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Evaluating Liposomes for the Delivery of Oxytetracycline to Oyster Larvae (*Crassostrea gigas*) and Subsequent Effects on Resistance to *Vibrio coralliilyticus*

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ABSTRACT

Despite ongoing research, improved methods for preventing and treating bacterial infections in aquaculture are needed to improve hatchery production of oysters. Present bacterial treatments often require the use of large quantities of antibiotics dissolved in culture water, a method that is wasteful and may promote antibiotic-resistant bacteria. In this research, we investigated the use of microparticles (liposomes) for direct delivery of antibiotics to the Pacific oyster (*Crassostrea gigas*) and evaluated subsequent effects on larval resistance to *Vibrio coralliilyticus* (*V. cor*). By feeding larval oysters fluorescently-tagged liposomes, we determined that two-day old and nine-day old larvae readily ingest and at least partially digest liposomes, releasing water-soluble antibiotics into their guts. Oxytetracycline (OTC), a water-soluble antibiotic shown to be highly effective against *V. cor.* at low concentrations, was successfully encapsulated within liposomes. However, when oyster larvae were fed liposomes containing OTC and exposed to *V. cor.*, larval survival did not increase. Moreover, while larvae were shown to take up dissolved OTC from the culture water, pretreatment with dissolved OTC did not increase larval resistance to *V. cor.* These results indicate that more research is needed to evaluate if prophylactic ingestion of antibiotics is an effective protection against bacterial infections. Despite the inability of antibiotic liposomes or pretreatments with dissolved OTC to increase larval survival, this research suggests that liposomes may be used to efficiently deliver other water-soluble compounds, such as dietary supplements, to young oyster larvae.

1. Introduction

Molluscan aquaculture, valued at US$19 billion globally, accounts for over 21.8% of the world's total aquaculture production by weight (FAO, 2016). The global production of the Pacific oyster (*Crassostrea gigas*) alone is over 4.38 million tonnes, greater than any other species of fish or molluscs (FAO, 2014). However, the outbreak of bacterial pathogens in hatcheries frequently results in the losses of entire stocks of young fish and shellfish, including Pacific oyster larvae. In particular, many bacteria are opportunistic pathogens and affect young animals suffering from stresses in the environment, such as changes in temperature, pH, salinity, feeding, upwelling, or overcrowding (Richards, 2014). Controlling these conditions and containing infections in crowded hatcheries is difficult, making cultures of young fish and shellfish especially susceptible to bacterial pathogens.

Recent studies have linked changing environmental conditions, such as climate change and ocean acidification, to the proliferation of opportunistic pathogens. In 2007, the upwelling of cold, nutrient-rich seawater into warm, surface seawater on the North American Pacific Coast

was associated with an outbreak of vibriosis that decreased larval oyster production in West Coast hatcheries by as much as 59% (Elston et al., 2008). A similar upwelling event and *Vibrio* epidemic occurred in a Netarts Bay hatchery in 1998 (Elston et al., 2008). The species of marine bacteria credited with these outbreaks was *Vibrio tubiashii,* now renamed *Vibrio coralliilyticus* (*V. cor.*). *V. cor.* has also been associated with mortality events in Eastern oyster larvae and the European flat oyster (Richards et al., 2015). The infection of oyster larvae with *V. cor.* typically results in detached vela, necrotic soft tissue, and mortality within 48 hours of exposure (Hasegawa et al., 2008).

Antibiotics are widely used in aquaculture to prevent and treat bacterial infections. However, the use of antibiotics in aquaculture can be problematic in several ways, much as it has been in agriculture. Dissolving large quantities of antibiotics in seawater allows bacterial pathogens to develop resistance to these antibiotics over time, making future outbreaks difficult to treat (Kümmerer, 2009). Furthermore, according to the World Health Organization, 39 of the 51 antibiotics most used in agriculture and aquaculture are also important in human medicine, and developed resistance may pose serious issues for treating infections in humans (Done et al., 2015). Given these issues, it is important to find more efficient methods for delivering antibiotics to oyster seed, especially in anticipation of upwelling events or bacterial blooms. Oxytetracycline hydrochloride, sulfadimethoxine, and florfenicol are the only three antibiotics approved for use in aquaculture by the U.S. Food and Drug Administration (FDA, 2017). Oxytetracycline (OTC) is currently the most widely used antibiotic for all major seafood species raised in aquaculture (Done et al., 2015). OTC is a bacteriostatic antibiotic that binds the 30S ribosomal subunit and inhibits protein synthesis in a wide range of bacteria, including *Vibrio* spp. (Romero et al., 2012). Doxycycline, another tetracycline antibiotic, and streptomycin also block protein synthesis in bacteria (Chopra and Roberts, 2001; Luzzatto et al., 1968). Finally, sulfadimethoxine inhibits the production of dihydrofolic acid and, thus, DNA and RNA synthesis (Romero et al., 2012). This project evaluated the use of microparticles (liposomes) for delivering these four antibiotics to *C. gigas* larvae in attempt to increase larval survival of *V. cor.*

Liposomes are microparticles made of one or more phospholipid layers surrounding an aqueous core. Barr and Helland (2007) demonstrated that liposomes can be easily produced and can store water-soluble nutrients, such as vitamins and free amino acids, without aggregation, disintegration, and with minimal leakage (Barr and Helland, 2007). Liposomes are ~4 μm in diameter (Hawkyard et al., 2015), an appropriate size to be consumed by young and old oyster larvae, which ingest particles between 0.5 μm and 12 μm in size (Baldwin and Newell, 1995). Given these properties, liposomes may provide a means for encapsulating nutrients in concentrated, neutrally buoyant packets for uptake by organisms, rather than dissolving nutrients into seawater. Parker and Selivonchick (1986) used radioactive labels and fluorescence microscopy to reveal the uptake of liposomes by juvenile Pacific oysters (Parker and Selivonchick, 1986). Parker and Selivonchick further demonstrated the incorporation of glucose and amino acids from liposomes into polysaccharides and proteins, suggesting that the liposomes were not only ingested but also metabolized (Parker and Selivonchick, 1986). Other studies have shown that liposomes are effective at delivering dietary supplements to fish larvae: Hawkyard *et al.* (2014) demonstrated that California yellowtail larvae fed on rotifers enriched with taurine

liposomes showed increased growth and greater dry weights than larvae fed on unenriched rotifers. In a different study, Hawkyard *et al.* (2015) compared the "dissolved method," in which taurine was dissolved in rotifer enrichment water, to enrichment with taurine liposomes; feeding liposome-enriched rotifers to rock sole larvae increased larval dry weights by 10-fold as well as whole body taurine concentrations compared to larvae fed unenriched rotifers.

The objective of this project was to apply the concept of encapsulating water-soluble antibiotics in liposomes to the problem of boosting larval resistance to bacteria. We investigated the impact of liposome-encapsulated oxytetracycline hydrochloride (OTC) on Pacific oyster larvae survival when faced with bacterial pathogens. Specifically, the objectives of this study were to 1) determine that OTC could be encapsulated in liposomes, 2) verify the activity of 3 antibiotics (OTC, doxycycline, and streptomycin) against *V. cor.*, 3) observe the ingestion and partial digestion of liposomes by oyster larvae, 4) investigate the impact of pretreatment with dissolved OTC on the survival of larvae subsequently exposed to *V. cor.*, and 5) investigate the effects of antibiotic-filled liposomes on larval resistance to *V. cor.*

2. Material and methods

GENERAL METHODS

2.1. Production of liposomes

Freeze-dried empty liposomes (FDELs) were produced by methods described by Hawkyard *et al.* (2015). To encapsulate sodium fluorescein in FDELs, 100 g L^{-1} FDELs were hydrated in a preheated (65°C) solution of 10 g L^{-1} sodium fluorescein and 34.8 g L^{-1} NaCl. The concentration of NaCl was calculated such that the core solution within the liposomes was isosmotic with respect to the seawater used in the oyster larval culture. This solution was stirred at 65°C for 15 min, until all crystals dissolved and FDELs were uniformly mixed throughout the solution. The suspension was then extruded using a peristaltic pump (Heidolph pumpdrive 5201; Heidolph Instruments Schwabach, Germany) for 30 min at a rate of 12 mL min^{-1} through a 22-gauge smooth-flow tip (Nordson EDF, Westlake, OH, USA).

Similar methods were used to encapsulate various antibiotics in FDELs. Liposomes containing doxycycline were prepared using a 20 g L^{-1} doxycycline hyclate and 32.7 g L^{-1} NaCl solution, and liposomes containing streptomycin were prepared using a 50 g L^{-1} streptomycin sulfate and 30.9 g L^{-1} NaCl solution. Liposomes containing sulfadimethoxine were prepared using a 50 g L^{-1} sulfadimethoxine sodium salt and 25.5 g L^{-1} NaCl solution. In the case of oxytetracycline liposomes, given the acidity of aqueous oxytetracycline hydrochloride (OTC), a 4 g L^{-1} OTC solution was first buffered to a pH between 8.00 and 8.10 using 0.2 M aqueous NaOH. Saline liposomes were produced using a 35 g L^{-1} NaCl solution. In each case, FDELs were added and heated, and the solution was extruded as detailed above.

