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THE ROLE OF BACTERIAL INFECTION IN EARLY BENTHIC PHASE MORTALITY OF MARINE INVERTEBRATES

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THE ROLE OF BACTERIAL INFECTION IN EARLY BENTHIC PHASE MORTALITY OF MARINE INVERTEBRATES

by

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We accept this thesis as conforming to the required standards:

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ABSTRACT

Marine invertebrates experience a substantial amount of mortality during the early benthic phase which can influence a population's future abundance and distribution as well as exert selective pressure, driving its evolution. Some causative agents of intertidal juvenile marine invertebrate mortality have already been elucidated, such as predation and desiccation stress, but the influence of bacterial infection on early benthic phase mortality is not known. The purpose of this study is to evaluate the role of infectious agents in generating the high mortality rates observed amongst wild populations of juvenile invertebrates living in the intertidal zone.

In this study, five antibiotics were administered to juvenile *Mytilus trossulus* (mussels) and Nucella ostrina (snails): oxytetracycline (OTC), chloramphenicol (CM), kanamycin sulfate (KS), and trimethoprim and sulfamethoxazole (TxS). The juveniles were placed in small cages and were constantly submerged off of the Bamfield Marine Sciences Centre docks. *M. trossulus* and *N. ostrina* were soaked in a mixture of the antibiotics for a half hour three times per day for five days. After five days the mortality in the treatment and control groups was compared.

Mortality was low in the control treatment for both species, ranging from 0 - 3.7% (*M. trossulus*) and 0.5 - 4.0% (*N. ostrina*), indicating that infectious stress is not a major cause of mortality of juvenile marine invertebrates. Further, no significant difference in mortality was observed between the antibiotic treatment and control treatment for either *M. trossulus* or *N. ostrina* (Friedman block test $P>0.99$ $S<0.01$ (*M. trossulus)*; Friedman block test $P=0.564$ $S=0.33$ (*N. ostrina*)), indicating that bacterial infection is not causing mortality of juvenile marine invertebrates.

Thesis Supervisor: Professor, Louis Gosselin, *PhD*

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INTRODUCTION

During the early benthic phase at the beginning of juvenile life, many marine invertebrate species experience a substantial bottleneck effect, often with more than 80% of individuals dying within the first 4 months. In fact, mortality is often highest within the first few hours or days of benthic life (Gosselin and Qian 1997). A number of factors are known to play a role in causing this high mortality rate, including predation, temperature and desiccation (Gosselin & Chia, 1995a, 1995b; Griffiths & Gosselin, 2008; Jenewein & Gosselin, 2013); wave action (Naylor & McShane, 2001); and ultraviolet (UV) radiation (Gosselin & Jones, 2010). Other factors may be involved, however, and infections such as those caused by bacteria or by viruses, fungi, and protozoa are likely candidates that have yet to be examined.

Many infectious agents are known to cause mortality of adult marine invertebrates in the wild, although mortality rates are often recorded only during the occurrence of an epidemic. The sea star wasting disease currently sweeping along the coast of North America is linked to a densovirus infection (Hewson et al. 2014). With regard to bacteria, there are reports of bacterial infections sweeping through wild invertebrate populations and causing substantial mortality amongst adult animals (reviewed in Fey et al., 2015), such as the 1999 and 2003 mass mortality occurrences in Mediterranean Sea caused by a Vibrio spp. bacterium which impacted invertebrate phyla as diverse as Ascidiae, Bryozoa, Cnidaria, Mollusca, and Porifera (Cerrano et al., 2000; Perez et al., 2000; Bonhomme et al., 2003). However, the information on mortality during reported epidemics refers only to the adult animals, and there is no information on the impact of epidemic or basal levels of bacterial infections on juvenile marine invertebrate mortality rates in a natural setting.

