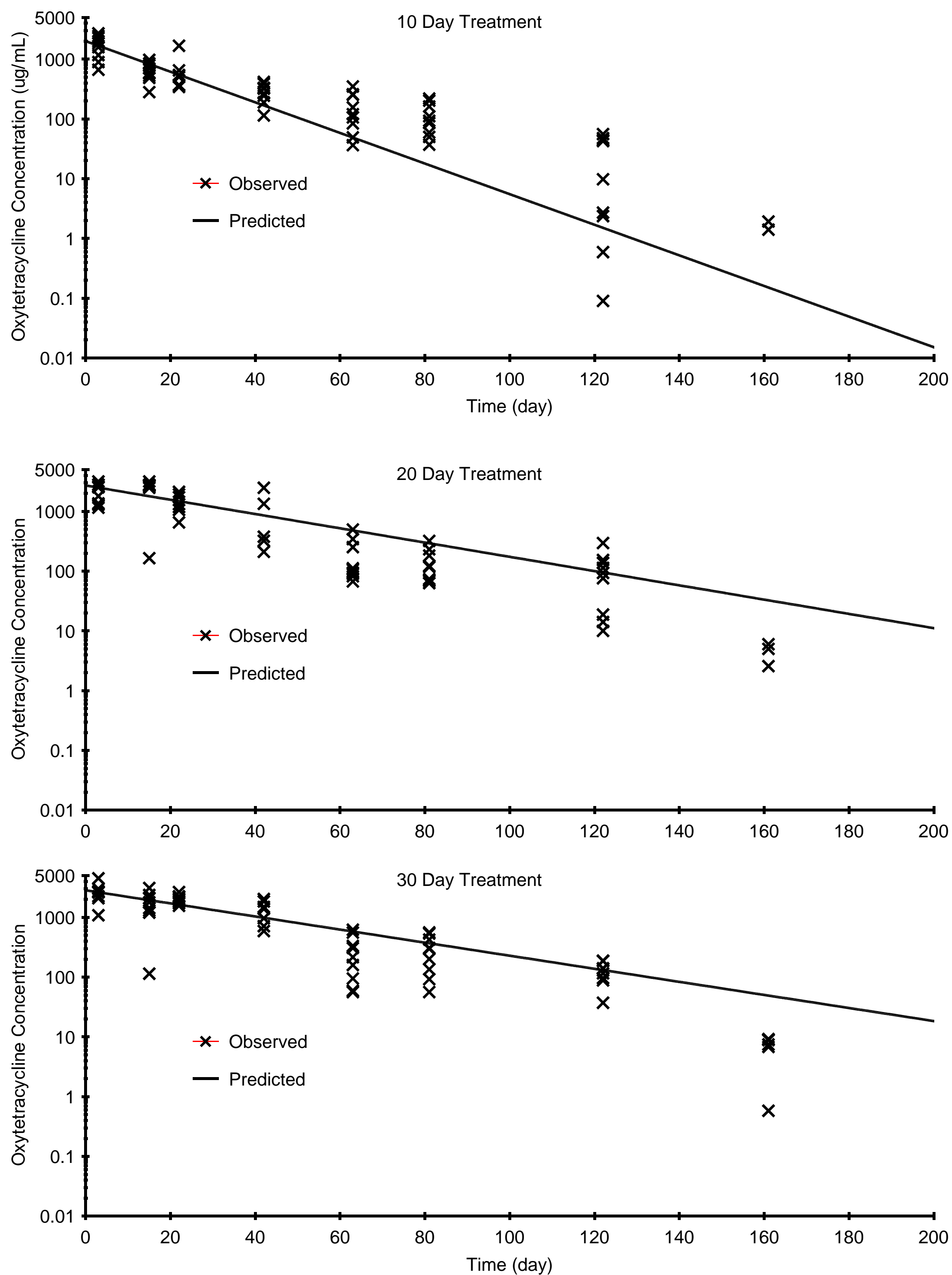

Figure 3. Oxytetracycline concentrations in the digestive gland of abalone medicated for 10 days, 20 days, and 30 days, Experiment 1. The predicted values are based on one-compartment pharmacokinetic model parameters.