2.2.Production of DiI Liposomes

Liposomes fluorescent stained with the lipophilic membrane dye DiI (1,1'dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; D282; Molecular Probes, Oregon, USA) were made by the methods described by Hawkyard *et al* (Hawkyard et al., 2015), and were modified as follows: the fluorescent stain DiI was added to the phospholipid-chloroform solution prior to rotary evaporation at a concentration of 1 mg DiI in 10 g of phospholipids. The FDELs stained with DiI were hydrated in a 35 g L^{-1} NaCl solution. This solution was heated for 15 min at 65°C and extruded for 30 min as described above.

2.3. Washing and counting of liposomes

After extrusion, the liposome suspensions were transferred to 50 mL conical tubes. The suspensions were centrifuged at 3900 RCF for 5 min, after which 5 mL distilled water was added to the tubes and the tubes were centrifuged at 3900 RCF for another 10 min. The supernatant containing unincorporated fluorescein or antibiotics was discarded. The conical tube was refilled with 20 mL of 35 g L^{-1} NaCl solution and centrifuged at 3900 RCF for 15 min. After centrifugation, the supernatant was discarded, new NaCl solution was added, and the pellet was resuspended. The suspension was centrifuged twice more for 15 min each time, with the supernatant discarded and fresh NaCl solution added after each round of centrifugation. The pellet was stored in darkness at room temperature (~21°C).

The liposomes constituting the pellet were suspended in 35 g L^{-1} NaCl solution and fed to oyster larvae in various feeding trials. The concentrations of liposomes (particles mL^{-1}) in the suspensions were counted using a hemocytometer in order to determine the appropriate volume of liposome suspensions to add to oyster larvae enrichment water.

2.4. Bacterial culture

Two strains of *V. coralliilyticus* isolated from shellfish hatchery environments in the Pacific Northwest of the U.S., RE98 and RE22, were used in this study. *V. cor.* was cultured in broth prepared from dehydrated Difco Luria-Bertani Broth supplemented with additional NaCl to a final concentration of 30 g L^{-1} NaCl. *V. cor.* was grown in a 25°C incubator on a culture rotator. Before use in trials with *C. gigas*, *V. cor.* was grown in a liquid culture to an optical density (λ=600 nm) between 0.750 and 0.810 and diluted in autoclaved seawater before addition to shell vials or well plates containing larvae at a final concentration of 64,000 cells mL^{-1}.

2.5. Pacific oyster husbandry

Pacific oysters (*C. gigas*) obtained from Oregon Oyster Farms (Yaquina Bay, OR, USA) and Oregon State University's Molluscan Broodstock Program (Newport, OR, USA) were conditioned at 18°C for several months. Fertilized oyster embryos were obstained using a strip spawning method. Briefly, eggs were stripped from two *C. gigas* adults, washed, and allowed to harden for 30 min, after which they were combined with sperm from two *C. gigas*

males. After checking for successful fertilization, zygotes were washed on a 25 µm sieve and stocked at a concentration of ~200 egg mL^{-1} in 30 L of 25°C sand-filtered seawater from Yaquina Bay, Oregon. Larval buckets were outfitted with airlines, and seawater was kept between pH 7.99 and 8.20. After 2 days, larvae were collected on a 45 µm sieve and restocked at a concentration of ~5 larvae mL^{-1}. At 7 days old, larvae were re-stocked to a concentration of ~1 larva mL^{-1}. A water change was performed on the cultures every other day. On water change days, larvae were fed a full ration of C-iso algae (*Isochrysis galbana*), and on days without water changes, larvae were fed a half-ration of algae in the morning and a half-ration in the evening. A full ration for two-day old larvae was 50,000 algae mL^{-1}, and the concentrations of algae fed to larvae increased as the larvae became older.

SPECIFIC METHODS

2.6. Ingestion of liposomes by C. gigas larvae

C. gigas larvae were offered fluorescently-stained liposomes to determine if young oyster larvae would ingest liposomes. Two-day old *C. gigas* larvae were spawned and their larvae reared according to the methods described in Section 2.5. For the feeding trial, two-day old larvae were stocked in shell vials at a concentration of 25 larvae mL^{-1} in 20 mL filtered seawater. Shell vials were assigned one of four treatment groups receiving different concentrations of DiI liposomes: 0, 5000, 10,000, and 25,000 liposomes mL^{-1}. Larvae were fed 25,000 algae mL^{-1} in addition to their assigned concentration of DiI liposomes and incubated at 25°C. After 15 min, a drop of 10% formalin was added to duplicate vials from each liposome treatment group to preserve the larvae. After 0.5, 1, 2, 3, and 4 hours, duplicate vials from each treatment group were preserved with formalin for image analysis.

Preserved larvae were collected on a 40 µm sieve, rinsed with filtered seawater, and imaged with an epifluorescent microscope (Leica DM 1000 microscope with DFC 400 camera; Leica Microsystems, Wetzlar, Germany). The optimal excitation and emission wavelengths for DiI are 549 nm and 565 nm, respectively, so DiI liposomes consumed by larvae were imaged using an Endow GFP Bandpass Emission filter set (Chroma Inc., VT, USA). Digital images of larvae fed fluorescent liposomes were analyzed using Image-Pro Premier (Media Cybernetics, Inc., MD, USA). In order to exclude background fluorescence from the gut of larvae, a minimum threshold intensity was selected that was at least twice the background levels observed in images of algae-fed oyster larvae. The surface areas of ingested liposomes were measured for ~25 larvae from each vial. For each set of duplicate vials fed a known concentration of liposomes for a known period of time, the average surface area of ingested liposomes (gut fullness) as well as the percent of larvae which had ingested any liposomes (feeding incidence) were calculated.

Similar methods were used to verify the ingestion of liposomes by older larvae. Nine-day old larvae were stocked in well plates at a concentration of ~25 larvae mL^{-1} in 4 mL filtered seawater and were assigned one of six treatments with DiI liposomes: 0, 10, 100, 1000, 10,000, and 40,000 liposomes mL^{-1}. Larvae were fed a diet of 40,000 algae mL^{-1} in addition to their assigned concentration of DiI liposomes. Over a 4 hour feeding period,

every 30 min, a vial from each liposome treatment group was preserved with a drop of formalin. Larvae were imaged as before with an epifluorescent microscope and filter set. Approximately 15 larvae from each vial were analyzed for fluorescent surface area. Based on this data, the gut fullness and feeding incidence of nine-day old larvae fed various concentrations of liposomes was measured over 4 hours.

2.7. Digestion of liposomes by C. gigas larvae

To determine if young *C. gigas* larvae could digest liposomes, two-day old *C. gigas* were fed liposomes containing a water-soluble fluorescent tracer, sodium fluorescein. Two-day old larvae were reared according to methods described in Section 2.5 and stocked in shell vials at a concentration of 25 larvae mL^{-1} in 20 mL filtered seawater. Larvae were fed a concentration of 25,000 fluorescein liposomes mL^{-1} and 25,000 algae mL^{-1}. After 1.5, 2.5, 3.5, and 18 hours of feeding, larvae were pooled on 40 µm sieves, washed with seawater, and imaged with an epifluorescent microscope and TRITC filter set (Chroma Inc., VT, USA).

2.8. Activity of four antibiotics against V. coralliilyticus

The process of preparing liposomes involves heating the aqueous core solution at 65°C for 45 min. To determine if the process of liposome encapsulation would render OTC ineffective against *V. cor.*, two solutions of 4 g L^{-1} OTC, one unbuffered (pH 2.48) and the other buffered with 0.2 M NaOH to a pH of 7.96, were heated for 45 min at 65°C. After heating, the OTC solutions were diluted and added to cuvettes containing LB broth at a concentration of 0.01 g L^{-1} OTC. *V. cor.* (strain RE98) was added to 4 cuvettes containing low pH OTC solution and 4 cuvettes containing buffered OTC. The optical densities (λ=600 nm) of the cuvettes were taken using a UV/Vis spectrophotometer (Beckman Coulter DU530 Life Science UV/Vis Spectrophotometer, Fullerton, California, USA) after 0, 0.5, 2, 4, and 22 hours of incubation at 25°C to measure the growth of *V. cor.* in each solution.

