AN ABSTRACT OF THE THESIS OF

Ephraim E. Temple for the degree of Master of Science in Fisheries Science presented on January 2, 2007.
Title: Delivering Oxytetracycline to First-Feeding Zebrafish *Danio rerio* (Hamilton) and Goby *Asterropteryx semipunctata* (Rüppell) Larvae Using Lipid Spray Beads

Abstract approved: _____
Chris J. Langdon

Effective treatment of larval bacterial diseases is a difficult problem when culturing ornamental fish. Oral administration of antibiotics using existing microparticle types is not effective due to high leakage rates; furthermore, injection of larvae is not practical. Treatment is currently limited to use of antibiotic baths, . In this study, we evaluated lipid spray beads (LSB) for delivery of the low-molecular weight, water-soluble antibiotic, oxytetracycline·HCl (OTC) to fish larvae. Lipid spray beads composed of menhaden stearine have shown good retention of water-soluble core materials and high acceptability by larval fish. Various OTC core-to-lipid ratios and OTC core concentrations were evaluated to maximize OTC delivery efficiencies. Acceptability and digestion of LSB containing riboflavin by larval zebrafish, *Danio rerio* (Hamilton), and larval gobies, *Asterropteryx semipunctata* (Rüppell), were also evaluated.

Increasing LSB core-to-lipid ratios from 1:3 v/v to 1:1 v/v resulted in an increase of encapsulation efficiency from 2.33% w/w to 3.68% w/w. LSB prepared with a ratio of 1:1.25 v/v were then used to determine the effect of core concentration on encapsulation, retention and delivery efficiencies. Increasing concentrations of OTC in the core from 0.1 to 0.5 g OTC mL^{-1} H_2O caused encapsulation efficiencies to

increase from 3.95% w/w to 18.77% w/w, respectively. This increase did not affect retention efficiencies and improved delivery efficiencies. Lipid spray beads prepared with a core concentration of 0.5 g OTC mL^{-1} H_2O produced the highest delivery efficiencies (7.9 ± 0.7% w/w) after suspending the beads in water for 60 min60 min aqueous suspension

Consumption of LSB containing OTC by first-feeding zebrafish and goby larvae was confirmed by analysis of feeding incidence and gut fullness. Visual observations of larvae fed on LSB containing riboflavin indicated that LSB were digested by larvae of both species. Particles were compacted and brightly fluorescent, freely-dissolved riboflavin filled the gut lumen and exited the anus in plumes. These results suggested that LSB composed of menhaden stearine could be used to deliver OTC to larval fish and may be useful in treating larval fish infected with bacteria susceptible to OTC. Oxytetracycline dosage could be controlled by either varying the concentration of administered LSB or the concentration of core solution.

Delivering Oxytetracycline to First-Feeding Zebrafish *Danio rerio* (Hamilton) and Goby *Asterropteryx semipunctata* (Rüppell) Larvae Using Lipid Spray Beads

by
Ephraim E. Temple

A THESIS

submitted to

Oregon State University

In partial fulfillment of
the requirements for the
degree of

Master of Science

Presented January 2, 2007

Commencement June 2007

Master of Science thesis of Ephraim E. Temple presented on January 2, 2007.

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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ACKNOWLEDGEMENTS

Dr. Chris Langdon served as my advisor during the past three years. His guidance and efforts have improved my ability to design, perform, and report on scientific research. I thank him graciously for the multiple edits of this manuscript. He had faith in my ability to accomplish the goals of this project. When I initially approached him regarding an open graduate research assistant position, he hesitated because my experience in aquaculture and research was weak. He must have seen something in me that caused the change of mind he experienced. I owe him my gratitude for allowing me the opportunity to apply myself to this challenging project and for exercising patience with my occasional stubbornness and ignorance.

I thank Dr. Paul Reno (Oregon State University) for his many hallway conversations filled with counsel, insight, and much needed humor. Doctor Ford Evans (Oregon State University) allowed me to occupy hours of his time exploring the inner workings of the mixed model ANOVA. William Gaeuman (Oregon State University) scrutinized my experimental design and its statistical robustness as a Statistics Department Consultant and provided far more than was probably expected of him. I thank Paul Lang and Dave Stick (both Doctoral candidates at Oregon State University) for their advice on creating effective visual presentations. I also thank Dr. Mary Hagedorn (Smithsonian Institution) and Robert Nelson (University of Hawaii) for supplying this project with blue-spotted goby broodstock. Hatfield Marine Science Center provided facilities. Oregon Sea Grant provided the bulk of the funding for this project. And thanks to the Mamie Markham Research Award, Walter G. Jones Fisheries Development Award, and Hatfield Student Organization for providing me

the opportunity to attend AQUA 2006 and Marine Ornamentals 2006 to experience the world of scientific conferences.

I feel I should thank everyone who stopped in the hallways and shared with me their experiences while allowing me the same favor. Each perspective has afforded me an increased understanding of science, the scientific method, and their application or importance in the world.

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DEDICATION

To Melinda, Ella, Isaac and life. Lachaim!

Delivering Oxytetracycline to First-Feeding Zebrafish *Danio rerio* (Hamilton) and Goby *Asterropteryx semipunctata* (Rüppell) Larvae Using Lipid Spray Beads

Introduction

Diseases are often a major problem in the culture of marine fish larvae, especially when densities are high, resulting in mass mortalities (Skjermo & Vadstein 1999; Conceição et al. 2001; Touraki et al. 2001; Katharios et al. 2005). Disease management for larvae can be more complex than for adults because water changes cannot be performed frequently due the greater susceptibility of larvae to handling stress (Skjermo & Vadstein 1999). Development of high bacterial populations in egg incubators and larval cultures compounds the effects of poor water quality (Olafsen 2001).

Therapeutic compounds can eliminate many bacterial diseases but treatment methods for larvae need to be improved. Oxytetracycline (OTC) is a low molecular weight (497 Da), broad-spectrum antibiotic that inhibits protein synthesis in bacteria by interfering with the functioning of 30S ribosomal subunits (Plumb 1995). Immersion treatments with this compound can be effective but are rendered less efficient in marine applications where OTC activity is inhibited due to binding with divalent cations in seawater (Lunestad & Goksøyr 1990; Treves-Brown 2000). This binding may cause a change in molecular charge (Lunestad & Goksøyr 1990), which in turn could inhibit diffusion through lipid-rich cell membranes (Kim et al. 2003). Injection, as an alternative treatment method to immersion, is impractical with larval fish. A more feasible and effective method may be to combine OTC with feeds for oral delivery to fish larvae.

Oxytetracycline is widely used in aquaculture and has been shown to persist both biologically in animal tissues and environmentally in sediments (Bjorklund & Bylund 1990; Lunestad & Goksøyr 1990; Bebak-Williams et al. 2002). Though estimates vary, OTC was detectable in animal tissues (excluding bones) up to 70 d after treatment (Treves-Brown 2000; Campbell et al. 2001; Rigos et al. 2002; Bernardy et al. 2003; Ueno et al. 2004; Uno 2004; Wang et al. 2004). In sediments, OTC was reported to have a half-life from 9 to 419 d (Jacobsen & Berglind 1988; Bjorklund & Bylund 1990; Bjorklund et al. 1990; Bjorklund et al. 1991). There is a need to reduce the amount of OTC used in aquaculture to minimize its presence in both animal tissues and their environment.

Bioencapsulation and microencapsulation are two possible approaches for oral delivery of therapeutic substances. Brine shrimp *Artemia franciscana* (Kellogg) can be enriched by immersion in a suspension of OTC (Roque et al. 1998). However, *A. franciscana* rapidly lose enrichment substances through defecation. Indeed, a gut retention time of 7 min was reported for actively feeding juveniles (Smith et al. 2002). An alternative to the use of *A. franciscana* for OTC delivery is via eggs of the polychaete worm, *Nereis virens* (Sars). These were osmotically loaded with OTC and delivered to Dover sole larvae, *Solea solea* (L.) (Katharios et al. 2005). Interestingly, unloaded eggs demonstrated inherent antimicrobial properties by inhibiting the growth of *Vibrio anguillarum*. These methods are effective but may not be appropriate for treatment of the early life stages of many fish species due to the large size of *A. franciscana* nauplii (487-491 µm; Shirdhankar et al. 2004) and *N. virens* eggs (180 µm; Katharios et al. 2005). For example, Cahu and Zambonino Infante (2001)

reported that early sea bass, *Dicentrarchus labrax*, larvae preferred particles between 50 and 125 μm in diameter. Gilthead seabream, *Sparus aurata*, larvae less than 4 mm in length preffered hard microparticles between 25 and 50 μm in diameter (Fernandez-Diaz et al. 1994). Furthermore, cultures of live prey are susceptible to "crashing" leading to interrupted supplies or the possible spread of disease to other organisms (Borowitzka 1997; Lubzens et al. 2001). Artemia nauplii were shown to carry *V. anguillarum* that later infected turbot, *Scophthalmus maximus*, larvae (Grisez et al. 1996)

Microencapsulation techniques have shown promise for delivery of antibiotics to suspension feeders (Touraki et al. 1995; Buchal & Langdon 1998; Langdon 2003). Microparticles do not carry pathogens, are not susceptible to "crashing", and can be made within the size range preferred by larvae. Although various microparticle types have been developed, very few are effective in delivering low molecular weight, water-soluble (LMWS) substances, such as OTC, to suspension feeders (Lopez-Alvarado et al. 1994; Roennestad, Thorsen & Finn 1999; Langdon 2003). Alginate, gelatin, carrageenan, and zein have been used to create microbound particles, but they have shown high leakage of amino acids (Lopez-Alvarado et al. 1994; Baskerville-Bridges & Kling 2000; Yúfera et al. 2002; Önal & Langdon 2004a; Önal & Langdon 2004b). Although past work has shown poor retention of amino acids by protein-walled microcapsules, recent work has improved retention by modifying the manufacturing process (Yúfera et al. 1996; Yúfera et al. 2002). Of the various particle types, microparticles made with lipids show the greatest retention of LMWS (Yúfera et al. 2002; Langdon 2003). Liposomes exhibit high retention of amino acids when

suspended in seawater but are expensive to produce, easily damaged, and generally too small for direct capture by larval fish (Özkizilcik & Chu 1994). These particles are much smaller in size than other microparticles mentioned here, ranging from 10 nm to 20 μm in diameter (Kulkarni et al. 1995). This characteristic has been exploit as liposomes have been used to enrich *A. franciscana* with oxytetracycline (Touraki et al. 1995). Lipid-walled microcapsules and lipid spray beads (LSB) retain high levels of LMWS over time and are able to encapsulate these substances in both particulate and aqueous form (Buchal & Langdon 1998; Langdon & Buchal 1998; Önal & Langdon 2004b). Leakage rates are affected by both the water solubility of the core material and whether it is encapsulated in particulate or aqueous form affect leakage rates (Langdon & Buchal 1998; Önal & Langdon 2004b).

One of the goals in microparticle production is to improve delivery efficiency (DE) by increasing inclusion, encapsulation, and retention efficiencies (IE, EE, and RE terms defined below; Koven et al. 2001; Önal 2002; Yúfera et al. 2002). Lipid spray beads have demonstrated high DE for low molecular weight, water-soluble substances, such as OTC. Buchal & Langdon (1998) reported RE of 98% w/w and DE of 45 mg core g^{-1} lipid for LSB composed of tripalmitin containing a core of aqueous OTC after 24 h suspension in seawater. Delivery efficiency of LSB have been reported to be significantly affected by changes in core concentration; for example, Langdon & Buchal (1998) showed that increasing core concentrations from 12 to 300 mg mL^{-1} OTC·HCl decreased RE by 13% w/w but increased DE by 9 mg core g^{-1} lipid after 24 h suspension of beads in seawater. Delivery efficiency of amino acids by LSB also showed significant responses to changes in lipid composition. Önal & Langdon

(2004b) reported that the RE of LSB composed of menhaden stearine (MS) alone (34.05% w/w at 60 min) exceeded that of beads composed of a mixture of MS and coconut oil (12.26% w/w) or spermaceti (18.56% w/w) for the delivery of glycine.