The fact that no studies have yet attributed juvenile mortality events in the wild to bacterial infection does not mean they are not occurring - bacterial infections do have the ability to affect juveniles and are known to cause significant health problems to invertebrates reared in the aquaculture industry, causing millions of dollars in damage every year (Wilkenfeld, 1992; Paillard et al., 2004). In particular, four bacterial infections are known to cause mortality specifically of juvenile bivalves: juvenile oyster disease (Proteobacteria), hinge ligament erosion disease (Cytophaga), chronic abcess syndrome (Vibrio), and an event referred to as summer oyster mortality (bacterial species not determined) (reviewed in (Paillard et al., 2004). Thus, juveniles are susceptible not only to adult infections but also to some infections unique to this life stage. When these diseases affect aquaculture stock, up to 100% of the juveniles may die, although this number is highly variable depending on the infectious agent (Goulletquer et al., 1998; Paillard et al., 2004). These high infection and mortality rates likely result from the concentrated monoculture nature of the aquaculture systems (Spaargaren, 1998). Juveniles of species reared in aquaculture, other than bivalves, are also affected by bacterial infection, including sea cucumbers, crabs, and shrimp.

To curb the appearance and spread of bacterial infection in invertebrate aquaculture, antibiotics are commonly used (Holmstrom et al., 2003; Thuy et al., 2011; de la Cruz et al., 2014; Wang et al., 2014). They apparently cause no adverse effects to the health of the animals and increase crop yields (Bray et al., 2006). In addition, antibiotics are commonly used in invertebrate research to elucidate the cause of certain diseases, improve animal health, or evaluate disease responses (Sutton & Garrick, 1993; Boettcher et al., 1999; Banerjee et al., 2007; Azam & Narayan, 2013). As such, antibiotics are an appropriate choice

to confer invertebrates protection against bacterial infection, and a selection of antibiotics has been chosen for use in this study based on those currently in use.

Mytilus trossulus, a marine bivalve, and *Nucella ostrina*, a marine snail, were chosen as model organisms for the present study due to the availability of early juveniles, their importance in intertidal communities, and also because they have different dispersal mechanisms before and during the juvenile phase. *M. trossulus* and *N. ostrina* can both be found abundantly in the intertidal zone from California to Alaska (Palmer et al. 1990; Rawson and Hilbish 1995). Both of these species also affect the community structure in their habitat (Menge et al. 1994). *M. trossulus* larvae disperse through the water column and settle on filamentous algae where they metamorphose into juveniles (Strathmann 1987). *N. ostrina* juveniles, on the other hand, emerge from egg capsules and crawl away as small juveniles (Gosselin and Chia 1995a). Both species reside in the intertidal zone where they are exposed to a variety of stressors.

The purpose of this study was to evaluate the role of infectious agents, and more specifically bacterial infections, in generating the high mortality rates observed in wild populations of juvenile invertebrates living in the intertidal zone. The specific objectives of this project were to determine 1) how much mortality of early benthic phase *M. trossulus* and *N. ostrina* occurs when these animals are protected from intertidal stressors such as predation, desiccation, UV radiation, and wave action, but remain exposed to bacteria, fungi, viruses, and parasites, and 2) if the administration of broad-spectrum commercial antibiotics [oxytetracycline (OTC), trimethoprim and sulfamethoxazole (TxS), chloramphenicol (CM),

and kanamycin sulfate (KM)] produces a significant decrease in early benthic phase mortality rates of *M. trossulus* and *N. ostrina* held in a natural setting.

MATERIALS AND METHODS

Study site and organisms

This study was conducted at the Bamfield Marine Sciences Center (BMSC) in Barkley Sound on the west coast of Vancouver Island, from 26 May to 17 August, 2014. Before experimentation began, early benthic phase specimens of the model organisms being used (*Mytilus trossulus* and *Nucella ostrina*) were collected. *M. trossulus* juveniles were obtained by collecting filamentous algae (*Cladophora columbiana*) from the upper intertidal zone at Prasiola Point (N 48° 81’ 76.0”; W 125° 16’ 84.1”) throughout the summer. *C. columbiana* was gathered and placed into Ziploc™ bags, which were taken back to the laboratory for processing. The algae were gently shaken and torn apart in 60% seawater diluted with freshwater (3 parts seawater to 2 parts freshwater). This diluted seawater caused *M. trossulus* to close, which helped to separate them from the algae. After the algae had been torn, the particulates that had fallen from the algae (and which were suspended in seawater) were passed through a series of three sieves: 3 mm, 610 µm, and 102 µm. Particles remaining on the 102 µm filter, including small juvenile *M. trossulus*, were rinsed off into a bowl with full strength seawater, and this bowl was placed under the dissection microscope. The smallest *M. trossulus* were removed from the filtrate using a combination of pipette and insect tweezers and placed in small cages, 10 mussels to a cage. The cages were made from microcentrifuge tubes and 102 µm mesh.