Trials verifying the activity of doxycycline hyclate, streptomycin sulfate, and sulfadimethoxine against *V. cor* were also carried out using *V. cor,* strain RE22. Doxycycline was added to cuvettes of LB broth at concentrations of 0, 0.001, 0.01, 0.1, 1 g/L. *V. cor.* was added to quadruplicate cuvettes, and optical densities were taken after 0, 2, 4, and 16 hours of incubation at 25°C. To check for loss of activity due to heat sensitivity, solutions of streptomycin and sulfadimethoxine were heated at 65°C for 45 min. Unheated and heated antibiotic solutions were added to LB broth at concentrations of 0, 0.001, 0.01, 0.1, 1 g/L. *V. cor.* was added to quadruplicate cuvettes of each solution, and optical densities were taken after 0, 2, 4, and 16 hours of incubation.

2.9. Verifying encapsulation of OTC in liposomes

Samples (0.5 g) from washed OTC and saline liposomes were collected in cryovials and suspended in 0.5 mL 35 g L^{-1} NaCl solution. Liposome samples were frozen at -80°C for 30 min, thawed in a warm water bath, and re-suspended. Liposomes were frozen, thawed,

and re-suspended twice more, after which liposomes were centrifuged at 15,000 *g* for 10 min. The supernatants were diluted and filtered using syringes outfitted with 0.2 μm filters. The filtered supernatants were diluted again and their fluorescence was measured at an excitation wavelength of 342 nm and an emission wavelength of 471 nm using a fluorometer (Horiba Scientific Aqualog, Edison, NJ, USA). A standard curve plotting dilute concentrations of heated and buffered OTC solutions against their fluorescence (342 nm excitation, 471 nm emission) was prepared. The measured intensities were compared to the standard curve, and the concentration of OTC within the OTC liposomes was calculated accordingly.

2.10. *Treatment of C. gigas larvae with OTC liposomes*

C. gigas were spawned and their larvae reared according to methods in Section 2.5. Two-day old and nine-day old larvae were stocked in well plates at a concentration of 25 larvae mL^{-1} in 4 mL filtered seawater. Larvae were assigned one of five treatments shown in Table I.

Treatment	**Treatment before exposure to *V. cor.***	**Treatment during exposure to *V. cor.***
None/None	No liposomes	No liposomes
Saline/None	Saline liposomes	No liposomes
OTC/None	OTC liposomes	No liposomes
Saline/Saline	Saline liposomes	Saline liposomes
OTC/OTC	OTC liposomes	OTC liposomes

Table 1. Five liposome treatments administered to two-day old and nine-day old oyster larvae to determine effects of OTC liposomes on larvae faced with *V. cor.*

Two-day old larvae receiving liposome treatments were fed 25,000 liposomes mL^{-1} and nine-day old larvae were fed 40,000 liposomes mL^{-1}, split up into 2, equal-sized rations. Two-day old larvae were also fed 25,000 algae mL^{-1} each day across 2, equal-sized rations, and nine-day old larvae were fed 40,000 algae mL^{-1} across 2 rations. After overnight pretreatment with liposomes, *V. cor.* (strain RE98) was cultured by methods detailed in Section 2.4. *V. cor.* was introduced to 8 replicate wells of each liposome treatment, and 8 wells of each treatment were left as no *V. cor.* controls. After 58 hours of incubation at 25°C, 10% formalin was added to preserve the larvae, and the numbers of live and dead larvae in each well at the time of preservation were counted.

2.11. *Treatment of C. gigas larvae with four antibiotic liposomes*

Two-day old larvae were stocked in well plates at a concentration of 25 larvae mL^{-1} in 4 mL filtered seawater. Eight replicate wells of larvae were assigned to one of six liposome treatments: no liposomes, saline liposomes, oxytetracycline liposomes, doxycycline liposomes, streptomycin liposomes, and sulfadimethoxine liposomes. Larvae were fed a concentration of 25,000 liposomes mL^{-1} of their assigned liposome type in addition to a concentration of 25,000 algae mL^{-1}. The "no liposome" treatment received 25,000 algae mL^{-1}

only. The larvae were incubated at 27°C for 14 hours, after which larvae were fed 40,000 algae mL^{-1}, and, as a booster, another 10,000 liposomes mL^{-1} of their assigned type of liposome. *V. cor.* (strain RE22) was and added to eight replicate wells of each treatment. Eight replicate wells of each treatment received no *V. cor.* and served as controls. After 24 hours, larvae were again fed 40,000 algae mL^{-1} and 10,000 liposomes mL^{-1} of their assigned liposome type. 48 hours after initial exposure to *V. cor.*, larvae were preserved with formalin, and the numbers of live and dead larvae at the time of preservation were counted.

This trial was repeated with seven-day old larvae. Several changes were made from the procedure described above. Since the larvae were older and larger animals, larvae were stocked at a concentration of ~12 larvae mL^{-1} in 4 mL seawater. In addition, larvae were fed an initial treatment of 40,000 liposomes mL^{-1} with 40,000 algae mL^{-1}, followed by a booster treatment of 16,000 liposomes mL^{-1} with 64,000 algae mL^{-1} prior to exposure to *V. cor.* After 24 hours of incubation, larvae were fed another 16,000 liposomes mL^{-1}. However, given the rapid mortality of the larvae, the trial was ended only 28 hours after introduction of *V. cor.*, and live and dead larvae were counted.

2.12. *Uptake of dissolved fluorescein and OTC by C. gigas larvae*

C. gigas larvae were soaked in solutions of fluorescent compounds to determine if larvae would take up dissolved compounds from enrichment water and if these substances would permeate the tissues of the larvae. Three replicate shell vials of larvae were assigned one of three treatments: no treatment, dissolved OTC, and dissolved fluorescein. Larvae assigned the dissolved OTC treatment were stocked in 30 mL of 0.1 g L^{-1} OTC buffered to a pH between 8.00 and 8.20 in seawater. Larvae assigned the dissolved fluorescein treatment were stocked in 30 mL of 0.1 g L^{-1} sodium fluorescein dissolved in seawater. Larvae assigned to a control treatment were stocked in seawater only. All larvae were fed a concentration of 50,000 liposomes mL^{-1} and incubated at 27°C for 18 hours. After 18 hours, larvae from each treatment group were imaged using an epifluorescent microscope and filter cubes either optimized for ultraviolet light (to visualize OTC) or optimized for GFP (to visualize fluorescein). Remaining shell vials were drained and refilled with clean seawater. After 4 and 8 hours of incubation in clean seawater, the larvae were imaged to visualize the retention or depletion of fluorescence after removal from OTC and fluorescein solutions.

2.13. *Treatment of C. gigas larvae with dissolved OTC*

Two-day old *C. gigas* larvae were stocked at a concentration of 25 larvae mL^{-1} in well plates. Larvae were assigned one of six treatments: 0, 0.0001, 0.001, 0.01, 0.1, and 1 g L^{-1} dissolved OTC. Two solutions of OTC were prepared, one buffered to pH 8.01 and the other left at pH 2.20. The solutions were diluted and added to larval enrichment water such that eight replicates of each treatment received buffered OTC solution and another eight received unbuffered OTC. *V. cor.* (strain RE22) was added to four replicates of each treatment. The remaining replicates served as no *V. cor.* controls. The wells were incubated at 27°C for 24

hours, after which formalin was added to each well. The numbers of larvae which were alive, dead, and abnormal at the time of preservation with formalin were counted.

2.14. *Pretreatments and concurrent treatments of C. gigas larvae with dissolved OTC*

In order to determine the effect of pretreatment with dissolved antibiotics on larval resistance to *V. cor.*, larvae were administered none, low, or high concentrations of dissolved OTC prior to or during exposure to *V. cor.* (strain RE22). 3-day old *C. gigas* larvae were stocked in shell vials at a concentration of ~10 larvae mL^{-1}. Eight replicate vials of larvae were assigned one of seven treatments. These treatments are listed in Table 2.

Treatments	Pretreatment Concentration of OTC ($g\ L^{-1}$)	Concurrent Treatment Concentration of OTC ($g\ L^{-1}$)
0/0	0	0
0/0.1	0	0.1
0/0.5	0	0.5
0.1/0	0.1	0
0.5/0	0.5	0
0.1/0.1	0.1	0.1
0.5/0.5	0.5	0.5

Table 2. Treatments with dissolved OTC administered to *C. gigas* larvae prior to and during exposure to *V. cor.*

Solutions containing OTC (0.1 g L^{-1} and 0.5 g L^{-1}) were prepared and buffered to pH 8 using 0.2 M NaOH. Larvae receiving pretreatments were incubated in dissolved OTC solutions for 18 hours. Larvae not receiving pretreatments were incubated in filtered seawater. After 18 hours, all shell vials were washed, and OTC solutions were added for larvae receiving treatments concurrent with exposure to bacteria. *V. cor.* (strain RE22) was cultured by methods described in Section 2.4 and added to four replicate vials of each treatment at a concentration of 64,000 CFU mL^{-1}. An additional four replicate vials of each treatment served as no *V. cor.* controls. After 24 hours of incubation, formalin was added, and preserved larvae were counted as live, dead, or abnormal. The abnormal classification referred to larvae that were likely alive at the time that formalin was added. The tissues of these larvae appeared full and intact, unlike the larvae affected by *V. cor.* However, the vela of the larvae protruded from their shells after addition of formalin, indicating that the larvae were likely unable to respond normally to the environment.