Lipid spray beads prepared with MS or other triacylglycerides could only be dispersed in seawater after incorporation in zein-bound complex particles because a zein coating reduced their hydrophobicity (Villamar & Langdon 1993; Önal & Langdon 2000, 2004a, 2004b). However, coating LSB with zein requires that all components be combined in an alcohol solution, making this approach impractical for LSB containing alcohol-soluble substances such as OTC (solubility 1 mg mL^{-1} at 20ºC; unpublished data) due to high leakage losses during the coating process.

This study aimed to improve methods for producing LSB with high IE, EE, RE, and DE for OTC by manipulating core-to-lipid ratios, core concentrations and dispersion of beads in seawater. In addition, the acceptability and digestibility of LSB containing OTC by fish larvae were determined.

Methods and materials

Experimental design

A series of experiments was designed to improve inclusion (IE), encapsulation (EE), retention (RE), and delivery (DE) efficiencies of lipid spray beads (LSB) made with menhaden stearine (MS; Omega Protein, Houston, TX; Önal 2002) and OTC·HCl (> 95% purity; Sigma-Aldrich, St. Louis, MO). Equations for the calculation of these characteristics are presented in Table 1. Oxytetracycline is a low-molecular weight (497 Daltons) molecule that is highly water-soluble (100 mg mL^{-1} at 25°C).

Oxytetracycline was dissolved in water for these experiments because encapsulation of solutions of OTC had been reported to result in higher EE, RE, and DE than encapsulation of OTC particles (Langdon & Buchal 1998). The OTC solution had a pH of 3.2 and pH was not adjusted in LSB production. Three batches of each LSB type were produced and compared to ascertain batch-to-batch variability. Different core-to-lipid ratios (v/v) in LSB were compared in the first experiment (Table 2). The LSB type with the highest EE was used in the second experiment, where the optimal core concentration of OTC was determined. Finally, RE and DE for LSB in Experiment 2 were determined.

Lipid spray bead production

Lipid spray beads were prepared following the method described by Önal & Langdon (2004a, 2004b). Oxytetracycline was dissolved in distilled water using a sonicator at 100 W output (Labsonic L, Braun Biotech International, Melsungen, Germany). This solution was then heated to 60°C and emulsified in molten (60°C) MS with sonication for 60 s. The resulting emulsion was sprayed through a heated (65°C) nozzle (system SU22 with nozzle assembly ¼ JBCJ; Spraying Systems Co., Wheaton, IL) pressurized with dry nitrogen gas into a stainless steel cone controleld to -40°C with liquid nitrogen vapor. Nozzle temperature was maintained with an insulated wire heater (FGR-080; Omega Engineering, Inc., Stamford, CT) attached to a temperature controller (CN9000A; Omega Engineering, Inc., Stamford, CT). Lipid spray beads formed when the lipid component solidified on contact with the chilled cone. They were collected and stored at -80°C under dry nitrogen.

Experiment 1: effect of core-to-lipid ratio of LSB on inclusion and encapsulation efficiencies

Lipid spray beads were created with different core-to-lipid ratios as shown in Table 2. Ratios above 1:1 were not created due to instability of the pre-spray emulsion. Total OTC content of LSB was defined as the combined weight of OTC contained within and on the surfaces of LSB. It was determined by dissolving triplicate 0.33 g samples of LSB in 10 mL aliquots of chloroform. There was no measureable solubility of OTC in chloroform. Oxytetracycline was then extracted from the organic phase by mixing with 10 mL 0.2 M HCl for 10 min on a tissue culture rotator (099A RD4512; Glas-Col, Terre Haute, IN). Samples were centrifuged at 8,000 rpm for 5 min (Allegra X-22R; Beckman Coulter, Fullerton, CA) and the aqueous phase was removed for analysis. The extraction process was repeated if the aqueous supernatant showed an absorbance reading (at 358 nm) higher than that of a blank control. Negative controls were created by analyzing LSB containing only water. Absorbance values of the combined aqueous extractions were converted to OTC concentrations using regression equations derived from standard curves. Chloroform was evaporated from the organic phase with dry nitrogen gas at room temperature and the remaining lipid was freeze-dried (FreeZone 12; Labconco Corporation, Kansas City, MO) to remove traces of chloroform and water so that total lipid weight could be determined.

Inclusion efficiency (IE) was defined as the percentage of OTC in the pre-spray mixture that was incorporated in LSB. Oxytetracycline that was on the surface

of LSB was determined by washing weighed quantities of beads held on filters (GF/A; Whatman) with 5 mL distilled water using vacuum filtration and spectrophotometrically (358 nm; DU 530; Beckman Coulter, Fullerton, CA) determining OTC concentrations in the filtrates. Oxytetracycline that was removed by the 5-mL rinse (considered to be external on the surface of the beads) was subtracted from the total amount associated with LSB after spraying. Inclusion efficiency was calculated as:

$$IE = [(W_1/W_2) / W_3] \times 100$$

where W_1 was the total extracted OTC dry weight minus external OTC dry weight, W_2 was the dry weight of lipid in the LSB sample, and W_3 was dry w/w ratio of OTC to lipid in the pre-spray formula.

The w/w ratio of water to LSB was determined by, first, weighing LSB samples in pre-weighed aluminum weigh boats and incubating them for 48 h at 60°C. Weigh boats with LSB samples were then weighed and water weight was determined by subtracting total post-incubation weight from total pre-incubation weight. Ratios were determined by dividing water weight by initial LSB weight.

EE was defined as the dry w/w percentage of OTC in LSB:

$$EE = (W_1 / W_2) \times 100$$

Where W_1 was the total extracted OTC dry weight minus external OTC dry weight, and W_2 was the wet weight of the LSB sample.

Experiment 2: effect of core concentration of LSB on inclusion, encapsulation, retention, and delivery efficiencies

Lipid spray beads were created with different core concentrations as shown in Table 2. Inclusion efficiency and EE were determined as described above using 0.25-0.05 g samples of each LSB type, 0.1 – 0.5 g OTC mL^{-1} H_2O, respectively. Core solutions were supersaturated by sonication and heating to 60°C until all OTC dissolved. Solutions with higher OTC concentrations than 0.5 g mL^{-1} resulted in solutions that were too viscous to allow preparation of LSB.

RE was defined as the percentage of initial total OTC, both internal and external, retained by LSB suspended in aqueous solution over time. To determine RE, triplicate 60 mg samples from each batch of each bead type were added to 3 mL of an aqueous solution of 2% w/v gum arabic (Sigma-Aldrich, St. Louis, MO) and 2% w/v REAX 85A (MeadWestvaco Corporation, Charleston, SC) (GR) at 4°C. Lipid spray beads were dispersed with five sonication bursts of less than one second each, using a sonicator at 100 W output. The temperature of the solution was raised to 28°C with the addition of 7 mL distilled water at 40°C and mixed for 10, 30, and 60 min. Lipid spray beads made with MS did not disperse well in water without the aid of GR. Gum arabic and REAX 85A, a sulfonated lignin, were selected for their ability to retain their dispersant properties under dilute conditions without foaming or increasing surface tension (Randall et al. 1988; Dickinson et al. 1989; Garti & Leser 2002; Meister 2002). As shown by Mulqueen et al. (2001), mixing surfactants is often useful in dispersion applications. The weight of LSB (60 mg) added to 10 mL of GR was chosen to ensure that all encapsulated OTC could potentially dissolve. Tubes were

sealed and placed on a tissue culture rotator at 20% speed in an incubator at 28°C without light. Bead suspensions were then sampled from one set of triplicate tubes per time interval and vacuumed-filtered through GF/A filters. Oxytetracycline concentrations in the filtrates were determined as described above.

RE at 0 min (rinse) was determined as follows: samples of LSB were weighed on tared GF/A glass microfiber filters (Whatman) and the beads washed with 10 mL dilute GR solution at 28°C using vacuum filtration. Filtrates were analyzed spectrophotometrically at 358 nm against a GR blank.

RE for each time interval was expressed as:

$RE = [(W_1 - W_2) / W_1] \times 100$

where W_1 was the initial OTC dry weight associated with the LSB (both external and internal fractions) and W_2 was the dry weight of OTC lost from the beads and present in the filtrate.

DE was defined as the percent dry weight of OTC remaining in LSB [(g core material g^{-1} LSB) x 100] after a period of suspension in GR.

Broodstock maintenance, spawning and embryo husbandry

Zebrafish, *Danio rerio* (Hamilton), broodstock were maintained at 28ºC in 2 L aquaria supplied with recirculating, dechlorinated freshwater at a density of 10 fish L^{-1}. Aeration was applied to the sump tank to maintain adequate oxygenation. Lighting was provided by 15 W fluorescent bulbs (AZOO Super Light; Aquatic Eco-Systems, Apopka, FL) on a 14L:10D cycle. Tanks were cleaned weekly and excess food and waste materials were removed every other day. Fish were fed manually three times a

day on flake food (TetraMin Tropical Flakes; Spectrum Brands, Inc., Atlanta, GA) or Zeigler Adult Zebrafish Diet (Zeigler Bros, Inc., Gardners, PA).

Zebrafish can spawn daily immediately after each dark period; therefore, the dark cycle was eliminated the day before a desired spawning event to prevent unwanted spawning. This precaution increased the quantity of eggs produced per spawn (Önal 1997). Twelve hours prior to spawning, adults were transferred to mesh-bottomed containers (3 mm pore size). The mesh allowed eggs to pass through to a separate chamber that prevented adults from eating the laid eggs. No effort was made to manipulate the male to female ratio in each spawning container. After spawning, adults were returned to broodstock tanks and eggs were collected using a plastic transfer pipette. The eggs were then treated for two min with a 30-ppm bleach solution to prevent mold growth. After removing the bleach solution, the eggs were treated with a 5-ppm solution of sodium thiosulfate for one min to neutralize the bleach. This solution was removed and the eggs were rinsed with sterile 0.5X E2 embryo medium (recipe from Zebrafish International Resource Center, University of Oregon, USA). Eggs were incubated in sterile, disposable Petri dishes of 100 mm diameter at 100 eggs $dish^{-1}$ with sterile E2 embryo medium at 28°C on a 14L:10D light cycle. Eggs hatched after 72 h post-fertilization (hpf) and larvae were used in experiments at approximately 170 hpf.

Broodstock of *Asterropteryx semipunctata* (Rüppell) were maintained at 26ºC in aquaria on a flow-through system supplied with 1-µm-filtered and UV-sterilized seawater at about 6 exchanges per day. Fish were fed manually twice a day on a gelatin-bound diet created in our lab. Lighting was provided by fluorescent bulbs on a

14L:10D cycle.

Adult gobies spawned every 2-3 weeks on the inner surface of 10 cm x 7 cm polyvinyl chloride tubes. Pipes with eggs were transferred to baskets floating in broodstock tanks the third night after spawning. Pipes were removed 4 h after the onset of darkness and hatching to improve survival. Larvae were retained in hatching baskets until used in experiments at approximately 150 hpf.

Experiment 3: acceptability and breakdown of LSB

Ingestion of LSB containing OTC (1:1.25 v/v core-to-lipid ratio) by zebrafish was compared with ingestion of either LSB containing distilled water (water LSB) at the same core-to-lipid ratio or Microfeast. Microfeast is a marine yeast (Torula sp.) supplement previously used to rear zebrafish larvae (Önal 1997). Starved larvae served as an additional control. Forty larvae at 170 hpf were stocked in 500 mL water per 1 L container and fed on each particle type for 2 h. Microfeast was delivered to larvae at a ration of 0.5 mg $larva^{-1}$ (0.04 mg mL^{-1}; Önal 1997) and all other particles were delivered at a ration of 2.5 mg $larva^{-1}$ (0.2 mg mL^{-1}). Due to the diverse behavior of the different particles, it was necessary to use different methods to disperse them prior to delivery to larvae. Distilled water, GR solution, and 2% w/v polyvinyl alcohol (cold water soluble; Sigma Aldrich, St. Louis, MO) were used to disperse Microfeast, OTC LSB, and water LSB, respectively. Particles were dispersed in 3 mL of the designated solution then fed to larvae. Similar protocols were followed to test the acceptability of OTC LSB by 150 hpf goby larvae. However, with these larvae, only water LSB and starved treatments were used as controls as no diet is known to be

acceptable for this species.