Ripe (unplugged) egg capsules of *N. ostrina,* containing fully developed juveniles that had not yet emerged, were also collected in the field at Prasiola Point and taken back to the laboratory from mid-July to August. The egg capsules were carefully removed from their attachment site by their base with tweezers. Egg capsules were placed in small cages lined with 660 µm mesh and submerged in seawater tables; newly hatched juvenile snails were removed from the cages 12-24 hours later, after they had crawled out of the egg capsule.

Size range of *M. trossulus* used in the experiments

Unlike early benthic phase *N. ostrina* which had just emerged from their egg capsules, *M. trossulus* individuals extracted from algae could not directly be aged. To ensure *M. trossulus* collected for experimentation were of the smallest size classes available in the field and had just settled, 235 mussels from field experiments 2, 3, and 4 were photographed and digitally measured after the completion of the 5 day field experiments. Their sizes were then compared to the full size frequency distribution of 208 *M. trossulus* individuals present in *C. columbiana* collected at our field site, Prasiola Point. The full size frequency distribution was obtained by photographing and digitally measuring all *M. trossulus* present in two samples of *C. columbiana* collected on different dates. The sizes of the mussels used in experimentation were then compared to the total size range present in the field to verify that only recently settled individuals were being used (Appendix Figure 1). Digital measurements were taken using ImageJ version 1.48.

Extent of *M. trossulus* exposure to the antibiotic solutions

Juvenile *M. trossulus* were also assessed to ensure they were being exposed to the antibiotic solution in the experiments described below. *N. ostrina* have an operculum that does not fully

cover the opening of their shell and the soft tissues of these juveniles are therefore continually exposed to the antibiotic solution when submerged in such a solution. However, *M. trossulus* can hermetically close their valves and thus needed to be evaluated to ensure they were opening their valves and being exposed to the treatment during the soaks. Visual assessment of the valve position of *M. trossulus* was carried out by placing 24 individuals in separate wells of a 24-well tray, each well containing 10 mL of the antibiotic cocktail. Every individual was observed separately under a dissecting microscope once every five minutes throughout the soak at which time their status, open or closed, was recorded.

Selection of antibiotics

Oxytetracycline hydrochloride (OTC), kanamycin sulfate (KS), a 1:10 mixture of trimethoprim:sulfamethoxazole (TxS), and chloramphenicol (CM) were the antibiotics selected for this study. Antibiotics were selected to provide a range of bacteriostatic and bactericidal protection, to work via different mechanisms of action, and to target different kinds of bacteria in order to create an effective broad spectrum bacterial treatment.

OTC was selected for use because it is effective against many bacteria known to infect juvenile marine invertebrates such as Vibrio spp., Rickettsia spp., and Chlamydia spp. (Paillard et al., 2004; Banerjee et al., 2007) and because it has been used safely on juvenile mud crabs and shrimp (Banerjee et al., 2007; Azam & Narayan, 2013). TxS combined in a 1:10 mixture were selected for use because they have a different mechanism of action than OTC and inhibit growth of different bacteria, and because sulfonamides such as sulfamethoxazole have been widely and successfully used in aquaculture (Boettcher et al., 1999; Liu et al., 2012; de la Cruz et al., 2014). CM was selected for use because it has a different mechanism of action from that of either OTC or TxS, and CM has been

administered to clams without causing mortality (Sutton & Garrick, 1993; Joyner et al., 2003). Finally, KS was selected because it has a different mechanism of action than the aforementioned antibiotics, and has been safely used in cephalopods (Meurant, 2012).