2.15. *Statistics*

Statistics were performed using JMP software, version 10.0.0 (SAS Institute Inc., Cary, NC, USA) and R, version 3.3.2 (R Foundation for Statistical Computing, Vienna, Austria). All percentage values were arc-sine square-root transformed before analysis. For challenge trials, differences between treatment groups were tested by ANOVA for significance. Tukey's

HSD with a $p<0.05$ threshold was used for further comparisons between treatment groups. Model selection for ingestion rates of liposomes by larvae was performed using AIC and BIC criteria.

3. Results

3.1. Ingestion of liposomes by C. gigas larvae

After 30 min of feeding on DiI liposomes, fluorescence was observed in two-day old *C. gigas* larvae fed DiI liposomes. Ingested liposomes appeared as clusters of fluorescent speckles in the gut-region of larvae. Fluorescence was also observed in nine-day old *C. gigas* larvae fed the three highest concentrations of liposomes: 1000 liposomes mL^{-1}, 10,000 liposomes mL^{-1}, and 40,000 liposomes mL^{-1}. Images of larvae fed DiI liposomes are shown in Fig. 1. No larvae from the treatment groups receiving lower concentrations of liposomes, 0 liposomes mL^{-1}, 10 liposomes mL^{-1}, and 100 liposomes mL^{-1}, were observed to ingest DiI liposomes.

For the two-day old and nine-day old oysters, the percent of larvae ingesting DiI liposomes (feeding incidence) and the average fluorescent surface area per oyster (gut fullness) were measured. For younger and older oyster larvae, feeding incidence and gut fullness for each liposome treatment were plotted against time, and the exponential models fitted to the data are shown in Fig. 3 and Fig. 4.

3.2.Digestion of liposomes by C. gigas larvae

After 1.5 hours of feeding on liposomes containing fluorescent dye (fluorescein), the gut of *C. gigas* larvae contained fluorescent clusters. Ingested fluorescein appeared as punctate, green speckles, as larger, smoother spheres, and as hazy clouds of fluorescence. However, the small clouds of fluorescence appeared only in the gut region of larvae.

3.3. Activity of four antibiotics against V. coralliilyticus

Based on optical densities (ODs) taken of *V. cor.* incubated in dissolved antibiotics, oxytetracycline, streptomycin, and sulfadimethoxine did not appear to be heat sensitive. The ODs of *V. cor.* cultures were significantly lower in cuvettes containing either heated or unheated sulfadimethoxine at 1 g L^{-1} concentration compared to cuvettes with lower concentrations of sulfadimethoxine or cuvettes without added antibiotics. Similarly, the ODs of cultures containing 1 g L^{-1} solutions of heated and unheated streptomycin after two hours of incubation were less than the initial ODs. However, oxytetracycline (OTC) appeared far more potent against *V. cor.* than streptomycin or sulfadimethoxine: ODs of cuvettes containing only 0.01 g L^{-1} solutions of either heated and unheated OTC were less than their initial ODs after two hours. While heated solutions of doxycycline were not tested, unheated doxycycline was highly effective, resulting in ODs less than initial ODs with concentrations as low as 0.001 g L^{-1} doxycycline.

3.4. *Verifying encapsulation of OTC in liposomes*

A dilute solution of OTC was buffered to pH 8, heated, and cooled, and its maximum excitation and emission wavelengths were found to be 342 nm and 417 nm, respectively. Using these wavelengths, the fluorescence intensities (Sc/Rc) of a series of dilute OTC solutions were measured and a standard curve relating concentration to fluorescence intensity was obtained (Fig. 7).

Two samples of OTC liposomes were taken from a pellet of OTC liposomes. When these samples were excited by 342 nm light, the most intense emissions occurred at 477 and 484 nm. Both of these wavelengths are within 75 nm of the maximum emission wavelength of the OTC solutions used in developing the standard curve in Fig 7. The concentration of OTC encapsulated in the liposome samples was found to be 5.64 g L^{-1} by interpolation from the standard curve. The liposomes were produced using a 4 g L^{-1} solution of OTC, lower than the interpolated value. However, due to the potential for high variability of the assay, these results should be interpreted with caution. As a control, the fluorescence intensity of the core solution extracted from saline liposomes was also measured. The fluorescence intensity of the saline liposomes was 6.022, a value indistinguishable from background noise, indicating that no OTC was contained in the saline liposomes.

3.5. *Treatment of C. gigas larvae with OTC liposomes*

When two-day old *C. gigas* larvae were pretreated with liposomes overnight and subsequently exposed to *V. cor.* (strain RE98), larval survival (raw data shown in Fig. 8) was slightly different among liposome treatments (two-way ANOVA, $p = 0.0118$). Larvae fed saline liposomes in the pretreatment followed by no treatment during exposure to *V. cor.* showed slightly higher survival than other treatment groups; larvae fed saline liposomes in the pretreatment and during exposure to *V. cor.* showed slightly lower survival than other treatment groups (Tukey's HSD, significance level $p = 0.05$).

When nine-day old larvae were challenged with *V. cor.*, differences in mean survivals (raw data shown in Fig. 9) between oysters fed daily liposomes and oysters fed only pretreatments or no liposomes were far more significant than in the case of two-day old larvae (one-way ANOVA, $p < 0.0001$). The survival of oysters fed daily OTC liposomes (survival: 56.5.1%, SD: 30.3%) and oysters fed daily saline liposomes (survival: 69.9%, SD: 27.4%) was lower and showed more variance than oysters in the other treatment groups. Since these two treatment groups showed much wider variances than other treatment groups, and assumptions of ANOVA may have been violated, Levene's test was used to test unequal variances. The Levene test outcome was < 0.0001, and therefore nonparametric comparisons for each pair of treatments were carried out. Based on this analysis, nine-day old larvae exposed to *V. cor.* and fed daily OTC liposome and daily saline liposome treatments demonstrated lower survival when compared to the other treatments (Wilcoxon's, $p < 0.00001$). Nine-day old larvae not fed liposomes daily showed much higher survival of *V. cor.* (strain RE98) than two-day old larvae and very similar survival as nine-day old larvae not

exposed to *Vibrio* bacteria. Two-day old and nine-day old larvae not exposed to *V. cor.* showed high survival with no significant differences between treatment groups.

3.6. Treatment of C. gigas larvae with four antibiotic liposomes

When two-day old *C. gigas* larvae were challenged with *V. cor.*, no significant differences in survival (raw data depicted in Fig. 10) were detected between oyster larvae treated with antibiotic liposomes, larvae treated with saline liposomes, and larvae not treated with liposomes. Control larvae not exposed to *V. cor.* showed significantly higher survival (97.4%, SD: 1.74%) than larvae challenged with *V. cor.* (38.9%, SD: 20.2%), although control larvae showed no significant differences in survival across treatments.

Similarly, when seven-day old *C. gigas* larvae were challenged with *V. cor.*, no significant differences in survival (raw data depicted in Fig. 11) were detected between oyster larvae treated with antibiotic liposomes, larvae treated with saline liposomes, and larvae not treated with liposomes after 38 hours of exposure to *V. cor.* As with two-day old larvae, control larvae not exposed to *V. cor.* showed significantly higher survival (99.6%, SD: 0.88%) than larvae challenged with *V. cor.* (0.28%, SD: 0.86%), but no significant differences were found between control larvae treatments.

3.7. Uptake of dissolved fluorescein and OTC by C. gigas larvae

After 18 hours of incubation in seawater containing sodium fluorescein, oyster larvae showed plumes of green fluorescence throughout their bodies (Fig. 12). Although the guts of the larvae fluoresced most intensely, clouds of fluorescein pervaded the outer tissues as well. 4 hours after removal from fluorescein solution and incubation in clean seawater, the majority of larvae only exhibited fluorescence in the outlines of their shells, and only a few larvae exhibited plumes of fluorescein throughout their tissues. After 18 hours of incubation in seawater containing OTC, larvae were weakly fluorescent (Fig. 13). Fluorescence was mostly observed in the outer rims of shells and as hazy clouds in the guts of larvae. After 4 hours of incubation in clean seawater, the rims of shells remained weakly fluorescent.

3.8. Treatment of C. gigas larvae with dissolved OTC

After 24 hours of exposure to *V. cor.*, significant differences in larval survival (raw data shown in Fig. 14) were observed between larvae receiving no or low concentrations (0.0001, 0.001, 0.01 g L^{-1}) of dissolved OTC and larvae receiving high concentrations (0.1 and 1 g L^{-1}) of OTC (ANOVA, $p < 0.0001$). Survival was significantly higher for larvae treated with 0.1 and 1 g L^{-1} OTC solution, with mean larval survivals of 99.5% and 100%, respectively.