Acceptability was quantified by determining both the percentage of larvae that ingested particles (% feeding incidence) and the cross-sectional area of food within the guts of larvae (gut fullness). Larvae were photographed using a digital camera (Spot Insight QE model 4.2; Diagnostic Instruments, Inc.) mounted on a compound microscope (Leica DM1000; Leica Microsystems, Wetzlar, Germany). Images were captured with Spot software (version 3.5.9.1; Diagnostic Instruments, Inc., Sterling Heights, MI) and analyzed with Image-Pro Plus (version 4.5.1.29, Media Cybernetics, Inc., Silver Spring, MD). Lipid spray beads were also photographed with Spot software and their diameter was measured with Image-Pro Plus software.

Lipid spray beads containing riboflavin were fed to zebrafish and goby larvae to qualitatively determine their digestibility (Önal & Langdon 2004a). They were prepared with a core consisting of 1 g ground riboflavin powder (< 10 μm particles; McCrone micronizing mill; McCrone Microscopes and Accessories, Westmont, IL) suspended in 10 mL distilled water. The riboflavin suspension was sonicated with 12.5 mL MS and LSB were created as described above. These LSB were suspended in 3 mL GR solution and fed to zebrafish larvae at 170 hpf and goby larvae at 150 hpf. After 2 h of feeding, larvae were sampled and examined under an epifluorescent microscope. Digestion was indicated by the release of free riboflavin in the gut together with misshapen or partly broken down LSB.

Statistical Analysis

Data were analyzed for normality using normal probability plots and

homogeneity of variances using Bartlett's test (5% level of significance). Transformation of data was unnecessary. For all regression analyses described below, terms preceded by “x” were regression terms. All other terms were used to determine lack-of-fit. A randomized complete block design was used to determine the effect of core-to-lipid ratio and core concentration on IE and EE, with 4 treatments in 3 blocks. Regression analysis of the means of these parameters was used within the mixed-model ANOVA with xtreatment, xtreatment*xtreatment, and treatment as fixed effects, and batch as a random effect. A split-plot design was used to determine the effect of core concentration on RE and DE. Batches were regarded as whole plots with individual treatments assigned as subplots. Four time intervals were assigned to each subplot. Regression analysis of RE means was used within the mixed model ANOVA with xtime, xtime*xtime, treatment, time, and treatment*time as fixed effects, and batch and batch*treatment as random effects. Regression analysis of DE revealed a significant lack-of-fit and non-significant linear and quadratic terms. Therefore, the mixed model ANOVA was used without regression terms to analyze DE. The significance of differences among treatments was determined for DE by comparing least squares means with the Tukey-Kramer adjustment. Gut fullness and % feeding incidence were analyzed using one-way ANOVA and Tukey’s Honest Significant Difference (HSD) to determine differences among treatments (5% level of significance). Analyses were performed using SAS 9.1 software (SAS Institute Inc., Cary, NC).

Results

Lipid spray bead characteristics

Lipid spray beads (LSB) had a mean diameter of 10.1 ± 5.4 (SD) μm, which was unaffected by changes in either core-to-lipid ratio or core concentration ($P > 0.05$; two-way ANOVA). Cyrstallized OTC was not observed within LSB. In general, LSB appeared spherical to ovoid.

Experiment 1: effect of core-to-lipid ratio of LSB on inclusion and encapsulation efficiencies

The mean inclusion (IE) and encapsulation (EE) efficiencies for LSB in this experiment are depicted in Fig. 1 (a and b). Core-to-lipid ratio had a significant effect on both IE ($P = 0.0045$, mixed model ANOVA) and EE ($P < 0.0001$, mixed model ANOVA). A negative correlation was observed between IE and core-to-lipid ratio (IE = -74.855x + 115.66, where x is core-to-lipid ratio), where IE decreased from 94.75% to 74.71% at ratios of 1:3 to 1:1, respectively. A positive correlation was observed between EE and core-to-lipid ratio (EE = 5.6106x + 1.1573, where x is core-to-lipid ratio), where EE increased from 2.33% to 3.68% at ratios of 1:3 to 1:1, respectively. The percentage of LSB composed of water increased from 25% to 48% as ratio increased from 1:3 to 1:1.

There was no evidence that EE for each treatment differed among batches of LSB for each core-to-lipid ratio ($P = 0.414$, mixed model ANOVA). Variance due to batch was 0.26% of total model variance. In contrast, batch variance for IE represented 46.6% of total variance and it had a significant effect on IE ($P = 0.024$).

Experiment 2: effect of core concentration of LSB on inclusion, encapsulation, retention, and delivery efficiencies

Mean IE and EE for LSB in this experiment are depicted in Fig. 1 (c and d). Core concentration had a significant effect on both IE and EE ($P > 0.05$, mixed model ANOVA). The relationship between IE and core concentration was best described by a quadratic equation ($IE = 450.27x^2 - 264.76x + 104.94$, where x is core concentration). Generally, the curve for IE across the range of core OTC concentrations was symmetrical with a maximum IE of 86.16% at 0.5 g mL^{-1} and a minimum of 68.07% at 0.3 g mL^{-1}. Encapsulation efficiency across the range of core concentrations was also best described by a quadratic equation ($EE = 30.8167x^2 + 17.631x + 2.0161$, where x is core concentration). An increase from 3.95% to 18.77% was observed across the range of 0.1 – 0.5 g mL^{-1}. Water-to-LSB ratios decreased from 0.46 – 0.3 across this same range of core concentrations. Batches of LSB made with different core concentrations were not significantly different for IE or EE ($P > 0.05$, mixed model ANOVA) with batch contributing less than 6% of total variance in both cases.

Regression analysis revealed a significant relationship for all treatments between RE and time period for LSB suspensions in water. The relationship was best described by a quadratic curve ($RE = 0.0192x^2 - 1.8566x + 77.9716$, where x is core concentration; Fig. 2). Retention efficiency was unaffected by treatment ($P > 0.05$, mixed model ANOVA). Generally, a rapid decrease in RE (~60%) was observed in the first 30 min of aqueous suspension followed by slight decrease after 60 min. The effect of time on DE was similar to its effect on RE, as shown by a progressive

decrease over 60 min ($P < 0.001$, mixed model ANOVA; Fig. 3). A significant batch x treatment interaction was found ($P = 0.027$). Increasing core concentration resulted in higher DE ($P < 0.001$, mixed model ANOVA). Lipid spray beads made with a core concentration of 0.5 g OTC mL^{-1} H_2O had the highest DE after 60 minutes of aqueous suspension (7.9 ± 0.7% w/w; $P < 0.05$, Tukey-Kramer multiple comparison). Regression analysis revealed a significant batch-to-batch variation in RE ($P = 0.0003$, mixed model ANOVA). There was weak evidence that these same batches differed concerning DE ($P = 0.0282$, mixed model ANOVA), where batch variance made up 20.7% of total variance. Table 2 shows RE and DE values after 60 min aqueous suspension.

Experiment 3: acceptability and breakdown of LSB

Lipid spray beads containing OTC were ingested readily by first-feeding zebrafish larvae. Percent feeding incidence of these particles was similar to that for water LSB and Microfeast ($P > 0.05$, Tukey's HSD; Fig. 4). Conversely, gut fullness differed by treatment ($P < 0.001$, one-way ANOVA). Larvae fed OTC LSB ate more particles than those fed Microfeast but less than those fed water LSB ($P < 0.05$, Tukey's HSD; Fig. 5). In contrast, feeding incidence and gut fullness for goby larvae did not differ between the two LSB treatments ($P > 0.05$, Tukey's HSD; Figs. 4, 5).

First-feeding zebrafish and goby larvae successfully digested LSB as indicated by free riboflavin in the gut lumen and release of free riboflavin from the anus (Fig. 6).

Table 1

Equations used to calculate inclusion, encapsulation, retention, and delivery efficiencies

LSB characteristic	Equation
Inclusion efficiency	$\left[\frac{\left(\frac{\text{g Encapsulated OTC}}{\text{g Post - spray lipid}}\right)}{\left(\frac{\text{g Pre - spray OTC}}{\text{g Pre - spray lipid}}\right)}\right] \text{x } 100$
Encpasulation efficiency	$\left(\frac{\text{g Encapsulated OTC}}{\text{g LSB}}\right) \text{x } 100$
Retention efficiency	$\left(\frac{\text{Initial OTC} - \text{Leaked OTC}}{\text{Initial OTC}}\right) \text{x } 100$
Delivery efficiency	$\left(\frac{\text{g Encapsulated OTC}}{\text{g LSB}}\right) \text{x } 100$, post aqueous suspension

Encapsulated OTC is total OTC content minus OTC removed in a quick rinse.

Table 2

Lipid spray bead compositions used in experiments 1 and 2

Exp. #	Core-to-Lipid ratio v/v	Core Concentration (g OTC mL^{-1} H_2O)
1	1:3	0.1
	1:2	0.1
	1:1.5	0.1
	1:1.25	0.1
	1:1	0.1
2	1:1.25	0.1
	1:1.25	0.2
	1:1.25	0.3
	1:1.25	0.4
	1:1.25	0.5

Table 3

Average retention efficiencies (RE) and delivery efficiencies (DE) ±1 SE (*n*=3) of LSB after 60 min aqueous suspension

Core concentration (g OTC mL^{-1} H_2O)	RE w/w (%)	DE w/w (%)
0.1	32.9 ± 8.6	1.5 ± 0.4
0.2	34.5 ± 5.3	3.1 ± 0.5
0.3	36.6 ± 2.0	4.7 ± 0.2
0.4	37.8 ± 3.2	6.5 ± 0.6
0.5	34.3 ± 3.4	7.9 ± 0.7

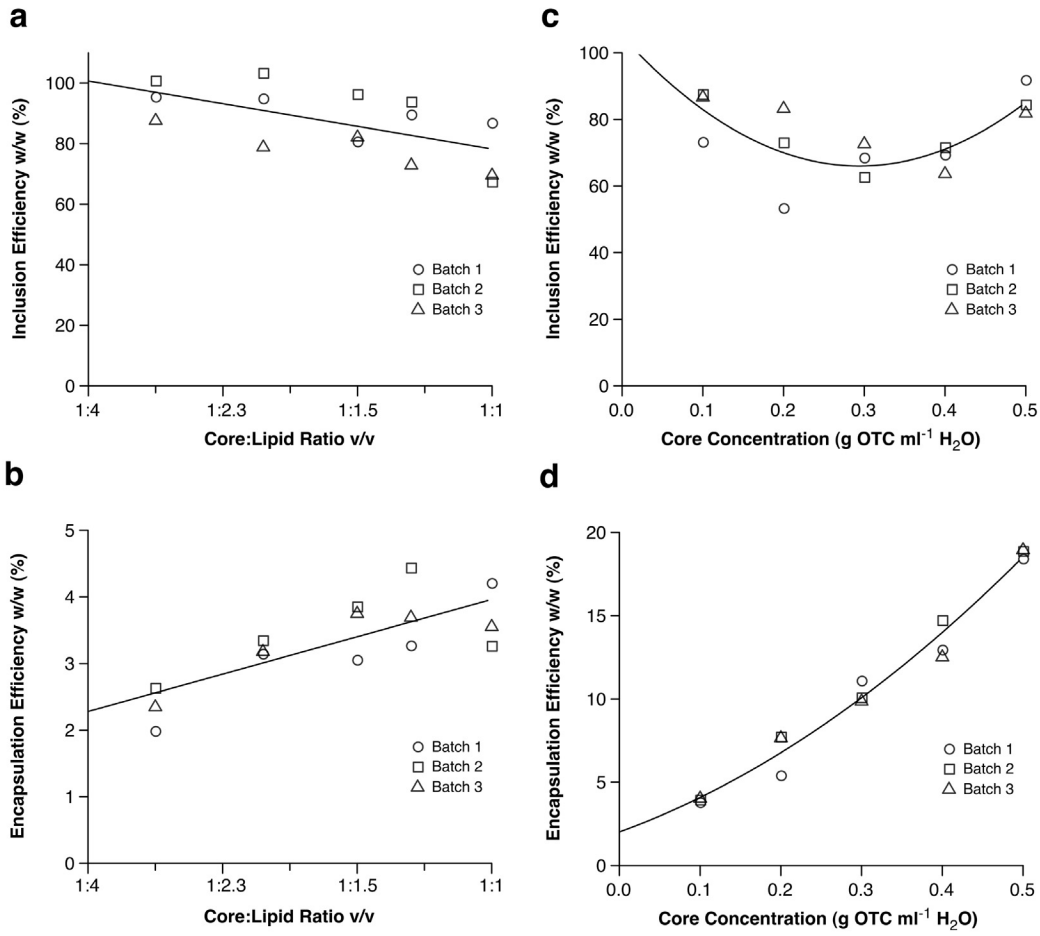

Figure 1
Average inclusion and encapsulation efficiencies for three LSB batches made in either a range of core-to-lipid ratios or core concentrations. (a and b) Three batches of particles were created with an OTC core concentration of 0.1 g mL^{-1} H_2O in a range of core-to-lipid ratios from 1:3 to 1:1 v/v. (c and d) Three batches of particles were created in a range of OTC core concentrations from 0.1 to 0.5 g mL^{-1} H_2O with a core-to-lipid ratio of 1:1.25.