Antibiotics were first tested separately at different concentrations to ensure they did not cause mortality of the juveniles, and the highest dosage of each antibiotic found to be nonlethal was then combined and tested in an 'antibiotic cocktail'. This was done to ensure there were no lethal additive effects of combining the antibiotics. This cocktail was then used in the field experiments.

Preparation of the antibiotic solutions

Antibiotic solutions were prepared by dissolving the aforementioned antibiotics in 0.2 µm filtered and autoclaved seawater. Concentrated stock solutions of OTC and KS were prepared and then subsequently diluted to create each dosage testing solution. Dosage testing solutions of the TxS and CM antibiotics were prepared each time from the original powdered compounds because they were only slightly water soluble. To dissolve the antibiotics, the solutions were mechanically mixed for one to two hours at room temperature and were then refrigerated until use. The final experimental solutions used in the field trials, which consisted of a cocktail of the antibiotics, were pH corrected with NaOH to a range of 8.2 to 8.4, matching the pH of local ocean surface water.

Experiments

Two sets of experiments were carried out: 1) dosage testing, to determine the highest concentration of antibiotics that would cause no detectable effect on the survival of juvenile *M. trossulus* and *N. ostrina*; and 2) field experiments, to determine if repeated short term

exposure to antibiotics leads to a reduction in juvenile mortality in a natural habitat. Dosage testing was performed in small cages in laboratory seawater tables, while field experiments were performed in cages attached to ropes hanging off of the BMSC docks.

Dosage trials: individual antibiotic solutions

Dosage testing was carried out from 19 June to 23 July 2014. Dosage trials of individual antibiotics were carried out on *M. trossulus*; those results were then used as a basis for determining the concentrations to be used in the cocktail for field experimentation with *M. trossulus* and *N. ostrina*.

Four sets of antibiotics, (OTC, KS, a 1:10 mixture of TxS, and CM) were separately tested in dosage trials. Each dosage trial included five treatment groups: a control treatment (no antibiotic), a high dose treatment, and three additional treatments in equal increments between the control and the high dose group. For each antibiotic, the highest dosage treatment was determined based on the Merck Veterinary Manual, previously published literature, or home marine aquarium dosage guidelines.

Juvenile *M. trossulus* and *N. ostrina* were kept in groups of 10 in small cages made of microcentrifuge tubes with 102 µm mesh screening. This set-up allowed the cages to be easily transferred from the seawater tables they were normally maintained in to the containers that held the antibiotic solution. At the end of the five day trial, mortality was counted in each cage.

In a preliminary trial with OTC and KS, *M. trossulus* juveniles were soaked for one hour per day for three days. OTC was tested at 0, 25, 50, 75, and 100 mg/L; and KS was tested at 0, 10, 20, 30, and 40 mg/L. No significant mortality was detected during this first trial (see

appendix Figures 2 and 3), and therefore concentrations were substantially increased and the method was modified to a half hour soak three times per day for five days. In the subsequent definitive dosage trials, OTC was tested at 0, 100, 200, 300, and 400 mg/L; KS was tested at 0, 5, 10, 15, and 20 mg/L; TxS was tested at 0, 7.5:75, 15:150, 22.5:225, and 30:300 mg/L; and CM was tested at 0, 20, 40, 60, and 80 mg/L on *M. trossulus* juveniles. These dosage trials ran from 20 June to 16 July 2014.

Dosage trials: combined antibiotic solutions

In the next dosage trials, the highest dose of each antibiotic that had been found to cause no mortality in the individual trials was combined into one treatment (the antibiotic cocktail). This was then tested to ensure there were no lethal additive effects of exposing the invertebrates to these combined antibiotics. The dosage trial for this antibiotic cocktail involved a control treatment (no antibiotic) and 4 cocktail concentrations: full strength (100%), as well as 75%, 50%, and 25% of full strength. The full strength cocktail included the following: OTC at 400 mg/L, KS at 20 mg/L, TxS at 22.5:225 mg/L, and CM at 80 mg/L. These represent the maximum dosages which caused no mortality during individual dosage testing with *M. trossulus*. Juvenile *M. trossulus* and *N. ostrina* were then exposed to these 4 treatment solutions to determine the most concentrated cocktail that caused no mortality for each species, and thus to determine which treatment would be used in the field experiments. This testing was carried out from 17 to 23 July, 2014.