3.9. Pretreatments and concurrent treatments of C. gigas larvae with dissolved OTC

After 24 hours of exposure to *V. cor.*, larval survival (data shown in Fig. 15) was significantly different between oysters treated with dissolved OTC before exposure to *V. cor.*

and oysters treated with OTC during exposure to *V. cor.* (ANOVA, $p < 0.0001$). Larvae receiving concurrent treatment of dissolved OTC during exposure to *V. cor.* demonstrated an average survival of 99.1% (SD: 0.75%), while larvae receiving no concurrent treatment demonstrated an average survival of only 4.65% (SD: 4.61%). Among larvae that did not receive concurrent treatment at the time of exposure to *V. cor.*, no significant differences in survival were found between larvae receiving low or high pretreatments of OTC, although larvae receiving no pretreatment showed slightly higher survival than those pretreated with OTC (ANOVA, $p < 0.05$). No significant differences in survival were found between larvae receiving low and high concentrations of OTC concurrent with *V. cor.* Control larvae not exposed to *V. cor.* showed no significant differences between in mean survivals between treatment groups.

4. Discussion

Fluorescent images taken of *C. gigas* larvae fed DiI-stained liposomes confirm that oysters ingest liposomes at young ages. Over 75% of two-day old larvae fed 5,000 liposomes mL^{-1} had ingested DiI liposomes after 2 hours of feeding. Larvae fed 25,000 liposomes mL^{-1} reached a 75% feeding incidence within 30 minutes of feeding. The high feeding incidence in short feeding periods indicates that *C. gigas* as young as 2 days old ingest liposomes quickly and at low concentrations. Nine-day old larvae were also observed to ingest liposomes (Fig. 1). Previous studies by Langdon and Siegfried (1984) investigated the use of microgel particles and lipid-walled capsules for replacing algal diets fed to juvenile *C. virginica* (Langdon and Siegfried, 1984). In addition, Parker and Selivonchick (1986) demonstrated ingestion and metabolism of liposomes by juvenile *C. gigas* (Parker and Selivonchick, 1986). The DiI liposome feeding trials conducted in this research build upon this existing research and suggest that liposomes may be used to deliver water-soluble nutrients not only to juvenile oysters but also to oysters as young as 2 days old.

While two-day old *C. gigas* ingest liposomes, it is uncertain if they can break down liposomes and liberate their contents. Larvae fed liposomes containing sodium fluorescein appeared to have small clouds of fluorescence in their guts (Fig. 2), suggesting at least partial digestion of liposomes. However, even after 18 hours of feeding on fluorescein liposomes, fluorescence was confined to the gut-region of larvae and not observed in outer tissues. In comparison, images of two-day old larvae soaked in dissolved fluorescein show green fluorescence throughout their bodies. Furthermore, fluorescent images of seven-day old *C. gigas* feeding on fluorescein liposomes show clouds of fluorescein suffusing the entire bodies of larvae (unpublished data collected by Carlos Gomes). The liposomes used in these trials were made of hydrogenated phosphatidylcholine, a saturated lipid that effectively retains small, water-soluble compounds within minimal leakage into surrounding seawater (Hawkyard et al., 2015). However, it is possible that two-day old *C. gigas* are unable to metabolize the sturdier, hydrogenated-phosphatidylcholine particles. Previous studies have investigated the use of fish oil and low-melting point lipids for softening lipid-spray beads and increasing their digestibility by fish larvae (Buchal and Langdon, 1998; Önal and Langdon, 2000). Further research is needed to

determine if the liposomes used in this study can also be softened by similar methods and thereby made more digestible for young larvae.

In this project, four antibiotics were encapsulated in liposomes: oxytetracycline (OTC), doxycycline, streptomycin, and sulfadimethoxine. When dissolved into growth media at high concentrations, all four antibiotics reduced the growth of *V. cor.* However, over 100 times more streptomycin and sulfadimethoxine were required to inhibit the growth of *V. cor.* as OTC or doxycycline. These results suggest that even though OTC and doxycycline are only slightly water-soluble at near-neutral pH, they are good candidates for encapsulation in liposomes, as low concentrations are highly potent against *V. cor.* Furthermore, OTC remained active against *V. cor.* after being buffered to pH 8 and heated, indicating that the process of encapsulating OTC in liposomes was unlikely to degrade the antibiotic. Finally, the fluorescent properties of OTC made it possible to verify the encapsulation of OTC into liposomes by estimating the concentration of the core solution (Fig.7).

Although OTC was found to be highly active against *V. cor.* and *C. gigas* larvae were shown to ingest liposomes, larvae fed OTC liposomes did not demonstrate increased resistance to *V. cor.* Larvae fed OTC liposomes prior to exposure to *V. cor.* showed similar survival to larvae fed only algae or saline liposomes. However, the survival of nine-day old larvae fed daily treatments of saline or OTC liposomes was significantly lower and had a wider variance than larvae in other treatment groups. In contrast, two-day old larvae fed daily saline liposomes showed only slightly lower survival than larvae fed only algae. One possible explanation for these results is that larvae fed daily liposome treatments were fed twice the number of particles (algae or liposomes) than larvae in other treatment groups, and the increased turbidity of the culture water may have encouraged blooms of *V. cor.* In addition, Elston and Leibovitz (1980) showed that *V. cor.* infects oysters through the alimentary track, as one of several mechanisms of infection (Elston and Leibovitz, 1980). As a result, larvae receiving daily liposome treatments may have been more susceptible to *V. cor.* due to increased feeding habits. This trend was not observed in two-day old larvae, who were fed a smaller total diet and eat considerably less than older larvae (Rico-Villa et al., 2006).

C. gigas larvae fed liposomes containing antibiotics (OTC, doxycycline, streptomycin, and sulfadimethoxine) did not show increased resistance to *V. cor.* compared to larvae fed saline liposomes or no liposomes. Moreover, larvae incubated in dissolved OTC before exposure to *V. cor.* were shown to take up dissolved OTC into their tissues (Fig. 13); yet when these larvae were reintroduced into clean seawater during exposure to *V. cor.*, nearly every larva was infected by *V. cor.* These results suggest that neither treatment with antibiotic liposomes nor pretreatment with dissolved OTC increase larval resistance against *V. cor.* There are many possible explanations for these results. It may be that the antibiotics were present in the larvae, but at concentrations too low to confer resistance to *V. cor.* During the time that the larvae were exposed to *V. cor.*, the OTC in their bodies may have been purged, leaving the larvae susceptible to infection. Another possible explanation is that the *V. cor.* was not affected by OTC during infection. Studies by Mersni-Achour *et al.* (2014, 2015) have identified several toxic factors, including an extracellular zinc metalloprotease, which are present in *C. gigas* infested with *V. cor.*, and whose genes are upregulated during infection. It is possible that the secretion of

extracellular proteases and toxins may allow *V. cor.* to damage larval cells without being affected by OTC, in which case oral ingestion of antibiotics may not be an effective protection against *V. cor.* at all.

The results of this study indicate that phospholipid-walled microparticles are readily ingested by *C. gigas* larvae and have the potential to improve the delivery of water-soluble nutrients to larvae. Such diets could significantly reduce the quantity of nutrients dissolved in larval culture water, potentially reducing costs and improving water quality. While antibiotics such as oxytetracycline can be encapsulated in these liposomes, we found that oyster larvae fed with antibiotic liposomes do not demonstrate increased resistance to *V. cor.* More research is needed to determine if ingestion of antibiotics by oyster larvae can confer increased resistance to *Vibrio* infections and if liposomes can be engineered to be more digestible by young larvae.

5. Figures

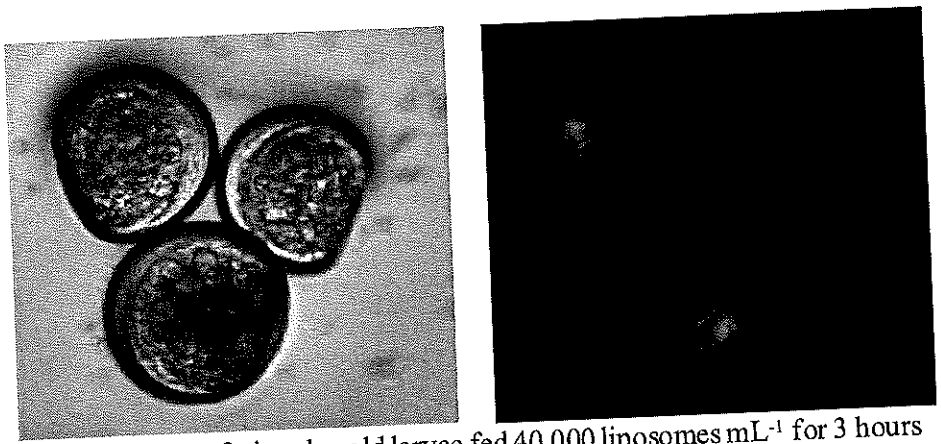


Fig. 1: Images of nine-day old larvae fed 40,000 liposomes mL^{-1} for 3 hours and visualized using an epifluorescent microscope and 40x objective. Liposomes were stained with DiI (0.5 mg g^{-1} w/w). Left: larvae viewed without fluorescence. Right: same larvae viewed with a filter cube optimized for fluorescence of DiI.