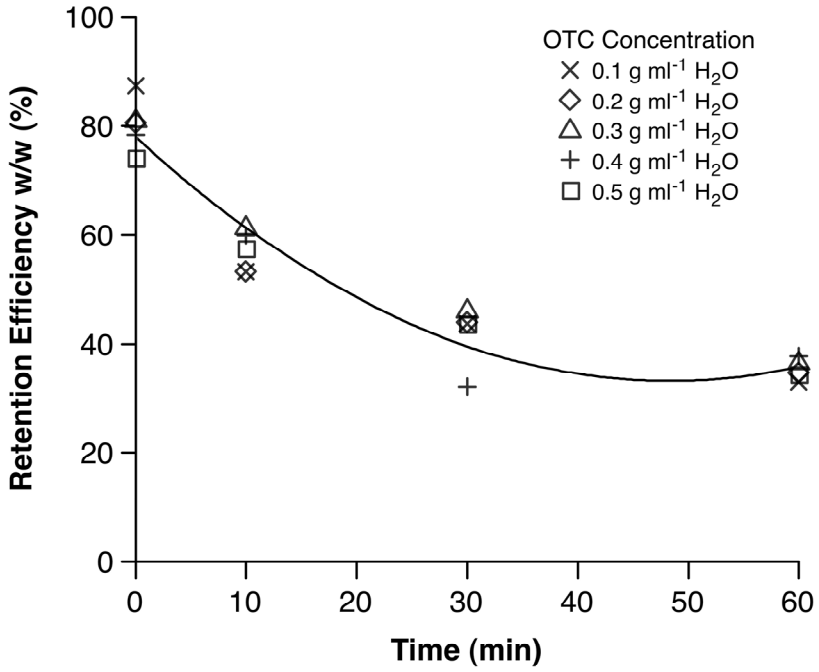

Figure 2
Average retention efficiencies (n = 3) of LSB containing a range of core OTC concentrations over a 1-h period of aqueous suspension. The resulting pattern describes the rate that core material leaked from within LSB. Though individual batches are not shown here, there was a significant batch effect on RE for each treatment over time (P = 0.0003, mixed model ANOVA). The regression line (RE = $0.0192x^2$ - 1.8566x + 77.9716) represents all data. There was no effect of treatment on RE over time (P > 0.05, mixed model ANOVA).

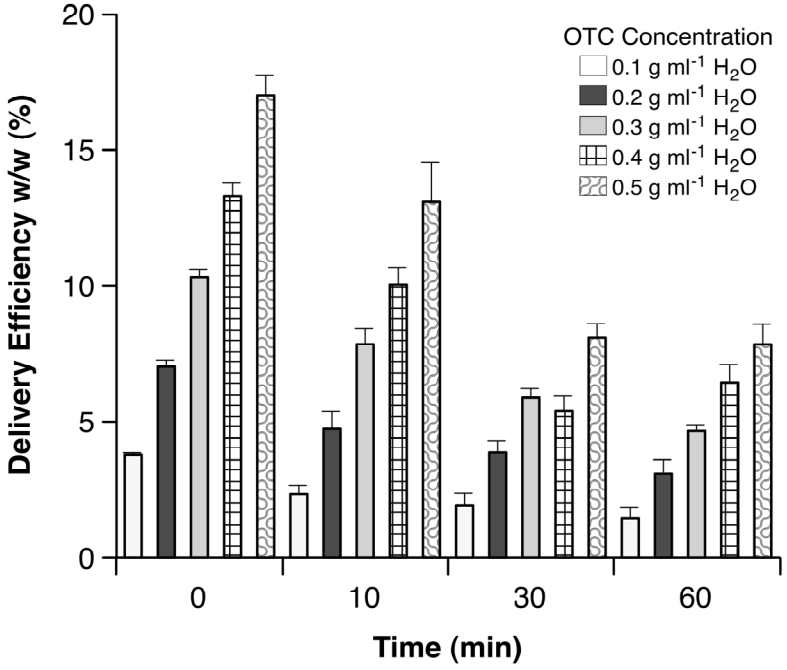

Figure 3
Average delivery efficiencies ± 1 SE (n = 3) of LSB containing a range of core OTC concentrations at various intervals of time for LSB in aqueous suspension. DE is the w/w percentage of LSB that is OTC.

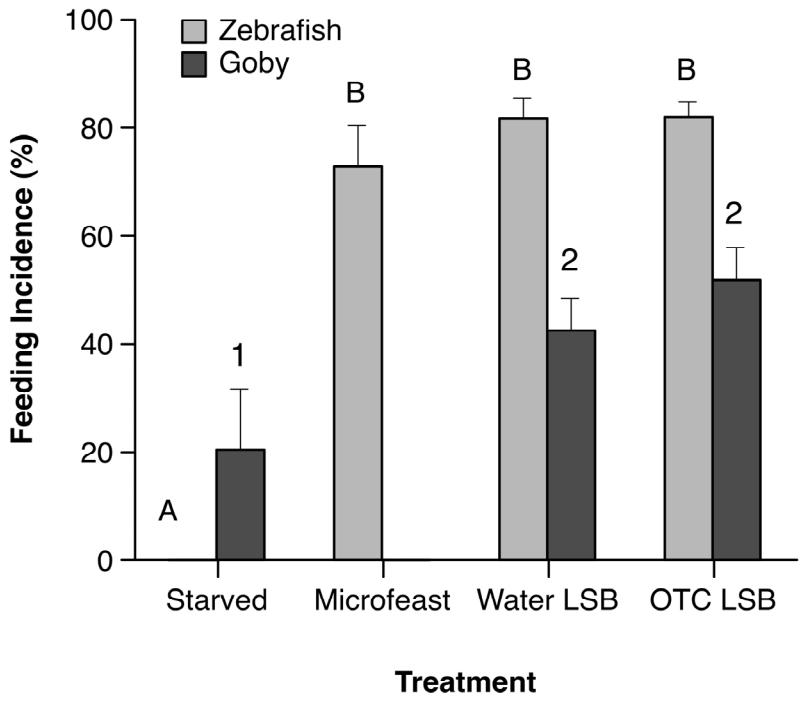

Figure 4
Average (n=3) feeding incidences ± 1 SE observed in larval zebrafish and gobies. Three particle types were separately fed to zebrafish larvae and two types fed to goby larvae in two-hour feeding trials. The observed feeding incidence of starved goby larvae is the result of pre-conditioning the larvae on a diet of the microalgae *Isochrysis galbana* 24 h prior to experimentation. Letters and numbers denote significant differences among zebrafish and goby treatments, respectively (Tukey's HSD, $P < 0.05$).

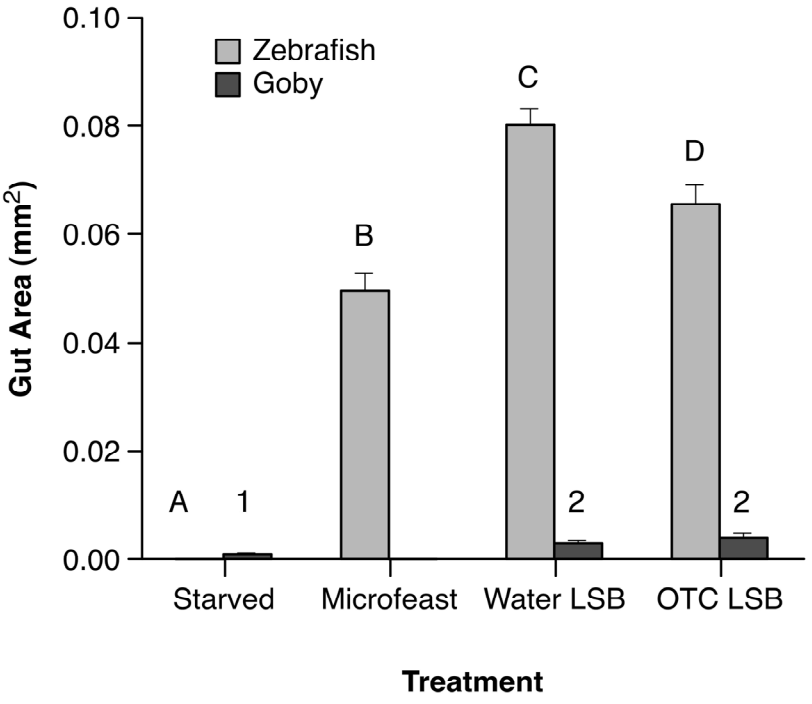

Figure 5
Average (n=50) areas ± 1 SE of food items in guts of larval zebrafish and goby treatment groups. Three particle types were tested with zebrafish larvae and two types with goby larvae in a two-hour feeding trial. Letters and numbers denote significant differences among zebrafish and goby treatments, respectively (Tukey's HSD, $P < 0.05$).

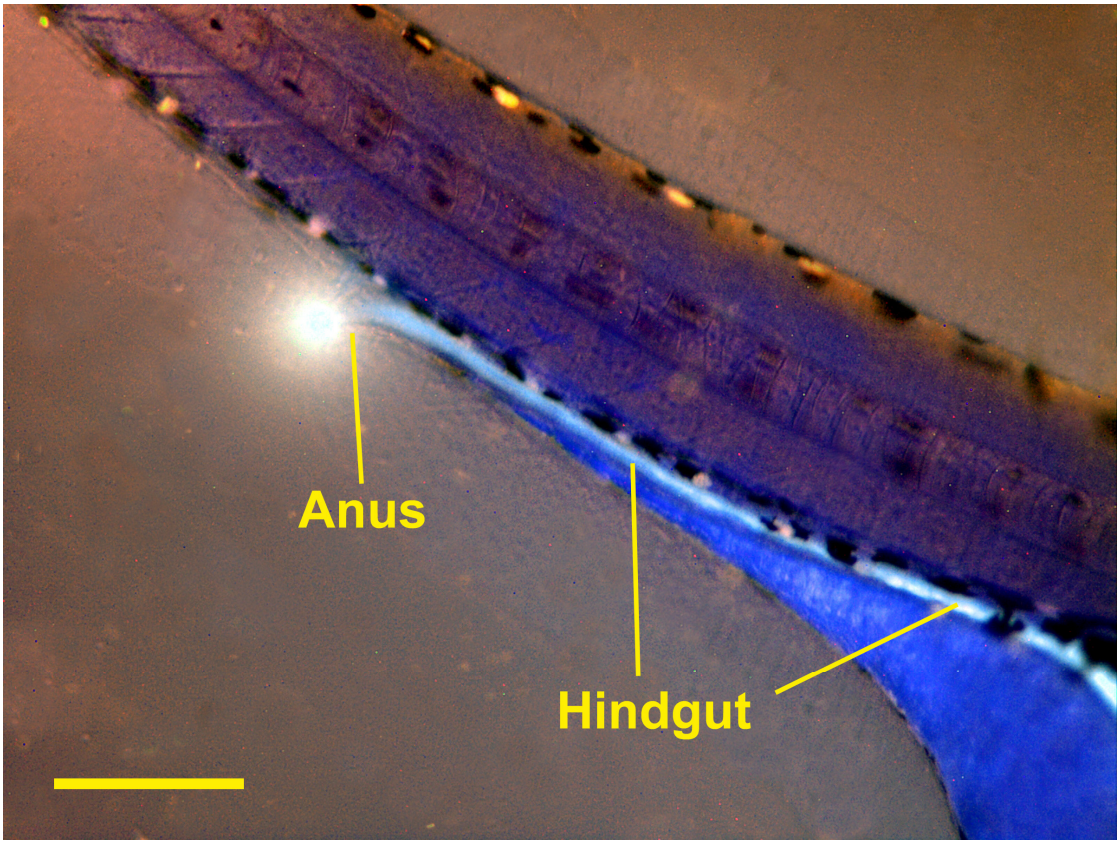

Figure 6
Lipid spray beads with a 1:1.25 core-to-lipid ratio of aqueous riboflavin and menhaden stearine were fed to 172 hpf *Danio rerio* for 2 h. Free riboflavin fluoresced white to bluish-white and was seen flowing through the hindgut and exiting the anus (bright spot) indicating digestion of LSB. Scale=200 µm.