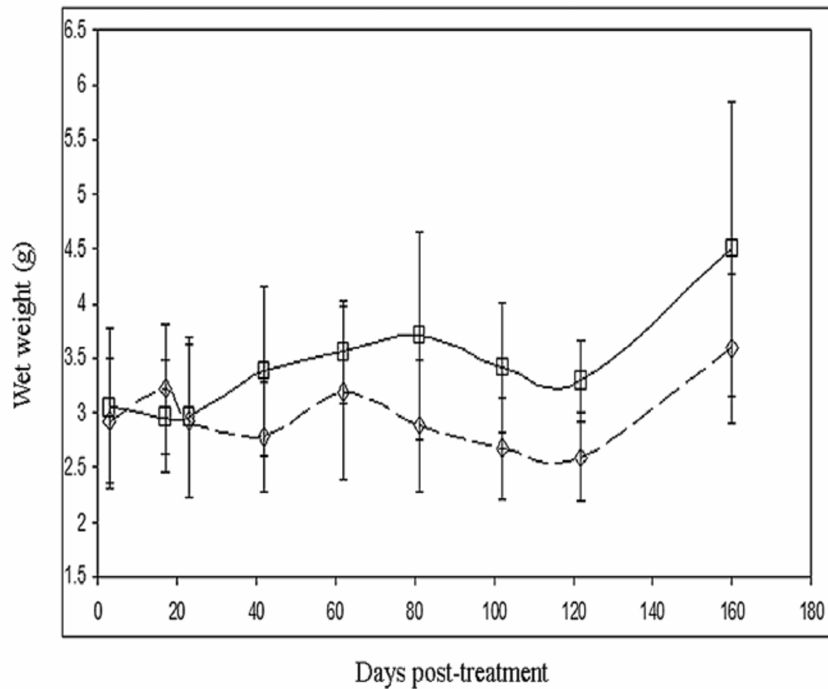

Figure 4. Wet weight over the 160 day post-treatment period for OTC treated and untreated animals, Experiment 2. Each time point is a composite of 10- , 20- and 30-day treatment durations and represents 27 animals. Significant increases in wet weights occur in both treated and untreated animals only at day 160 ($p \leq 0.05$). Symbols are □ OTC-treated animals; ◊ un-treated. Error bars represent standard deviations.

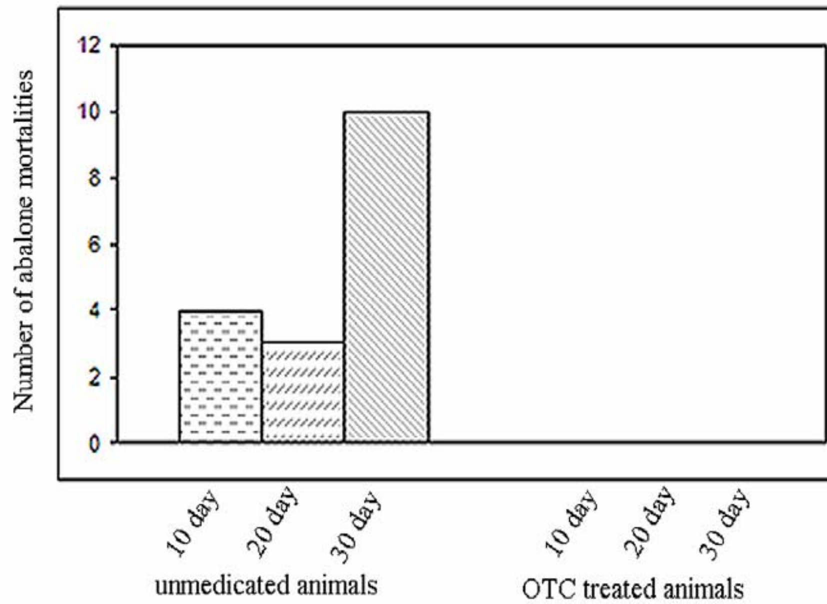

Figure 5. Mortalities observed within the 10- , 20- , and 30-day OTC-treated and un-treated groups over 160 days, Experiment 2. No mortalities occurred in the treated groups.