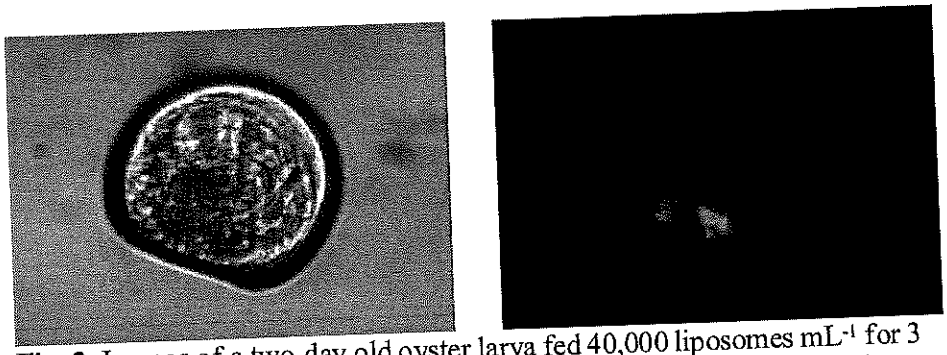


Fig. 2. Images of a two-day old oyster larva fed 40,000 liposomes mL^{-1} for 3 hours and visualized using an epifluorescent microscope and 40x objective. Liposomes contained sodium fluorescein solution (0.1 $g\,L^{-1}$). Left: larva viewed without fluorescence. Right: same larva viewed with a filter cube optimized for visualizing fluorescein.

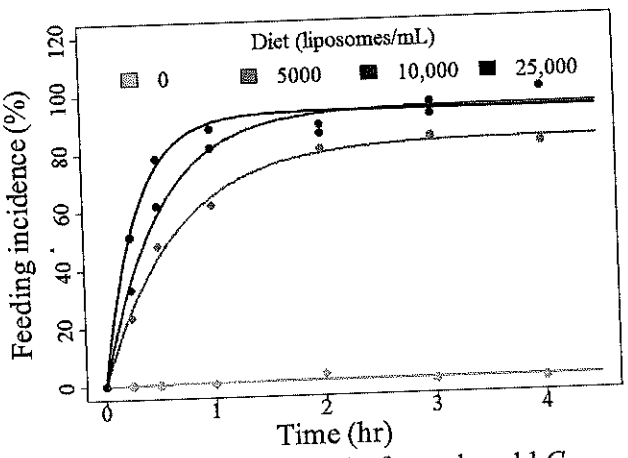


Fig 3. Feeding incidence (%) of two-day old *C. gigas* larvae fed on DiI liposomes over 4 hours. Colors denote different concentrations of liposomes fed to oysters in a mixed liposome-algal diet.

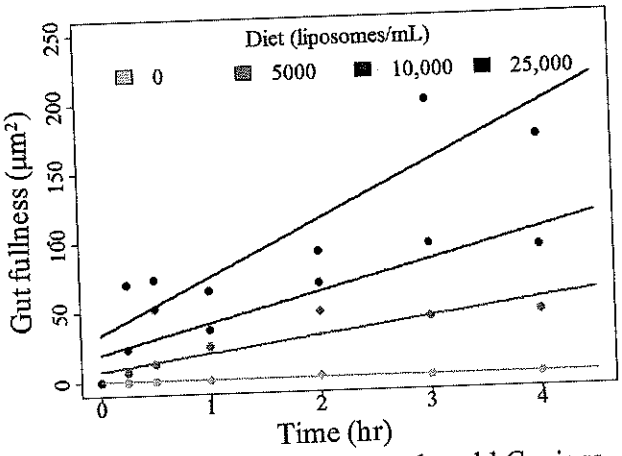
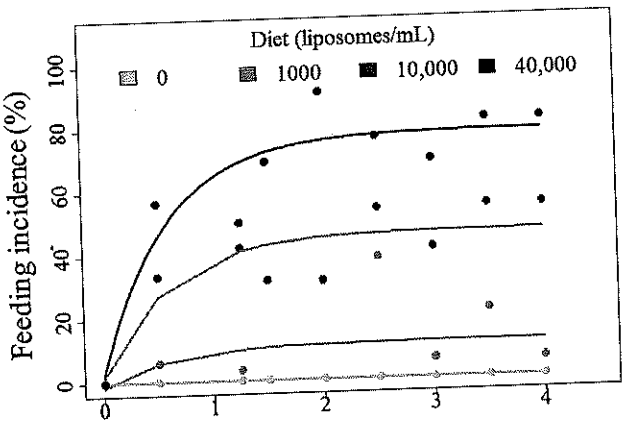
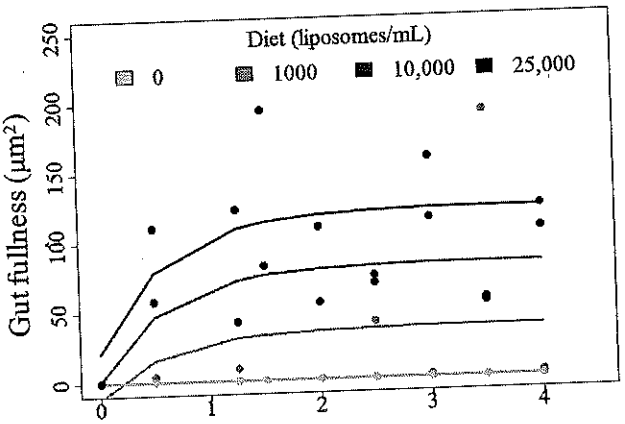


Fig. 4. Gut fullness (μm^2) of two-day old *C. gigas* larvae fed on DiI liposomes for 4 hours. Colors denote different concentrations of liposomes fed to oysters in a mixed liposome-algal diet.





Time (hr)

Fig. 5. Feeding incidence (%) of nine-day old *C. gigas* larvae on DiI liposomes over 4 hours. Colors denote different concentrations of liposomes fed to oysters in a mixed liposome-algal diet.

Time (hr)

Fig. 6. Gut fullness (μm^2) of nine-day old *C. gigas* larvae fed on DiI liposomes for 4 hours. Colors denote different concentrations of liposomes fed to oysters in a mixed liposome-algal diet.

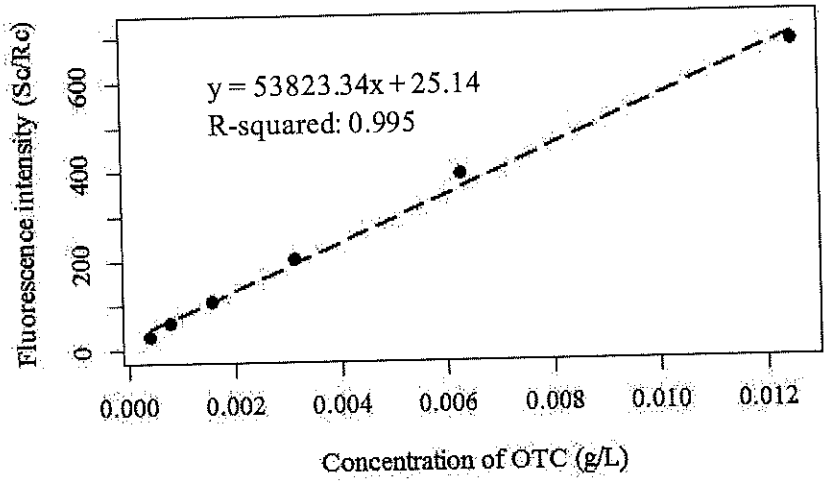


Fig. 7. Fluorescence intensity of OTC solutions prepared to known, dilute concentrations.