Discussion and Conclusion

One of the major goals in microparticle production is to increase delivery efficiency (DE) by improving inclusion, encapsulation, and retention efficiencies (IE, EE, and RE). We produced lipid spray beads (LSB) containing oxytetracycline·HCl (OTC) with a range of core-to-lipid ratios and core concentrations and determined which combination of these parameters ultimately produced the highest encapsulation and delivery efficiencies for OTC. Increasing the core-to-lipid ratio to 1:1.25 v/v (or 0.44) and core concentration to 0.5 g OTC mL^{-1} H_2O resulted in a DE of 7.9 ± 0.7% after 60 min aqueous suspension. A ratio of 1:1.25 v/v was selected because it was the highest ratio that provided a stable pre-spray emulsion. The core-to-lipid ratio of the pre-spray mixture could not be increased beyond 1:1 v/v because at these high core-to-lipid ratios, the mixture separated into lipid and aqueous fractions within 60 s of sonication and required constant agitation until sprayed. If not mixed well, the emulsion reversed to create LSB with the aqueous OTC solution surrounding droplets of menhaden stearine.

The concentration of the core solution was limited by the water solubility of OTC. At room temperature and without sonication, the maximum water solubility of OTC was 0.1 g OTC mL^{-1}. A solution at this concentration needed to be used within an hour because OTC would precipitate out of solution rendering it useless as an aqueous core. Oxytetracycline crystals were not observed within LSB suggesting that, once encapsulated, OTC did precipitate from solution. This is significant because OTC is taken up in the intestine in aqueous solution (Lunestad 1992).

Encapsulation efficiency values obtained here are comparable to those found in

the literature. Twelve-fold and 33-fold increases in EE were observed, compared with results reported by Langdon & Buchal (1998) and Buchal & Langdon (1998), respectively. Both papers defined EE as mg core material g^{-1} lipid at 0 h aqueous suspension, RE as percent of core material remaining after 24 h aqueous suspension, and DE as EE multiplied by RE. Buchal & Langdon (1998) created spray beads made of tripalmitin and triolein containing OTC and reported EE of 11.9 ± 2.3 mg core g^{-1} lipid , RE of 35.9 ± 10.6% w/w, and DE of 4.1 ± 0.8 mg core g^{-1} lipid. Langdon & Buchal (1998) found that lipid-walled microcapsules made with tripalmitin and fish oil had an EE of 31.5 ± 5.3 mg core g^{-1} lipid, RE of 30.1 ± 3.6% w/w, and DE of 9.4 ± 1.0 mg core g^{-1} lipid (Langdon & Buchal 1998). By fitting our data to their equations, LSB created in this study with a core concentration of 0.5 g OTC mL^{-1} H_2O had an EE of 401.6 mg core g^{-1} lipid, RE of 34.3 ± 3.4% w/w, and theoretical DE of 137.8 mg core g^{-1} lipid. Retention and delivery efficiencies in this study characterize LSB after 60 min aqueous suspension, which prohibits direct comparisons with other reports where particles were suspended in water for 24 h. It is possible, however, that RE and DE for LSB here after 24 h could exceed previously reported values. All bead types showed rapid leakage rates of OTC in the first 30 minutes after suspension but little loss from 30 to 60 min. It is believed that droplets of OTC near the surface of LSB diffused into ambient water during the first 30 min while droplets near the center remained intact and protected until 60 min. Considering its behavior between 30 and 60 min, RE could continue stable until 24 h resulting in a 13-fold increase in DE over results reported by Langdon & Buchal (1998).

Addition of OTC to LSB resulted in a small but significant decrease in

ingestion by larval fish. Zebrafish and gobies fed on OTC LSB demonstrated % feeding incidence similar to those fed on water-containing LSB. First-feeding goby larvae did not surpass 40% feeding incidence when fed on the microalgae *Isochrysis galbana* (Parke; clone T-Iso) for 2 h (personal observation) and, since their natural prey are unknown, we have no information on natural feeding incidence. Previous studies showed that OTC had a negative effect on diet palatability for adult fish (Rigos et al. 1999; Treves-Brown 2000). It has also been shown that many larvae respond to chemical cues during prey selection (Cahu & Zambonino Infante 2001; Kolkovski 2001). Larval gobies did not show either of these responses to the presence of OTC. However, the lower gut fullness observed in larval zebrafish fed on OTC LSB relative to those fed on water-containing LSB suggested that the chemical signal of OTC decreased LSB palatability. However, it did not prevent larvae from receiving the minimum recommended dosage of OTC.

A range of concentrations from 5 to 120 mg OTC L^{-1} H_2O is recommended for immersion treatment of fish (Treves-Brown 2000). Consider a hypothetical situation where 10 larvae are stocked in 500 mL H_2O. Treating these fish through immersion would require from 2.5 to 60 mg OTC. The recommended oral dose of OTC for adult fish is 75 mg kg^{-1} day^{-1} in saltwater and 50 mg kg^{-1} day^{-1} in freshwater (Treves-Brown 2000). Larval zebrafish, weighing about 200 ng (Jacob et al. 2002), would need to receive 0.01 ng OTC to receive such a dosage. The average diameter for LSB is 10.1 ± 5.4 μm. A LSB of this size would weigh 0.55 ng and contain 0.104 ng OTC. A zebrafish larva would need to ingest only 27.8% of one LSB to meet the minimum oral dosage of 0.01 ng OTC even after 60 minutes in aqueous suspension. The amount

of OTC added to the system in this hypothetical case is 10^6 times less than required using an immersion treatment. Lowering the amount of OTC added to the environment could aid in reducing the well-documented spread of antibiotic-resistant bacteria.

Oxytetracycline has been reported to be toxic to some fish while non-toxic to others. Bumguardner & King (1996) reported an LC_{50} of 597 mg L^{-1} for juvenile striped bass, *Morone saxatilis* (Walbaum), exposed to OTC for 6 h. Marking, Howe & Crowther (1988) showed that OTC was not toxic to lake trout, *Salvelinus namaycush* (Walbaum), when they were exposed to water baths with OTC concentrations ranging from 200 – 800 mg L^{-1}. This range provided OTC to the fish in excess of recommended therapeutic doses (55 mg OTC kg^{-1} day^{-1}). Similarly, preliminary experiments in our lab showed no mortalities of larval zebrafish when fed OTC LSB at a dose of 2.8 x 10^6 mg OTC kg^{-1} day^{-1} for three days. This dosage was well above standard recommendations and the results suggested OTC might not be toxic to larval zebrafish.

Particle size is an important characteristic of LSB. When added to water they form aggregations much larger than many first-feeding larvae can ingest. For this reason it is important to establish and maintain particle separation. The 2% gum arabic + 2% REAX 85A (GR) solution showed excellent dispersing properties for OTC LSB, reducing their hydrophobicity and allowing them to readily disperse in both fresh and saltwater. Once dispersed, LSB remained suspended in an undisturbed water column for up to 30 min. In this study, gentle agitation was applied every 30 min with a transfer pipette to resuspend LSB; however, other agitation systems could be used in large-scale facilities. The use of this dispersing mixture eliminated the need for further

processing of LSB, such as coating with zein (Önal & Langdon 2004a). The optimal dispersing agents may vary, depending on particle type; for example, while the GR solution effectively dispersed OTC and riboflavin LSB, water-containing LSB were better dispersed with a 2% solution of polyvinyl alcohol.

Gum arabic, a gum exudate from *Acacia senegal* (L.) trees, has been used in food, plastics, and other industries (Albihn et al. 1986). Gum arabic emulsifies and stabilizes suspensions and dispersions (Randall et al. 1988; Imagi et al. 1992; Sanchez, Renard et al. 2002). Though its structure is not yet fully understood, research has shown that it is an arabinogalactan-protein comprised of three fractions: a large, low-protein fraction, a smaller fraction with proteins responsible for emulsification, and a small, protein-deficient fraction (Akiyama et al. 1984; Vandevelde & Fenyo 1985; Anderson & McDougall 1987; Connolly et al. 1988; Randall et al. 1988; Dickinson et al. 1989; Randall et al. 1989; Islam et al. 1997; Garti & Leser 2001). The proteinaceous components give gum arabic good film-forming properties that are unaffected by dilution (Randall et al. 1988; Dickinson et al. 1989; Garti & Leser 2002) while the carbohydrate chains keep oil droplets from coalescing (Randall et al. 1988). Because it doesn't affect the viscosity of emulsions at high concentrations (Islam et al. 1997) and aggregates on solid surfaces (Sanchez et al. 2002), gum arabic could be appropriate to disperse and stabilize suspensions of LSB prior to delivery to larval fish.

Sulfonated lignins have been used in oil recovery (Ng et al. 2003), food and agriculture (Calsamiglia et al. 1996; Tarabanko et al. 2004), cement (Meister 2002), and plastics applications (Albihn et al. 1986). They are polyanionic and hygroscopic

making them useful in dispersion of organic particles where foaming and surface tension reduction are undesirable (Meister 2002). Mixtures of surfactants have previously been used to accomplish particle dispersion in a large variety of applications (Wieland et al. 1993; Mulqueen et al. 2001).

IE, EE, retention efficiency (RE) and delivery efficiency (DE) of different batches of beads were statistically compared in each experiment. Inclusion efficiency and EE in experiment 1 showed no significant batch-to-batch variation ($P > 0.05$). Encapsulation efficiency for LSB with various core concentrations also showed no batch-to-batch variation, whereas IE, RE and DE were significantly different among batches ($P < 0.05$). Figure 7 shows that the behavior of Batch 3 is the likely cause for the significant differences in RE among batches at 60 min for experiment 2. The batch x treatment interaction for DE was also probably due to the different behavior of Batch 3 compared with Batches 1 and 2. It is possible that the third batch was physically compromised during production affecting the final outcome. Handling of the LSB during analysis could have allowed the beads to partially melt resulting in poor bead integrity and faster leakage rates of OTC.

The particles described in this study may be useful in the treatment of bacterial disease in marine and freshwater larvae. Dosages can be controlled by manipulating the OTC core concentration or by changing the dose of administered OTC LSB. Lipid spray beads would be most useful for species where the mortality of an entire larval population represents significant financial loss. Other water-soluble antibiotics, such as flumequine and oxolinic acid, could potentially be encapsulated in LSB for enhanced treatment flexibility. Control of bacterial diseases in other suspension-

feeding marine animals, such as mollusks and crustaceans, could also benefit from treatment with antibiotic-containing LSB.

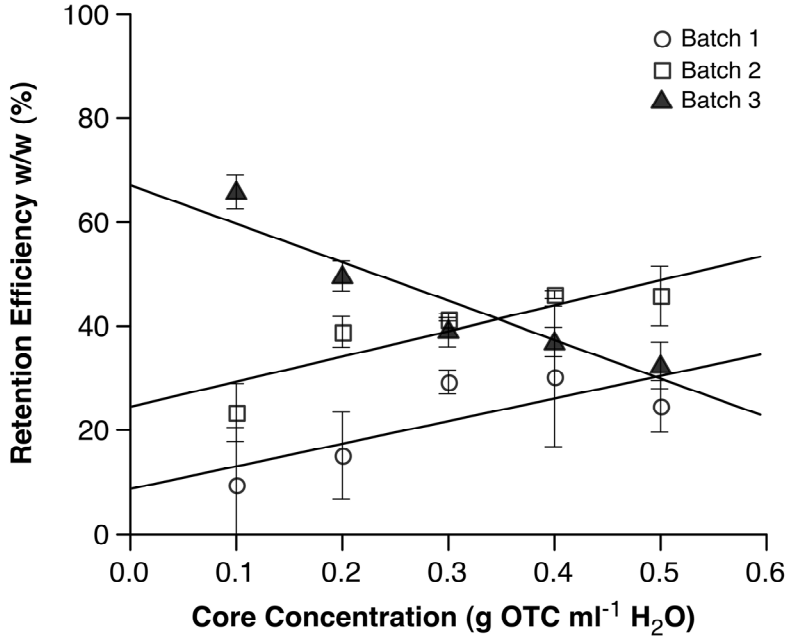

Figure 7
Retention efficiency ± 1 SE of three batches of LSB after 60 min aqueous suspension. Simple regression lines show major differences in leakage rate patterns among the three different batches

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APPENDICES

Appendix A

ANOVA tables and Tukey's tests for experiments described in thesis body

Table A.1

Results from the Mixed model ANOVA and estimates for fixed effects for encapsulation efficiencies of LSB prepared with a range of core-to-lipid ratios from 1:3 to 1:1. The lipid was menhaden stearine and the core was a solution of OTC in water at a concentration of 0.1 g OTC ml^{-1} H_2O.