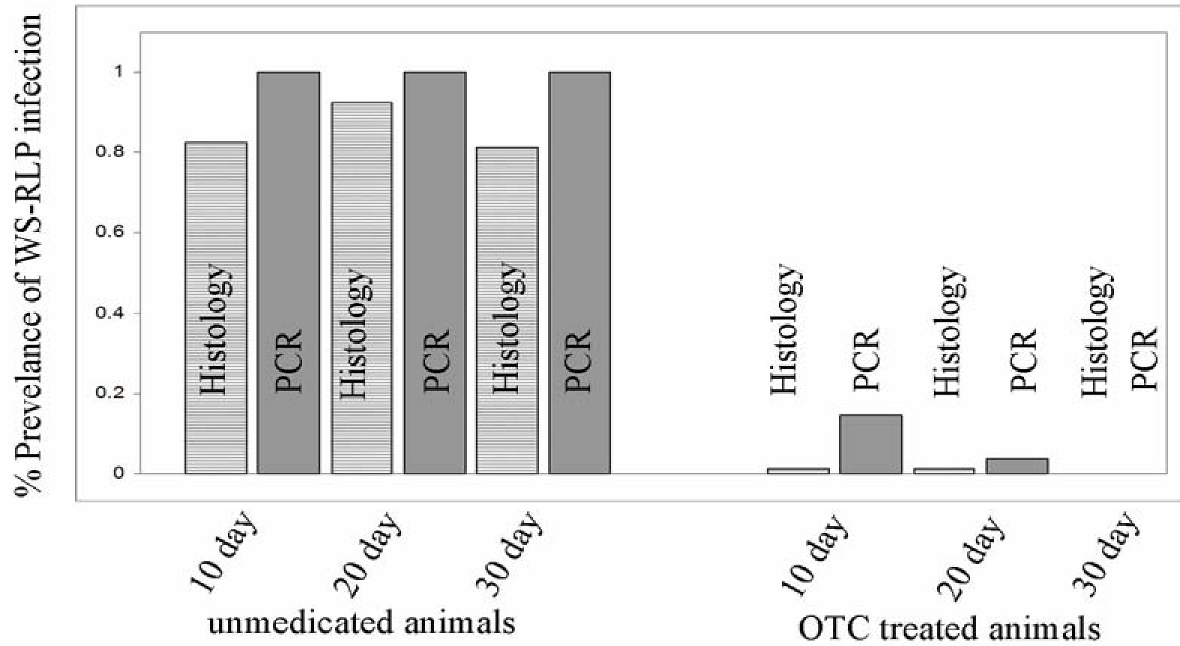

Figure 6. Prevalence of WS-RLP positive animals tested over the 160 day post-treatment period as detected by histology and PCR in the OTC-treated and untreated groups, Experiment 2.

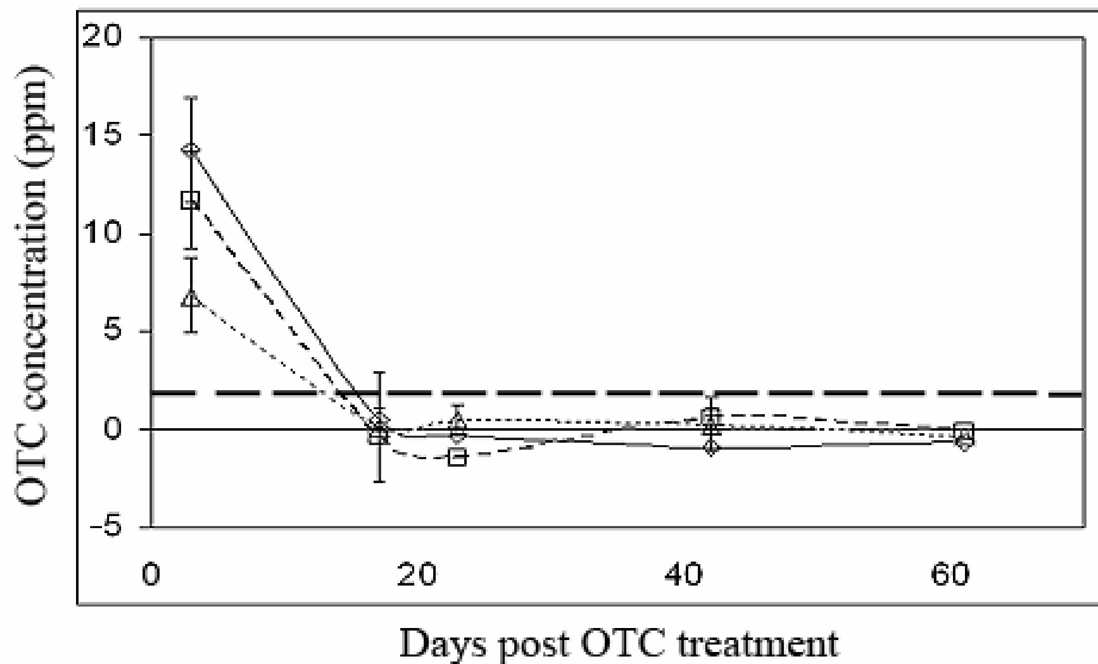

Figure 7 . Foot muscle oxytetracycline concentrations (ppm) in the abalone after 10-day, 20-day, and 30-day treatment durations, Experiment 2. The mean OTC foot muscle concentrations of all three treatment groups fell below 2 ppm 23 days post-treatment with no detectable OTC concentrations in any of the animals from 10- and 20-day treatments occurring after 42 days and after 63 days in 30-day treatments. Error bars are standard deviations and symbols represent □= 10-day treatments ◊ = 20-day treatments Δ= 30-day treatments. Dashed horizontal line represents tolerance level of 2ppm.