Effects of antibiotics on mortality in the field

Field testing was carried out from 25 July to 17 August 2014. Weighted ropes were hung off the BMSC docks and the cages containing juveniles were attached to these ropes at depths of

0.75-1.25 m. This setup maintained the animals constantly in the surface seawater, isolating the juveniles from heat, desiccation, predators, UV radiation, and variations in pH or salinity that are typical of intertidal habitats. Three times per day, for five days, one set of replicate cages containing juveniles was soaked for a half hour in 500 mL of filtered, autoclaved seawater (control treatment) and a second set of cages was placed in 500 mL of antibiotic cocktail (antibiotic treatment). *M. trossulus* were exposed to a 75% antibiotic cocktail solution, and *N. ostrina* were exposed to a 100% antibiotic cocktail solution as determined by the dosage testing. Four field trials, each with a different set of animals, were carried out. In the first field trial, the pH of the antibiotic solutions was not corrected, but in the three subsequent trials the pH of the antibiotic solution was corrected to that of surface water in the inlet by adding NaOH until the pH fell between 8.2-8.4, similar to the pH of ocean surface water. This pH correction served to counteract the acidifying effects of the antibiotic cocktail and eliminate any stress this may have put on the animals during the study. When uncorrected, the pH of the antibiotic solution was 6.0 in the 100% concentration treatment and 6.5in the 75% concentration treatment.

Data analysis

Data were not normally distributed and did not have homogenous variances, therefore nonparametric tests were used to analyze the data. The effect of the individual antibiotics and combined antibiotics on juvenile mortality was evaluated using Kruskal-Wallis tests; differences were considered to be significant if $p<0.05$. For the field experiment, mortality in the antibiotic and control treatments were compared using Friedman's nonparametric randomized block test; results were considered to be significant if $p<0.05$. All data analyses were carried out using Minitab software.

RESULTS

Size range of *M. trossulus* used in the experiments

Almost all (98%) *M. trossulus* used in this study were <0.75mm in shell length and belonged to the smallest size classes occurring in the field (Figure 1). Detailed size frequencies of *M. trossulus* juveniles used in field experiments, per imaging date, are reported Appendix Figure 1. These results confirm that only the smallest size classes of *M. trossulus* were used in this study. No *M. trossulus* smaller than 250 μm were ever collected from the field. Also note that measures of experimental *M. trossulus* were taken at the end of the five day field trials; these mussels would have been smaller at the start of the trials.