Source	df (num/den)	Mean Square	Exp MS	Error Term	F-value	*P*-value
xTreatment	1/8	3.577	Var(Residual) + Q(xTreatment, xTreatment*xTreatment,Treatment)	MS(Residual)	20.07	0.0021
xTreatment* xTreatment	1/8	0.641	Var(Residual) + Q(xTreatment*xTreatment,Treatment)	MS(Residual)	3.60	0.0944
Treatment	5/8	0.0057	Var(Residual) + (Q(Treatment)	MS(Residual)	0.03	0.993
Batch	2/8	0.176	Var(Residual) + 5 Var(Batch)	MS(Residual)	0.99	0.414
Residual	8	0.178	Var(Residual)			

Effect	Estimate	Standard error	df	*t*-value	*P* > |t|
Intercept	1.1573	0.5087	13	2.27	0.0405
xTreatment	5.6106	1.2871	13	4.36	0.0008

Table A.2

Results from the Mixed model ANOVA and estimates for fixed effects for inclusion efficiencies of LSB composed with a range of core-to-lipid ratios from 1:3 to 1:1. The lipid was menhaden stearine and the core was a solution of OTC in water at a concentration of 0.1 g OTC ml^{-1} H_2O.

Source	df (num/den)	Mean Square	Exp MS	Error Term	F-value	*P*-value
xTreatment	1/8	636.77	Var(Residual) + Q(xTreatment, xTreatment*xTreatment,Treatment)	MS(Residual)	11.04	0.0105
xTreatment* xTreatment	1/8	67.98	Var(Residual) + Q(xTreatment*xTreatment,Treatment)	MS(Residual)	1.18	0.3093
Treatment	5/8	5.006	Var(Residual) + (Q(Treatment)	MS(Residual)	0.09	0.993
Batch	2/8	270.71	Var(Residual) + 5 Var(Batch)	MS(Residual)	4.69	0.045
Residual	8	57.684	Var(Residual)			

Effect	Estimate	Standard error	df	*t*-value	*P* > |t|
Intercept	115.66	9.1647	12.6	12.62	< 0.0001
xTreatment	-74.8595	21.0621	11	-3.55	0.0045

Table A.3

Results from the Mixed model ANOVA and estimates for fixed effects for encapsulation efficiencies of LSB composed with a fixed core-to-lipid ratio of 1:1.25 and a range of core concentration from 0.1-0.5 g OTC ml^{-1} H_2O.

Source	df (num/den)	Mean Square	Exp MS	Error Term	F-value	*P*-value
xTreatment	1/8	391.42	Var(Residual) + Q(xTreatment,xTreatment*xTreatment,Treatment)	MS(Residual)	530.82	< 0.0001
xTreatment*xTreatment	1/8	3.989	Var(Residual) + Q(xTreatment*xTreatment,Treatment)	MS(Residual)	5.41	0.0485
Treatment	2/8	0.787	Var(Residual) + Q(Treatment)	MS(Residual)	1.07	0.3881
Batch	2/8	0.687	Var(Residual) + 5 Var(Batch)	MS(Residual)	0.93	0.433
Residual	8	0.737	Var(Residual)			

Effect	Estimate	Standard error	df	*t*-value	*P* > |t|
Intercept	2.0161	1.0633	12	1.90	0.0823
xTreatment	17.631	8.1031	12	2.18	0.0503
xTreatment*xTreatment	30.8167	13.25	12	2.33	0.0384

Table A.4

Results from the Mixed model ANOVA and estimates for fixed effects for inclusion efficiencies of LSB composed with a fixed core-to-lipid ratio of 1:1.25 and a range of core concentration from 0.1-0.5 g OTC ml^{-1} H_2O.

Source	df (num/den)	Mean Square	Exp MS	Error Term	F-value	*P*-value
xTreatment	1/8	8.744	Var(Residual) + Q(xTreatment,xTreatment*xTreatment,Treatment)	MS(Residual)	0.11	0.7457
xTreatment*xTreatment	1/8	851.508	Var(Residual) + Q(xTreatment*xTreatment,Treatment)	MS(Residual)	10.98	0.0106
Treatment	2/8	19.416	Var(Residual) + Q(Treatment)	MS(Residual)	0.25	0.7845
Batch	2/8	56.635	Var(Residual) + 5 Var(Batch)	MS(Residual)	0.73	0.5114
Residual	8	77.575	Var(Residual)			

Effect	Estimate	Standard error	df	*t*-value	*P* > |t|
Intercept	104.94	9.9365	12	10.56	< 0.0001
xTreatment	-264.76	75.7227	12	-3.5	0.0044
xTreatment*xTreatment	450.27	123.82	12	3.64	0.0034

Table A.5

Results from the Mixed model ANOVA and estimates for fixed effects for retention efficiencies of LSB composed with a fixed core-to-lipid ratio of 1:1.25 and a range of core concentration from 0.1-0.5 g OTC ml^{-1} H_2O.

Source	df (num/den)	Mean Square	Exp MS	Error Term	F-value	*P*-value
xTime	1/30	14447	Var(Residual) + Q(xTime,xTime*xTime,Treatment*Time)	MS(Residual)	80.74	< 0.0001
xTime*xTime	1/30	3127.7	Var(Residual) + Q(xTime*xTime,Time,Treatment*Time)	MS(Residual)	17.48	0.0002
Treatment	4/8	37.704	Var(Residual) + 4 Var(Batch*Treatment)+ Q(Treatment,Treatment*Time)	MS(Batch*Treatment)	0.15	0.956
Time	1/30	439.41	Var(Residual) + Q(Time,Treatment*Time)	MS(Residual)	2.46	0.128
Batch	2/8	1485.8	Var(Residual) + 4 Var(Batch*Treatment) +20 Var(Batch)	MS(Batch*Treatment)	6.08	0.025
Treatment*Time	12/30	60.458	Var(Residual) + Q(Treatment*Time)	MS(Residual)	0.34	0.975
Batch*Treatment	8/30	244.25	Var(Residual) + 4 Var(Batch*Treatment)	MS(Residual)	1.37	0.251
Residual	30	178.92	Var(Residual)			

Effect	Estimate	Standard error	df	*t*-value	*P* > |t|
Intercept	77.9716	5.5347	3.05	14.09	0.0007
xTime	-1.8566	0.2735	55	-6.79	< 0.0001
xTime*xTime	0.0192	0.0043	55	4.46	< 0.0001

Table A.6

Results from the Mixed model ANOVA and unpaired *t*-tests of differences of least squares means with the Tukey-Kramer adjustment for delivery efficiencies of LSB composed with a fixed core-to-lipid ratio of 1:1.25 and a range of core concentration from 0.1-0.5 g OTC ml^{-1} H_2O.

Source	df (num/den)	Mean Square	Exp MS	Error Term	F-value	*P*-value
Treatment	4/8	151.175	Var(Residual) + 4 Var(Batch*Treatment)+ Q(Treatment,Treatment*Time)	MS(Batch*Treatment)	50.34	<0.0001
Time	3/30	101.444	Var(Residual) + Q(Time,Treatment*Time)	MS(Residual)	38.08	<0.0001
Batch	2/8	17.313	Var(Residual) + 4 Var(Batch*Treatment) +20 Var(Batch)	MS(Batch*Treatment)	5.77	0.028
Treatment*Time	12/30	6.319	Var(Residual) + Q(Treatment*Time)	MS(Residual)	2.37	0.027
Batch*Treatment	8/30	3.003	Var(Residual) + 4 Var(Batch*Treatment)	MS(Residual)	1.13	0.374
Residual	30	2.664	Var(Residual)			

Treatment Comparison	Estimate	Standard error	df	*t*-value	Significance
0.1, 0.2	-2.3023	0.7075	8	-3.25	
0.1, 0.3	-4.8098	0.7075	8	-6.8	S
0.1, 0.4	-6.4228	0.7075	8	-9.08	S
0.1, 0.5	-9.1395	0.7075	8	-12.92	S
0.2, 0.3	-2.5074	0.7075	8	-3.54	S
0.2, 0.4	-4.1205	0.7075	8	-5.82	S
0.2, 0.5	-6.8372	0.7075	8	-9.66	S
0.3, 0.4	-1.6131	0.7075	8	-2.28	
0.3, 0.5	-4.3298	0.7075	8	-6.12	S
0.4, 0.5	-2.7167	0.7075	8	-3.84	S

Time Comparison	Estimate	Standard error	df	*t*-value	Significance
0, 10	2.685	0.596	30	4.51	S
0, 30	5.2495	0.596	30	8.81	S
0, 60	5.5905	0.596	30	9.38	S
10, 30	2.5645	0.596	30	4.3	S
10, 60	2.9055	0.596	30	4.88	S
30, 60	0.3409	0.596	30	0.57	

Table A.7

Results from the one-way ANOVA and Tukey's HSD for feeding incidence observed in zebrafish populations fed on different particle types. S denotes a significant difference at $P < 0.05$.

Source	DF	Squares	Mean Square	F-value	*P*-value
Treatment	3	14154.611	4718.204	79.21	<0.0001
Residual	8	476.532	59.567		

Treatment	Significance
Water LSB, OTC LSB	
Water LSB, Microfeast	
Water LSB, Starved	S
OTC LSB, Microfeast	
OTC LSB, Starved	S
Microfeast, Starved	S

Table A.8

Results from the one-way ANOVA and Tukey's HSD for gut fullness observed in zebrafish populations fed on different particle types. S denotes a significant difference at $P < 0.05$.

Source	DF	Squares	Mean Square	F-value	*P*-value
Treatment	3	183559.98	61186.66	153.07	<0.0001
Residual	199	79544.71	399.72		

Comparison	Between Means	95 % Confidence Limits-lower	95 % Confidence Limits-upper	Significance
Water LSB, OTC LSB	14.518	4.261	24.776	S
Water LSB, Microfeast	30.682	20.424	40.94	S
Water LSB, Starved	80.131	69.822	90.44	S
OTC LSB, Microfeast	16.164	5.906	26.422	S
OTC LSB, Starved	65.612	55.303	75.921	S
Microfeast, Starved	49.449	39.14	59.758	S

Table A.9

Results from the one-way ANOVA and Tukey's HSD for feeding incidence observed in goby populations fed on different particle types. S denotes a significant difference at $P < 0.05$.

Source	DF	Squares	Mean Square	F-value	*P*-value
Treatment	2	1549.5	774.75	3.93	0.081
Residual	6	1182.1	197.02		

Treatment Comparison	Significance
Water LSB, OTC LSB	
Water LSB, Starved	
OTC LSB, Starved	
Microfeast, Starved	

Table A.10

Results from the one-way ANOVA and Tukey's HSD for gut fullness observed in goby populations fed on different particle types. S denotes a significant difference at $P < 0.05$.

Source	DF	Squares	Mean Square	F-value	*P*-value
Treatment	2	0.0057	0.002848	6.1	0.004
Residual	59	0.0275	0.000467		

Treatment Comparison	Significance
Water LSB, OTC LSB	
Water LSB, Starved	S
OTC LSB, Starved	S
Microfeast, Starved	S

Appendix B

Gel diet recipe used for maintaining *Asterropteryx semipunctata* broodstock.

Table B.1

Gel diet recipe used for maintaining *Asterropteryx semipunctata* broodstock.