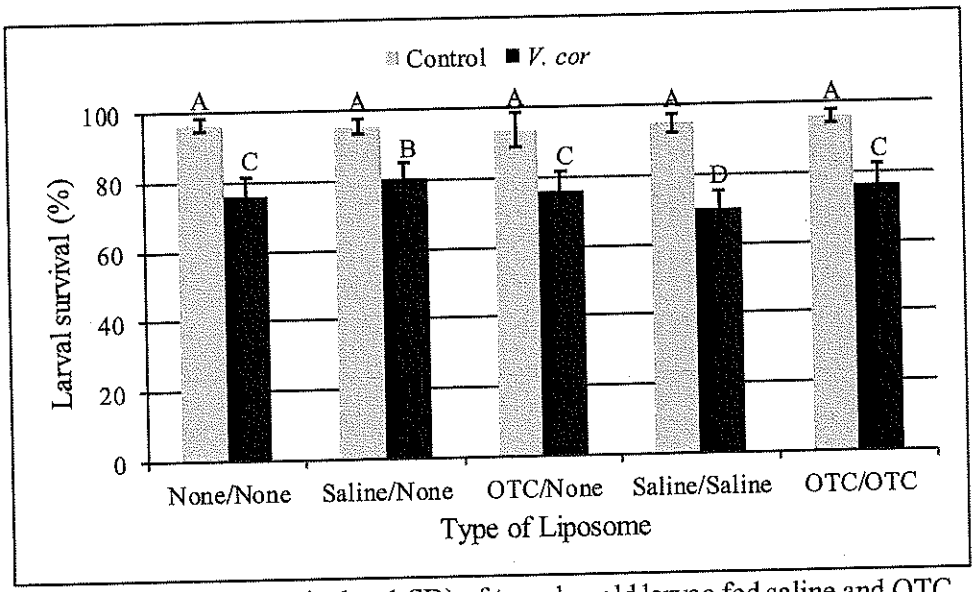


Fig. 8. Survival (% survival; ± 1 SD) of two-day old larvae fed saline and OTC liposomes when exposed to *V. cor.* (strain RE98). Oysters in the "Saline/None" and "OTC/None" treatments were fed saline and OTC liposomes, respectively, prior to introduction of *V. cor.* but fed only algae afterwards; oysters in the "Saline/Saline" and "OTC/OTC" treatments were fed saline and OTC liposomes, respectively, before and during exposure to *V. cor.*; oysters in the "None/None" were fed only algae throughout the trial. Letters indicate significant differences between treatments (Tukey's HSD, significance level $p = 0.05$).

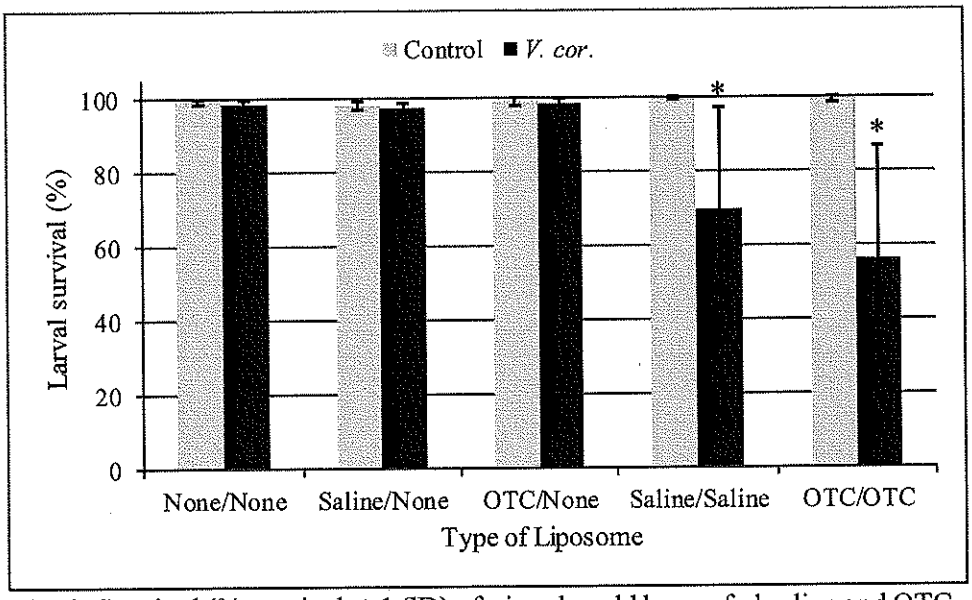


Fig. 9. Survival (% survival; ± 1 SD) of nine-day old larvae fed saline and OTC liposomes when exposed to *V. cor.* (strain RE98). Asterisks denote treatment groups determined to be significantly different from the control treatment (None/None) using the Dunn method for non-parametric comparisons (significance levels p = 0.0106 and 0.0005).

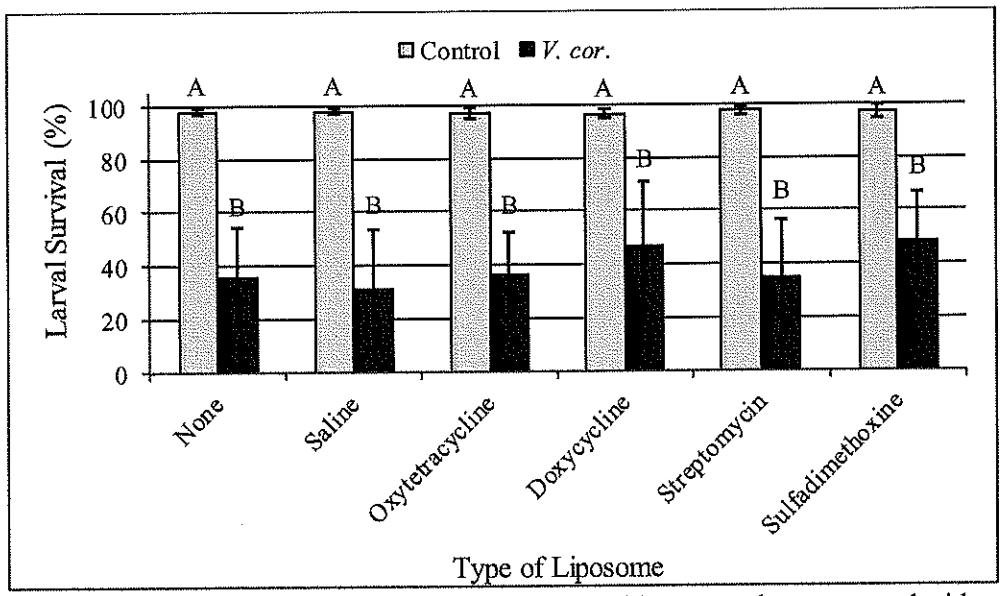


Fig. 10. Survival (% survival; ± 1 SD) of two-day old *C. gigas* larvae treated with no liposomes, saline liposomes, and liposomes containing four different antibiotics after exposure to *V. cor.* (strain RE22). Survival (% survival, ± 1 SD) of larvae not exposed to *V. cor.* also shown (control). Letters indicate significant differences between treatments (Tukey's HSD, significance level p < 0.0001).

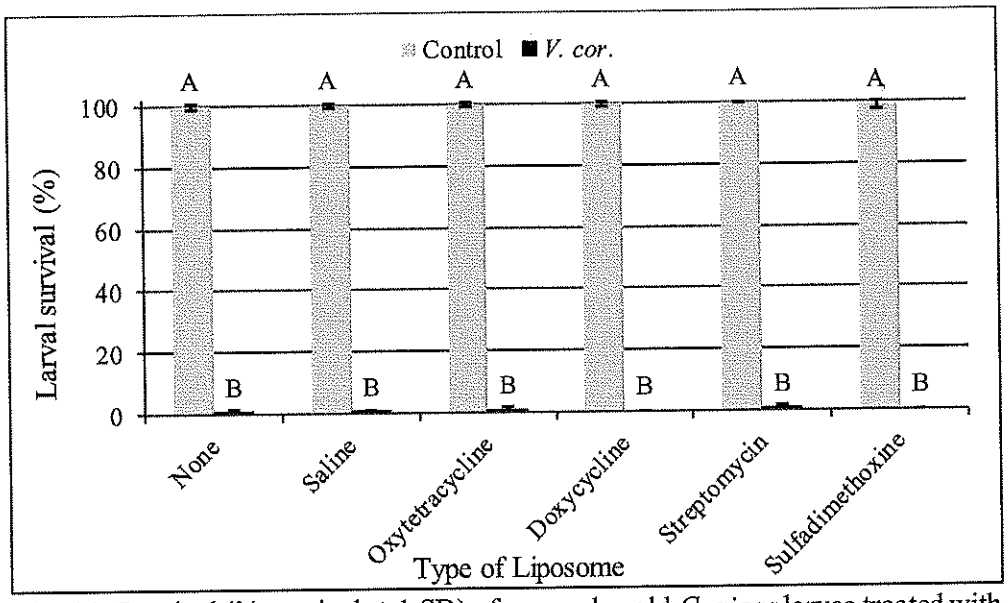


Fig. 11. Survival (% survival; ± 1 SD) of seven-day old *C. gigas* larvae treated with no liposomes, saline liposomes, and liposomes containing four different antibiotics after exposure to *V. cor.* (strain RE22). Survival (% survival, ± 1 SD) of larvae not exposed to *V. cor.* also shown (control). Letters indicate significant differences between treatments (Tukey's HSD, significance level $p < 0.0001$).