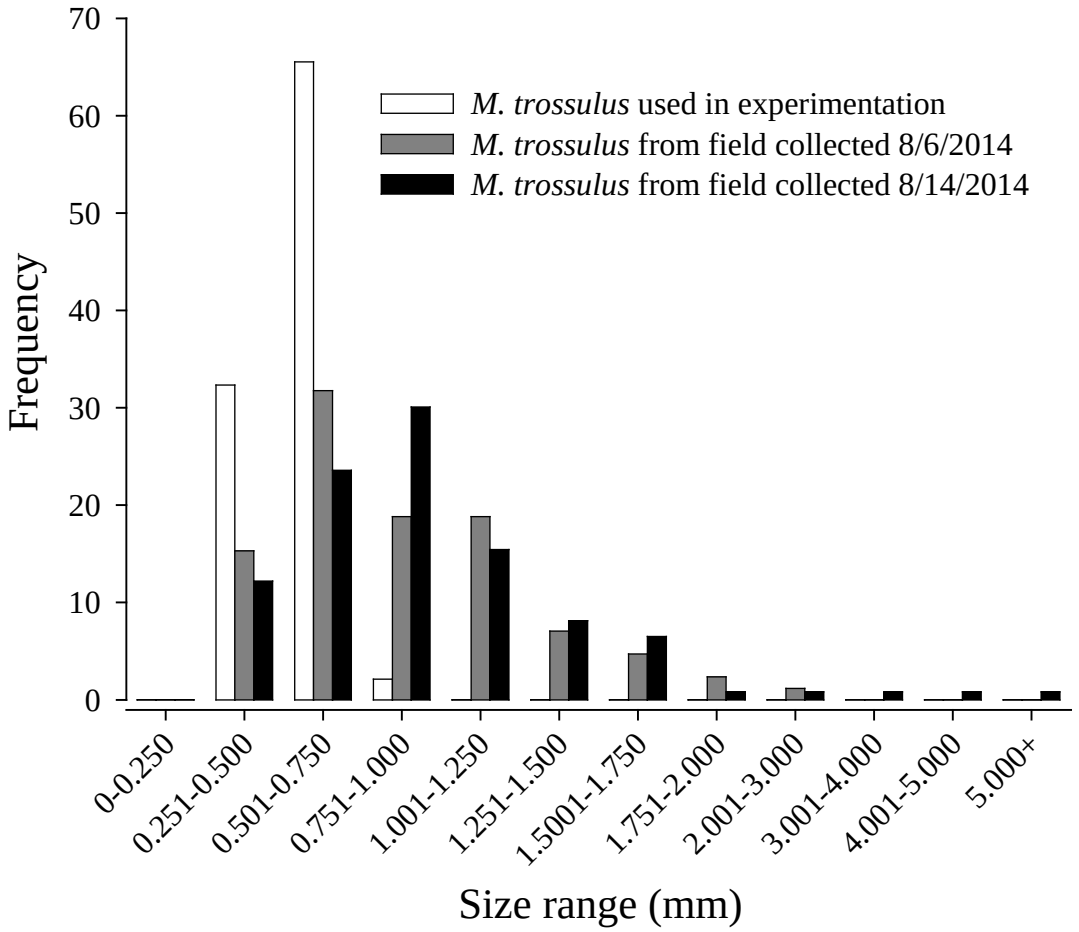

Figure 1: Size frequency distribution of *M. trossulus* collected in the field (found in filamentous algae) displayed by date of collection, and size frequency distribution of those used in field trials 2, 3 and 4 as measured at the end of the five day trials.

Extent of *M. trossulus* exposure to the antibiotic solutions

Juvenile *M. trossulus* were also examined to determine if they were opening their valves when exposed to the antibiotic solutions. Of the 24 mussels examined, 17 were observed to open at some point in the 30 minute trial (71%) while 7 were never observed open (29%). The number open at any one time varied throughout the 30 minute period (Figure 2).