Ingredient	Mass (g)
Lansy Breed Diet	350
Flatfish fillets	300
Squid	200
Krill/Euphasids	120
Fish eggs	150
Spirolina	50
Kelp meal/powder	150
Astaxanthin	3
ß-Carotene	1.5
Vitamins	26
Gelatin	250
Distilled water	1,500

Thaw any frozen items prior to weighing, grind Lansy Breed pellets, vitamins, and minerals, and heat water to 35°C prior to preparation. Add ingredients to a high-powered industrial blender a little at a time to avoid blender damage. Add gelatin as the last ingredient. The water can be added at any time during blending if needed but the bulk should be added with the gelatin to achieve the desired consistency. When desired consistency is reached, pour gel diet into zip lock bags, seal, date, and place in 0°C freezer until needed.

Appendix C

Report on experiments to establish bacterial disease in larval fish.

Introduction

The initial goals of this thesis were to develop lipid spray beads (LSB) to contain as much oxytetracycline (OTC) as possible and then administer them to diseased larvae to test their effectiveness in reducing mortalities due to bacterial disease. Zebrafish have shown susceptibility to *Vibrio anguillarum* via immersion treatment (O'Toole et al., 2004). The following experiments were carried out to confirm this and find a combination of bacteria and fish larvae that would result in observable disease. Bacteria were provided by Paul Reno at Oregon State University and were selected for their various modes of infection, virulence, and susceptibility to OTC.

Materials and Methods

All bacteria, except *Flavobacterium columnare* and *V. tubiashi*, were cultured in trypticase soy broth (TSB) and used after 24 h incubation at 26°C. *Flavobacterium columnare* was cultured in cytophaga broth and used after 48 h incubation at 26°C. *Vibrio tubiashi* was cultured in marine agar and used after 24 h incubation at 26°C.

Experiment 1: Exposure of zebrafish larvae to 5 different bacterial species via immersion

Forty larval zebrafish were stocked in 1 L dechlorinated H_2O at 28°C with

three replicates per treatment. Larvae at 170 h post fertilization (hpf) were exposed to *Streptococcus* sp., *V. anguillarum*, *Yersinia ruckeri*, *F. columnare*, and *Aeromonas hydrophila* for 6 h at 10^6, 10^5, 10^7, 10^4, and 10^6 cfu ml^{-1}, respectively, for 8 h. Larvae were rinsed twice and transferred to 1 L clean water. Mortality was the expected end point of disease and mortalities were counted and removed at day 2 and day 6 post exposure. Treatments were compared with a control consisting of exposure to sterile TSB for 8 h. For this and all other trials, observations ended when all control fish died.

Experiment 2: Exposure of zebrafish larvae to five different bacterial species via immersion after heat shock, sand shock, or both

Zebrafish larvae were stocked as described above. Treatments were duplicated. Fish were exposed to a stress type and then to bacteria. The first stress was exposure to silica sand via tumbling in a test tube. Each tube contained 1 g silica sand and 75 ml H_2O and larvae were tumbled by inverting the tubes 3 full rotations. Any further tumbling resulted in sufficient damage to cause mortality. Heat shock was the second stress. Larvae were exposed to 36°C for 5 minutes and returned to 28°C. The third stress was a combination of sand and heat shock following the above methods. All larvae were exposed to a bacterial species 2 h after exposure to physical stress. Sand, and heat/sand stressed fish were exposed to *F. columnare* and *A. hydrophila* for 8 h at 10^6 cfu ml^{-1} each. Heat stressed fish were exposed to *Streptococcus* sp., *V. anguillarum*, *Y. ruckeri*, *F. columnare*, and *A. hydrophila* for 8 h at 10^6 cfu ml^{-1} each. Controls consisted of larvae exposed to each stress and sterile TSB for 8 h. Mortalities were counted and removed daily

Experiment 3: Exposure of zebrafish larvae to *V. anguillarum* cultured in two ways

Zebrafish larvae were stocked as described above with 3 replicates per treatment and 20 larvae per replicate. Treatments included larvae exposed for 8 h to *V. anguillarum* at a concentration of 10^7 cfu ml^{-1} cultured with an autoclaved fish or bacteria cultured with autoclaved Microfeast (12 mg Microfeast L^{-1} TSB). The control consisted of larvae exposed to sterile TSB for 8 h. Mortalities were counted and removed daily.

Experiment 4: Exposure of zebrafish, glowlight tetra (*Hemigrammus erythrozonus*) and fancy guppy (*Poecilia reticulata*) adults to *V. anguillarum* via injection

To determine the susceptibility of zebrafish, glowlight tetra, and fancy guppies to *V. anguillarum* infection via methods other than immersion, adults were injected with this bacteria. Bacterial broths were diluted to 10^6 with 85 % saline. Fish were anesthetized with tricaine (80 mg L^{-1} H_2O) and injected midway between the anal and pectoral fins with 300 µl of diluted bacterial solution. Juvenile trout were injected similarly as a control. Two fish of each species were also injected with 300 µl of sterile 85 % saline as a control. All fish were fed daily during the experiment.

Experiment 5: Exposure of AB wild-type zebrafish larvae to *V. anguillarum* and *F. columnare* via immersion

Claudia Harper of the Cardiovascular Research Center at Massachusetts General Hospital informed me that they had success infecting AB wild-type zebrafish larvae with *V. anguillarum*. These fish were procured from ZIRC at the University of Oregon. Three replicates were used per treatment with 20 larvae per replicate. Larvae were exposed to *V. anguillarum* and *F. columnare* at 10^8 and 10^6 cfu ml^{-1}, respectively, for 18 h. The control consisted of exposure to sterile TSB for 18 h. Mortalities were counted and removed daily.

Experiment 6: Exposure of AB wild-type zebrafish larvae to *V. anguillarum* via immersion for 2 time periods

F. columnare had no affect on larval mortality and was excluded from further experimentation. Fifteen larvae were stocked in Petri dishes with 40 ml sterile, dechlorinated freshwater at 28°C with three replicates per treatment. Treatments included exposure to *V. anguillarum* at 10^6 cfu ml^{-1} for 4 h and 21 h. Following bacterial exposure, fish were placed in 1 L upweller Imhoff cones (Wheaton), supplied with non-recirculating dechlorinated water at 28°C with 6 exchanges per day. The control consisted of exposure to sterile TSB for 21 h. Mortalities were counted and removed daily.

Experiment 7: Exposure of AB wild-type zebrafish larvae to *V. anguillarum* via immersion for 4 hours

The previous trial suggested a 4 h exposure to *V. anguillarum* produced disease in larval AB zebrafish. Trial 5 was modified by exposing larvae to 10^5, 10^6, $10^{6.5}$, 10^7, $10^{7.5}$, or 10^8 cfu ml^{-1} for 4 h. Controls consisted of untreated fish and fish exposed to sterile TSB for 4 h. Mortalities were counted and removed daily.

Experiment 8: Exposure of Tübingen long fin (TL) zebrafish to *V. anguillarum* via immersion for 2 time periods

TL zebrafish larvae acquired from ZIRC were submitted to several treatments. Larvae were stocked at 50 per replicate with three replicates per treatment. The first treatments were exposure to *V. anguillarum* at 10^6 cfu ml^{-1} for 20 and 4 h. Other treatments were exposure to *V. anguillarum* at 10^6 cfu ml^{-1} for 20 and 4 h followed by feeding on LSB containing OTC (core of 0.5 g OTC ml^{-1} H_2O, core:lipid ratio of 1:1.25). 50 mg LSB were suspended in 3 ml GR (2% gum arabic + 2% REAX 85A in distilled H_2O) and fed to larvae three times daily for three days. The final treatments involved feeding on LSB containing OTC three times over 12 h followed by exposure to *V. anguillarum* for 20 or 4 h. Controls consisted of exposure to sterile TSB for 20 h or feeding larvae on LSB containing OTC three times over 12 h followed by exposure to sterile TSB for 20 h. Mortalities were counted and removed daily.

Experiment 9: Exposure of AB wild-type zebrafish larvae to *V. tubiashi* via immersion

The methods used in Trial 6 were repeated with a range of *V. tubiashi* concentrations. Larvae were exposed to 10^4, 10^5, 10^6, or 10^7 cfu ml^{-1} for 4 h. Controls consisted of untreated larvae and larvae exposed to sterile marine broth for 4 h. Mortalities were counted and removed daily.

Results

Experiment 1: Exposure of zebrafish larvae to 5 different bacterial species via immersion

All treatments demonstrated the same mortality rate as the control ($P > 0.05$, Kaplan-Meier survival analysis).

Experiment 2: Exposure of zebrafish larvae to 5 different bacterial species via immersion after heat shock, sand shock, or both

All treatments demonstrated the same mortality rate as the control ($P > 0.05$, Kaplan-Meier survival analysis).

Experiment 3: Exposure of zebrafish larvae to *V. anguillarum* cultured in two ways

All treatments demonstrated the same mortality rate as the control ($P > 0.05$, Kaplan-Meier survival analysis).

Experiment 4: Exposure of zebrafish, glowlight tetra (*Hemigrammus erythrozonus*) and fancy guppy (*Poecilia reticulata*) adults to *V. anguillarum* via injection

All control fish lived for at least 2 months showing no signs of disease. All juvenile trout died within 5 days showing signs of hemorrhaging. Control guppies showed no signs of disease and lived for at least 3 weeks. Treated guppies died within 3 days with major hemorrhaging below the skin near the gills and pectoral fins. Treated zebrafish lived for at least 2 months when observations ended showing no signs of disease. Treated glowlight tetra also lived for at least 2 months showing no signs of disease.

Experiment 5: Exposure of AB wild-type zebrafish larvae to *V. anguillarum* and *F. columnare* via immersion

Larvae treated with *F. columnare* demonstrated the same mortality rate as the control while those treated with *V. anguillarum* demonstrated a slightly steeper rate though not significantly different from the control (Fig. D.1; $P > 0.05$, Kaplan-Meier survival analysis).

Experiment 6: Exposure of AB wild-type zebrafish larvae to *V. anguillarum* via immersion for 2 time periods

I performed this experiment to verify the results of Trial 4. A near 50 % mortality was observed in all control and 21-h containers within the first 12 hours. These mortalities were censored for Kaplan-Meier survival analysis. The 4 h exposure

resulted in a higher mortality rate than the 21 h exposure (Fig. D.2).

Experiment 7: Exposure of AB wild-type zebrafish larvae to *V. anguillarum* via immersion for 4 hours

Kaplan-Meier survival analysis revealed a significant difference between treatments ($P < 0.0001$). However, the pattern was not consistent across the range of bacterial concentrations (Fig. D.3). It suggested a concentration of 10^6 cfu ml^{-1} caused a higher mortality rate than 10^7 cfu ml^{-1} and that the control caused a higher mortality rate than 10^4 cfu ml^{-1}.

Experiment 8: Exposure of Tübingen long fin (TL) zebrafish to *V. anguillarum* via immersion for 2 time periods

All treatments demonstrated the same mortality rate as the control ($P > 0.05$, Kaplan-Meier survival analysis). There was no evidence that TSB caused mortality of larvae.

Experiment 9: Exposure of AB wild-type zebrafish larvae to *V. tubiashi* via immersion

All treatments demonstrated the same mortality rate as the control ($P > 0.05$, Kaplan-Meier survival analysis).