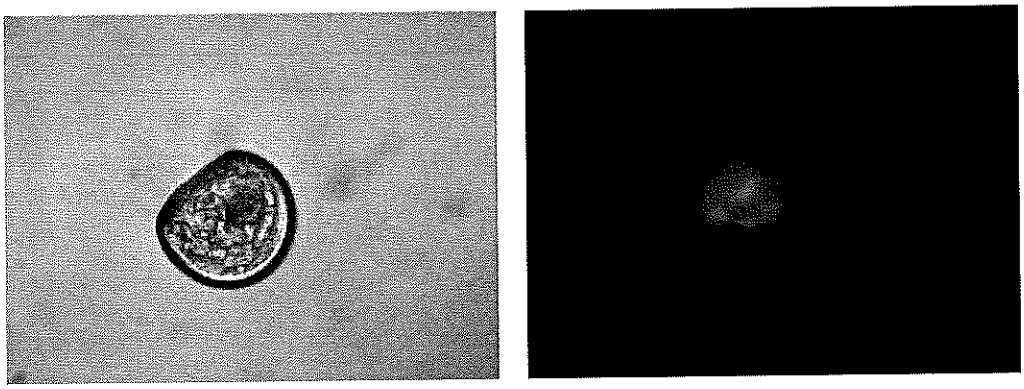


Fig. 12. Images of a two-day old larva incubated in dissolved fluorescein for 18 hours and visualized using an epifluorescent microscope and 40x objective. Left: larva viewed without fluorescence. Right: same larva viewed with a filter cube optimized for fluorescein.

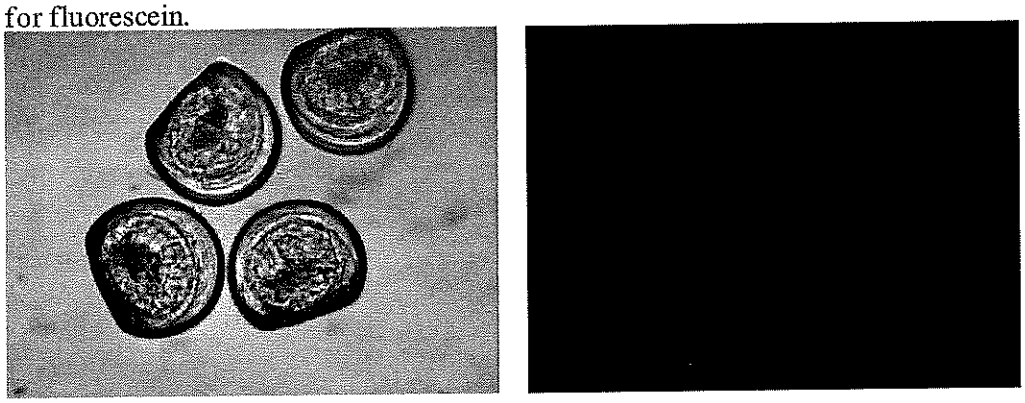


Fig. 13. Images of two-day old larvae incubated in dissolved OTC for 18 hours and visualized using an epifluorescent microscope and 40x objective. Left: larvae viewed without fluorescence. Right: same larva viewed with a filter cube optimized for OTC.

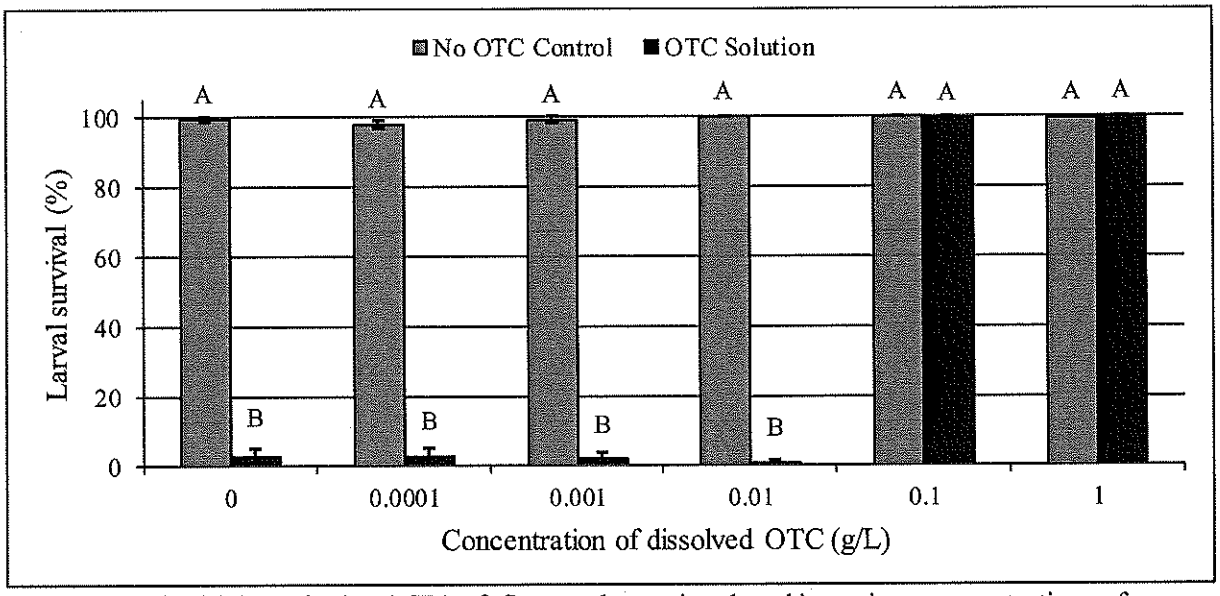


Fig 14. Survival (% survival; ± 1 SD) of *C. gigas* larvae incubated in various concentrations of buffered (pH ~8) and unbuffered (pH ~5-8) OTC solutions after exposure to *V. cor.* Percent survival for each well includes larvae considered abnormal in appearance but alive at time of preservation. Letters indicate significant differences between treatments (Tukey's HSD, significance level $p < 0.0001$).

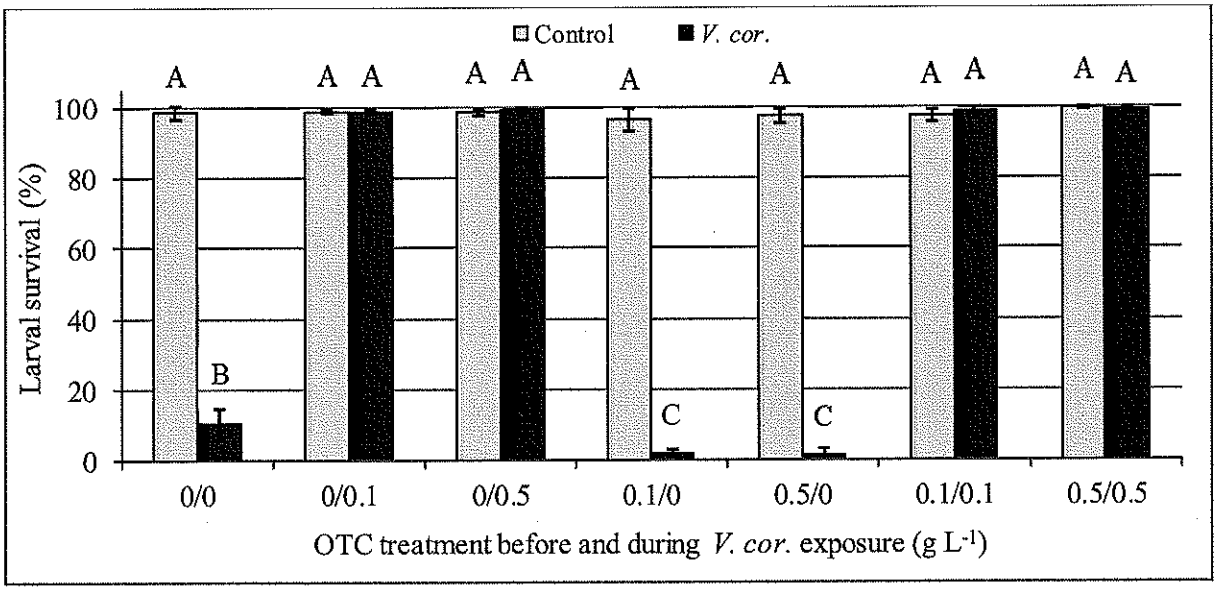


Fig. 15. Survival (% survival; ± 1 SD) of *C. gigas* larvae treated with different concentrations of OTC (0, 0.1, and 0.5 g/L) before exposure to and during exposure to *V. cor.*, as well as survival of larvae treated with OTC but not exposed to *V. cor.* (control). Oysters in the "0/0.1" and "0/0.5" treatments were incubated in 0.1 and 0.5 g L^{-1} OTC solutions, respectively, during exposure to *V. cor.*; oysters in the "0.1/0" and "0.5/0" treatments were incubated in 0.1 and 0.5 g L^{-1} OTC solutions, respectively, but moved into fresh seawater before introduction of *V. cor.*; oysters in the "0.1/0.1" and "0.5/0.5"

treatments were incubated in 0.1 and 0.5 g L^{-1} OTC solutions before and during *V. cor.* exposure. Letters indicate significant differences among treatments (Tukey's HSD, significance level $p < 0.0001$).

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