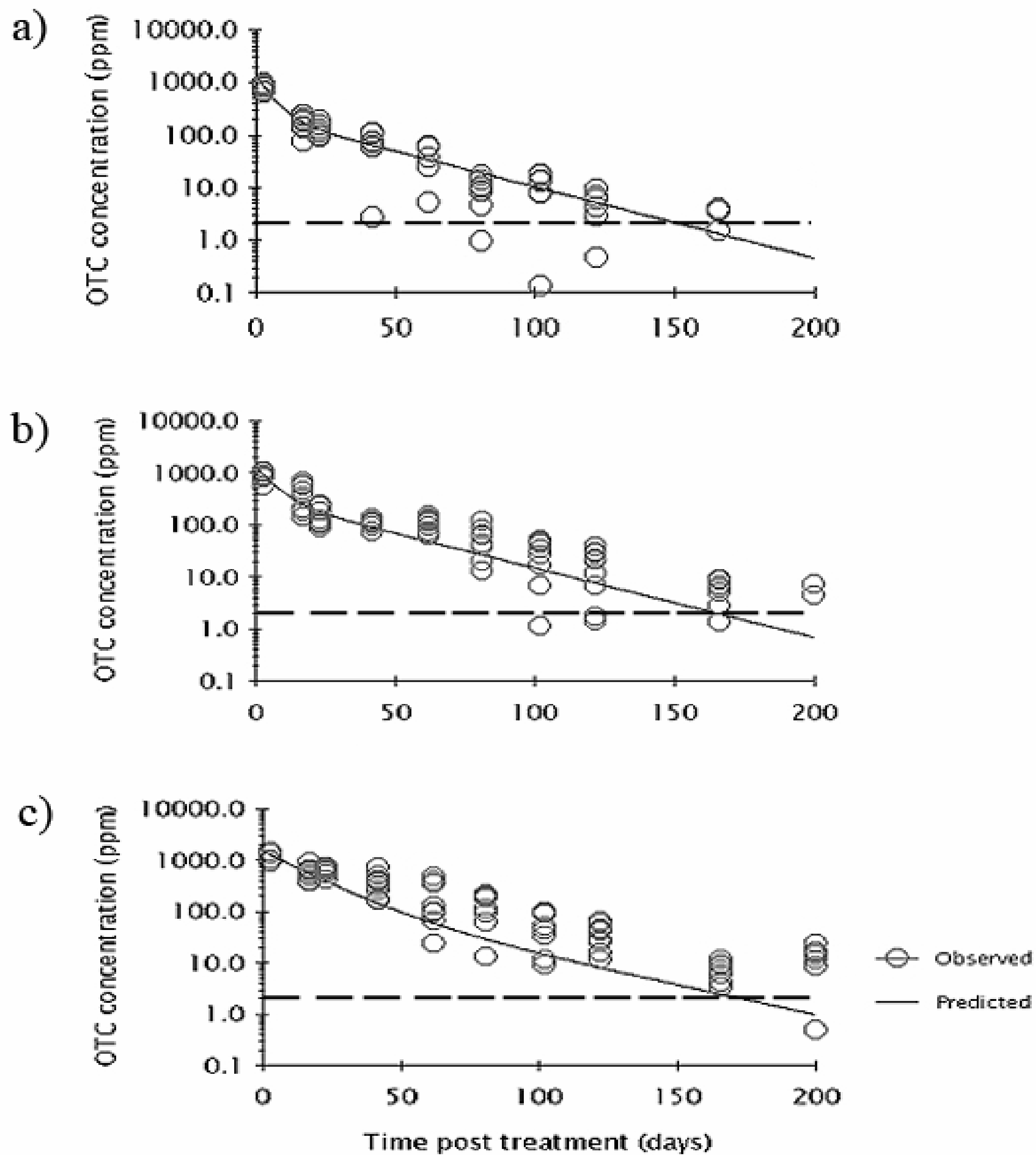

Figure 8. Semi-log plot of digestive gland oxytetracycline concentrations (ppm) in the abalone following a) 10-day, b) 20-day, c) 30-day treatment durations, Experiment 2. OTC depuration from the digestive gland best fit the two compartment model. The elimination half life ($t\ ½_{\beta}$) of the terminal part of the elimination phase was estimated to be 22.7, 22.4, and 22.4 days for the 10-, 20-, and 30-day treatments respectively. Each point represents one animal and the line is the predicted depuration curve. Dashed horizontal line represents tolerance level of 2ppm.