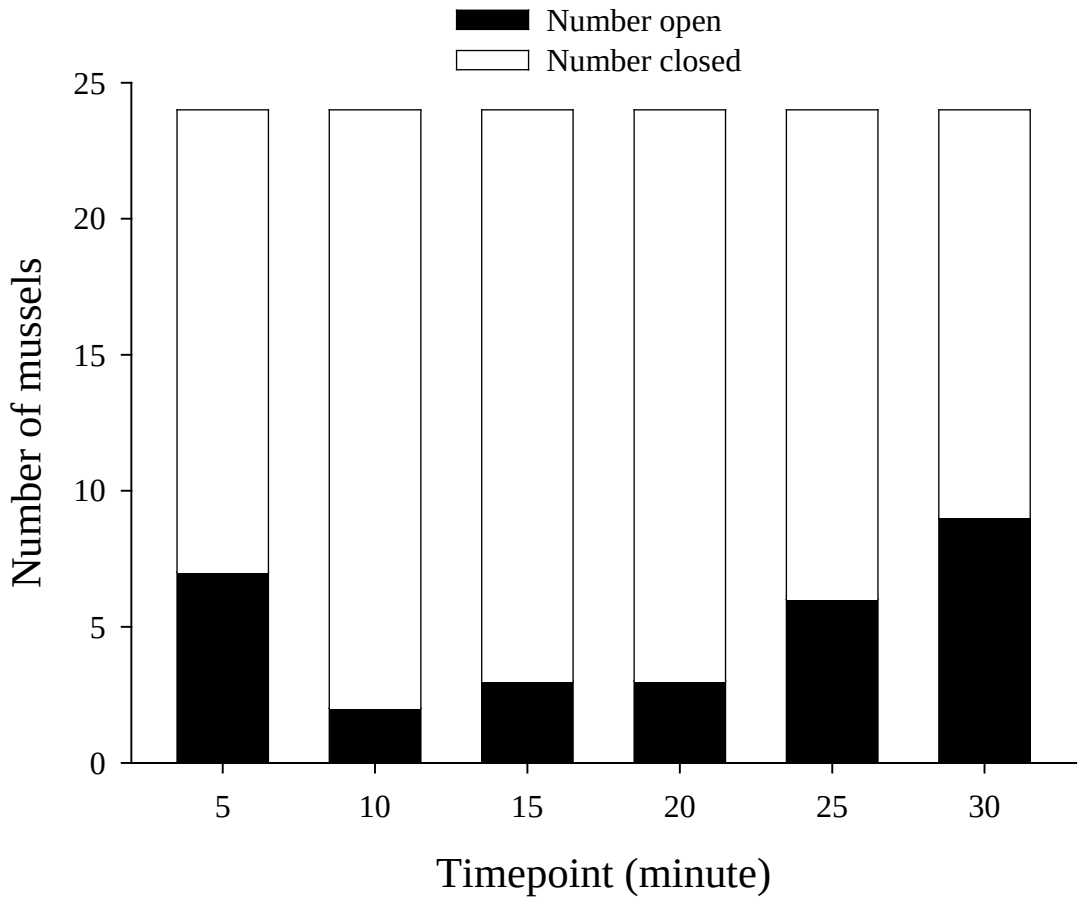

Figure 2: Proportion of *M. trossulus* juveniles that were either open (and thus exposed to the antibiotics) or closed during the course of a half hour soak. Twenty-four *M. trossulus* were individually tracked over the course of this evaluation. At each timepoint, each individual was observed for ten seconds.

Dosage trials: individual antibiotic solutions

At the beginning of the experimental period, antibiotics OTC and KS were separately administered to juvenile *M. trossulus* for one hour per day for three days in an effort to produce a mortality curve. No mortality resulted, and the detailed results of this experiment can be found in Appendix Figures 2 and 3.

For all four antibiotics that were tested, mortality levels in the dosage treatments (Figure 3) were not significantly different from that in the controls (Kruskal-Wallis tests; OTC:

$H_{4,4,4,4,4}$=3.34, df=4, p=0.502; CM: $H_{4,4,4,4,4}$=5.74, df=4, p=0.219; KS: $H_{4,4,4,4,4}$=3.68, df=4, p=0.451; TxS: $H_{4,4,4,4,4}$=3.00, df=4, p=0.558). Additionally, the second highest dosage of the trimethoprim and sulfamethoxazole mixture was selected for further use as this is almost exactly the maximum solubility of sulfamethoxazole in an aqueous solution of a slightly basic pH.