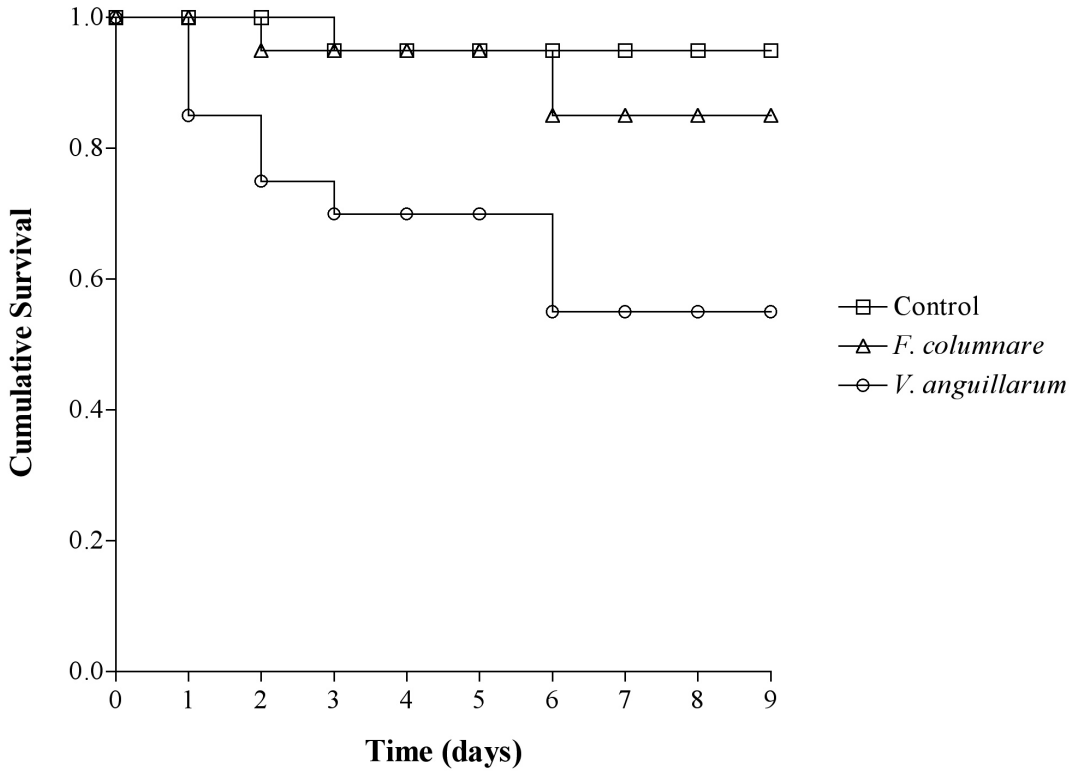

Figure C.1
Kaplan-Meier analysis of survival of AB wild-type larval zebrafish exposed to *V. anguillarum* and *F. columnare* at 10^8 and 10^6 cfu ml^{-1}, respectively, for 18 h.

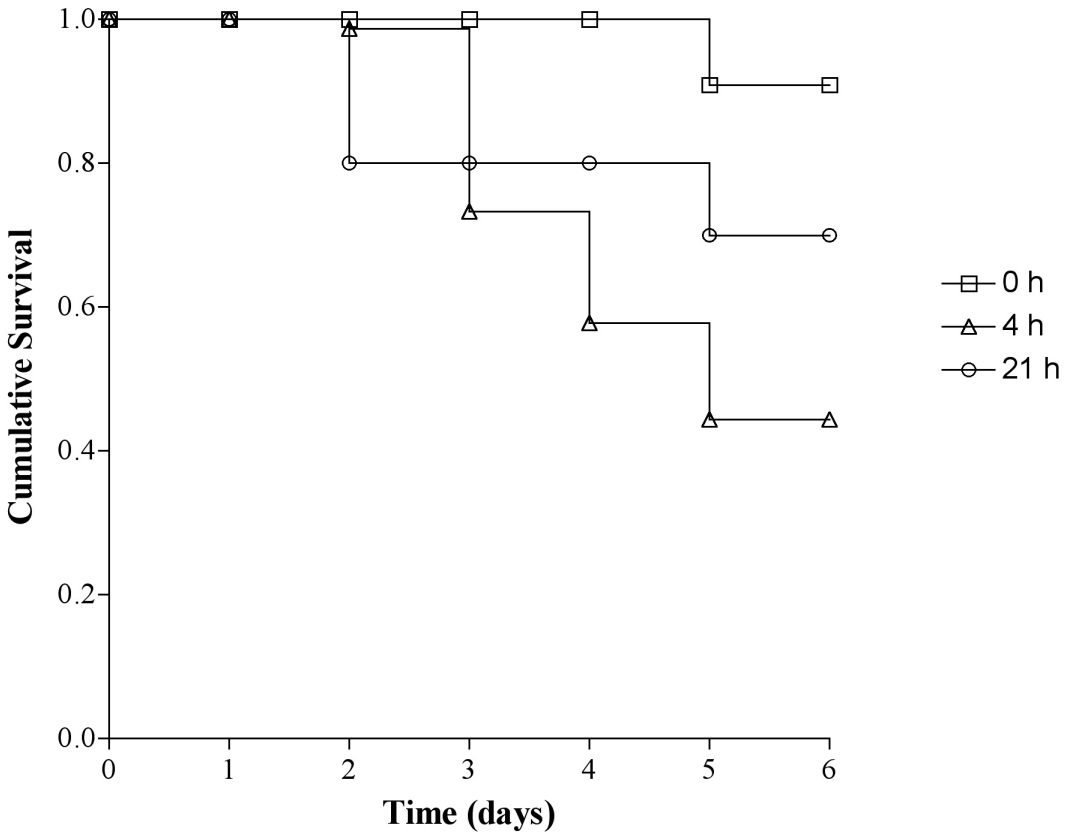

Figure C.2
Kaplan-Meier analysis of survival of AB wild-type larval zebrafish exposed to *V. anguillarum* for 4 and 21 h.

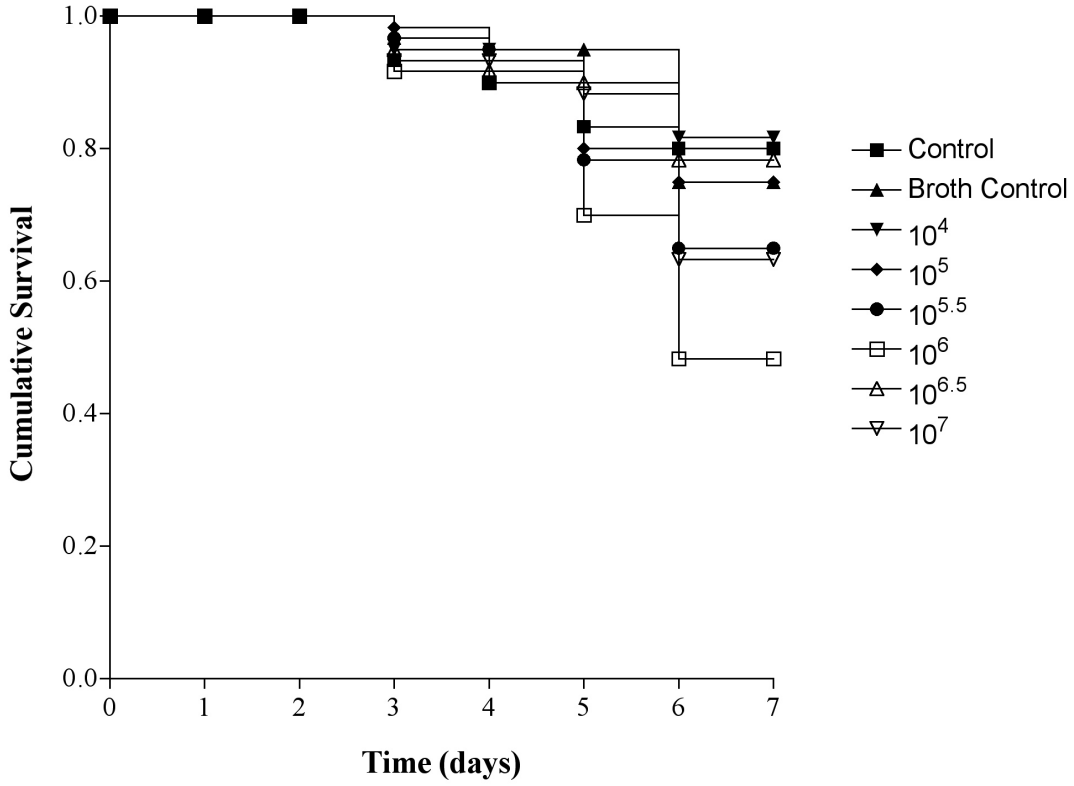

Figure C.3
Kaplan-Meier analysis of survival of larval AB wild-type zebrafish exposed to a range of *V. anguillarum* concentrations from 10^4 to 10^7 cfu ml^{-1} for 4 h.

Discussion

The first two trials attempted to find a bacterial species that would infect larval zebrafish. The five species selected invade fish either through the gills, skin, or ingestion and have all demonstrated high virulence and can cause high mortality rates in fish populations. However, none of the species was able to cause an observable disease in larval zebrafish. The reasons for this are unknown and baffling. I continued trials with zebrafish because of previous reports discussed their susceptibility to bacterial disease (O'Toole et al., 2004; van der Sar et al., 2004). In particular, O'Toole et al. discussed infection with *V. anguillarum*, which we had at our facilities and is susceptible to OTC.

Initial trials were performed on larvae from adults of an unknown strain purchased from a pet store. These trials were fruitless suggesting common zebrafish may be sufficiently hardy to resist bacterial infection. Claudia Harper (Cardiovascular Research Center, Massachusetts General Hospital, Boston, MA) suggested I try the AB wild-type strain of zebrafish because of their susceptibility due to decades of inbreeding resulting in weakened immune defenses. Her lab has been able to establish infections in AB larvae resulting in mortality. My trials involving AB larvae seemed to show that larvae were succumbing to exposure to *V. anguillarum*. However, these results were not replicable as a comparison of the curves in Figs. D.1, D.2, and D.3 demonstrates. The positive results shown in Figs. D.1 and D.2 were probably affected by the number of censored fish in the three analyses. Fish were censored if their mortality was not suspected of being caused by bacteria. In all the above trials, fish were censored if they died within 24 h of exposure to bacteria or on the same day as

the last control fish. The mortality rate of fish exposed to *V. anguillarum* was not significantly different from those exposed to *F. columnare* but the data suggested a possible difference (Fig. D.1). A second trial involved V. anguillarum alone at two exposure times and demonstrated a significant difference between treatments and the control. However, the analysis of these data was weakened by the large number of censored fish at the start of the experiment. The number of comparable mortalities was reduced and the results are probably falsely positive. This conclusion is supported by the results of the final experiment on AB zebrafish where a range of *V. anguillarum* concentrations was unable to produce an observable infection in larvae (Fig. D.3). ZIRC suggested I try TL zebrafish because they are also suspected of having weak immune systems. All trials involving this strain were also unsuccessful in demonstrating signs of bacterial disease.

I investigated other fish species' susceptibility to infectious bacteria by injecting adults with *V. anguillarum*. Of these, only fancy guppy adults and juvenile trout died of bacterial disease as demonstrated by mortalities and heavy hemorrhaging below the skin. Trials on guppy larvae were never performed due to difficulties in obtaining guppy larvae. Future research should investigate the use of guppy larvae further as a freshwater model for testing the utility of LSB containing OTC in reducing mortalities due to bacterial disease.

Bibliography

O'Toole R., von Hofsten J., Rosqvist R., Olsson P.-E. & Wolf-Watz H. (2004) Visualization of zebrafish infection by GFP-labelled *Vibrio anguillarum*. *Microbial Pathogenesis* **37**, 41-46.

van der Sar A.M., Appelmelk B.J., Vandenbroucke-Grauls C.M.J.E. & Bitter W. (2004) A star with stripes: zebrafish as an infection model. *Trends in Microbiology* **12**, 451-457.

Appendix D

Recipe for 0.5 x E2 embryo medium.

This recipe was acquired from the Zebrafish International Resource Center (ZIRC) at the University of Oregon, USA. Typically, three stock solutions are prepared, called E2A, E2B and E2C. These are easily stored and used to make a large volume of the 0.5X E2.

E2A:

140.0g NaCl
6.0g KCl
19.2g $MgSO_4$
3.3g KH_2PO_4
1.1g Na_2HPO_4

Add Millipore water to 1600 mL. Shake and stir to dissolve the reagents. Autoclave and stir to dissolve any precipitation that has formed during autoclaving. Store at 4°C.

E2B:

Dissolve 11.0 g $CaCl_2$ (or 14.6g $CaCl_2$ x $2H_2O$) in the final volume of 200 mL. Shake to dissolve the reagent. Autoclave and divide into 20 mL aliquots (in 50 mL tubes). Store at -20°C.

E2C:

Dissolve 6.0g $NaHCO_3$ in the final volume of 200 mL. Shake to dissolve the reagent. Autoclave and divide into 20 mL aliquots (in 50 mL tubes). Store at -20°C.

0.5X E2:

100 mL **E2A**
20 mL **E2B**
20 mL **E2C**

Add reverse osmosis water to 19 L. Adjust pH to 7.0 – 7.5 (with concentrated HCl or concentrated NaOH). Add reverse osmosis water to 20 L. Store at room temperature.