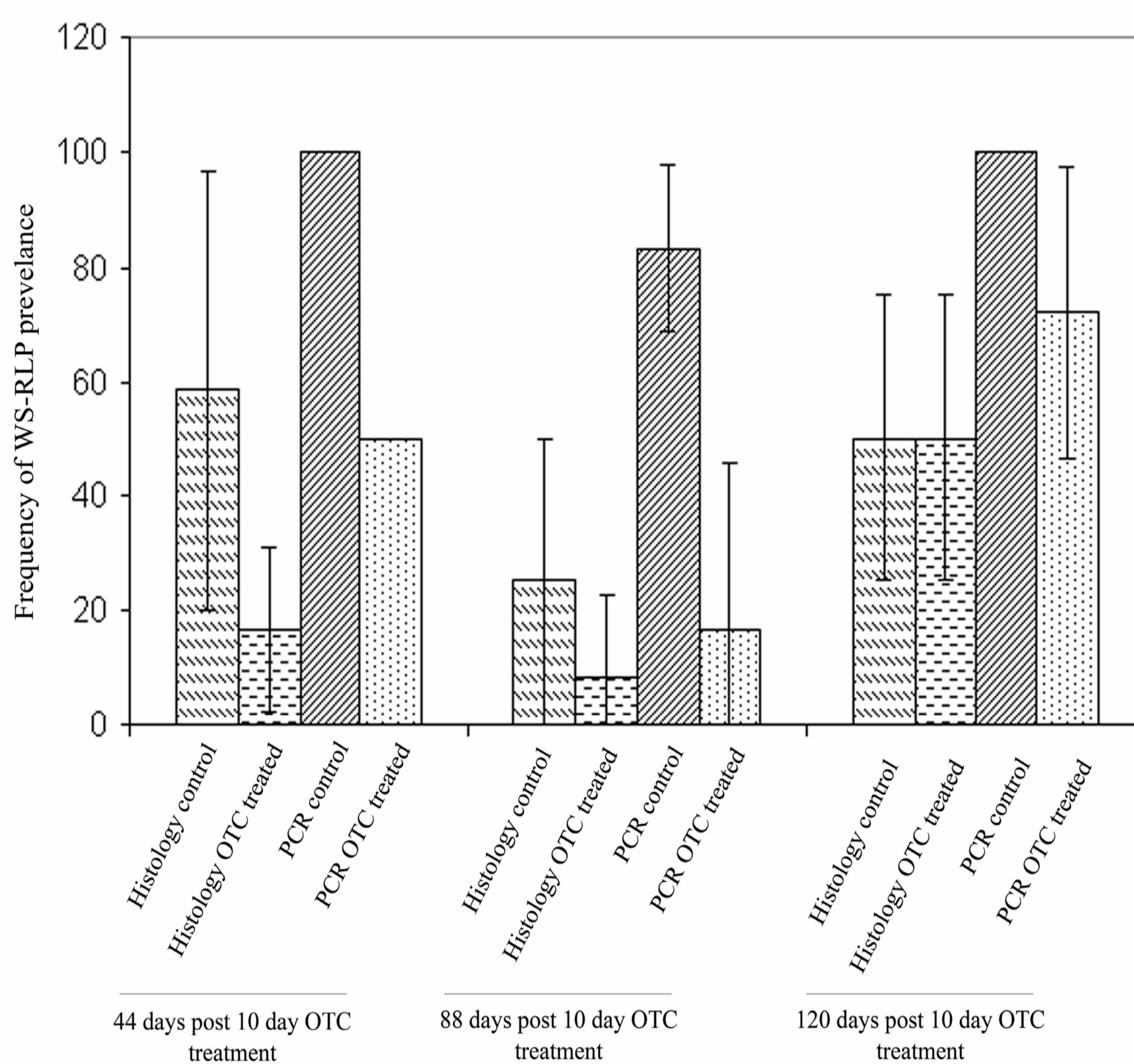

Figure 9. WS-RLP prevalence in cohabitation trails initiated 42, 81, and 122 days post 10-day OTC-treatments, Experiment 2. WS-RLP prevalence was detected by either histology or PCR within each cohabitation tank, 3 replicate tanks were used for control and OTC-treated animals. Error bars represent standard deviation.