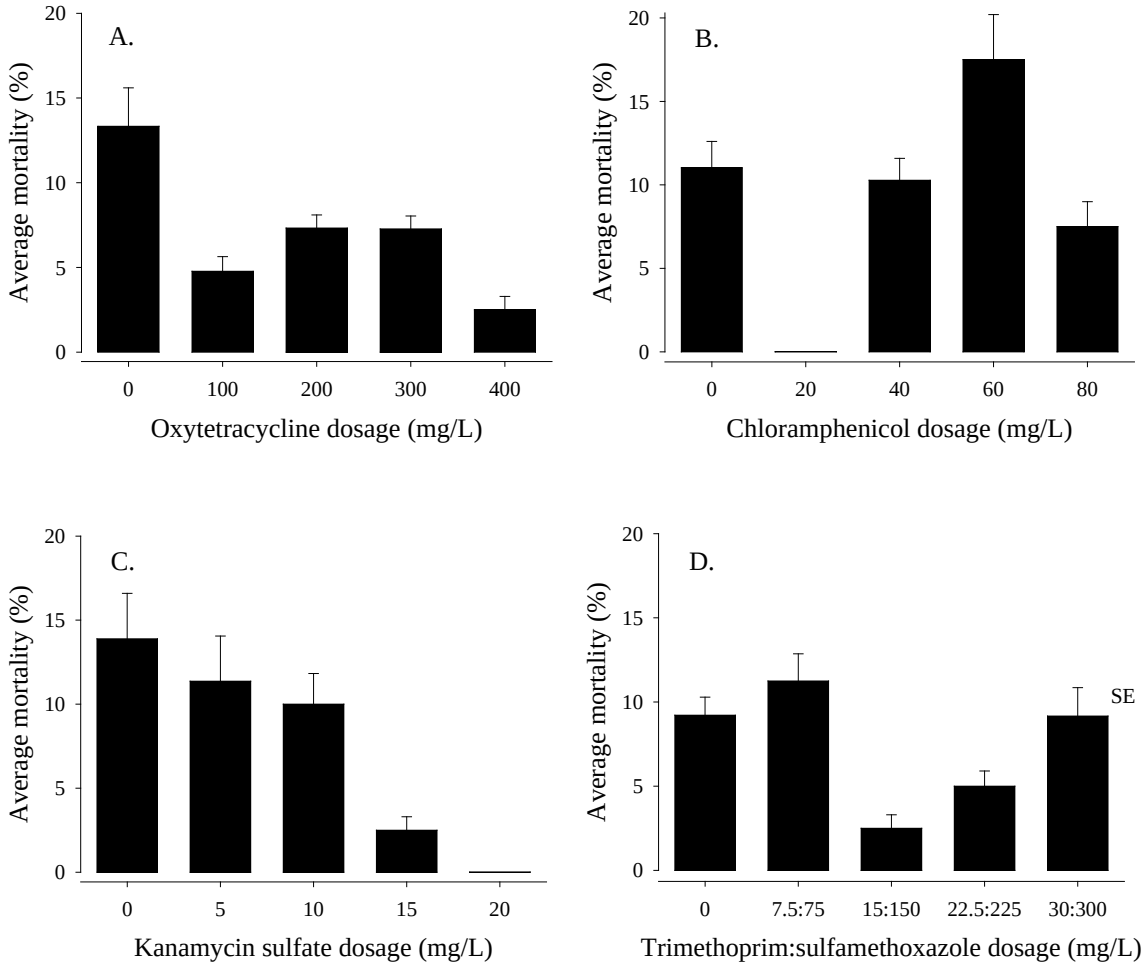

Figure 3: Percent mortality of newly settled *M. trossulus* in A) oxytetracycline, B) chloramphenicol, C) kanamycin sulfate, and D) trimethoprim:sulfamethoxazole antibiotic solutions administered for a half hour three times per day for five days. Each value is based on four replicates of 10 individuals.

Dosage trials: combined antibiotic solutions

Given that the highest concentrations of individual antibiotics used in the first set of dosage trials did not cause mortality, the highest dosage of each antibiotic (with the exception of TxS) were combined into one 'cocktail', which was tested on both *M. trossulus* and *N. ostrina* juveniles in a laboratory setting from 17 to 23 July 2014.

Mortality of juvenile *M. trossulus* and *N. ostrina* in the four dosages of the antibiotic cocktail were not significantly different from the control (no antibiotic). For *M. trossulus*, the 75% strength cocktail was selected for use in the field experiment (Figure 4) as it appeared that the 100% treatment may have caused some mortality that was undetectable by statistical analysis (Kruskal-Wallis test, $H_{4,4,4,4,4}$=5.38, df=4, p=0.251). In *N. ostrina*, there was no significant difference in mortality between any of the groups (Figure 5) and as such the 100% cocktail was selected for use in the field experiment (Kruskal-Wallis test, $H_{7,7,7,7,7}$=5.32, df=4, p=0.256).

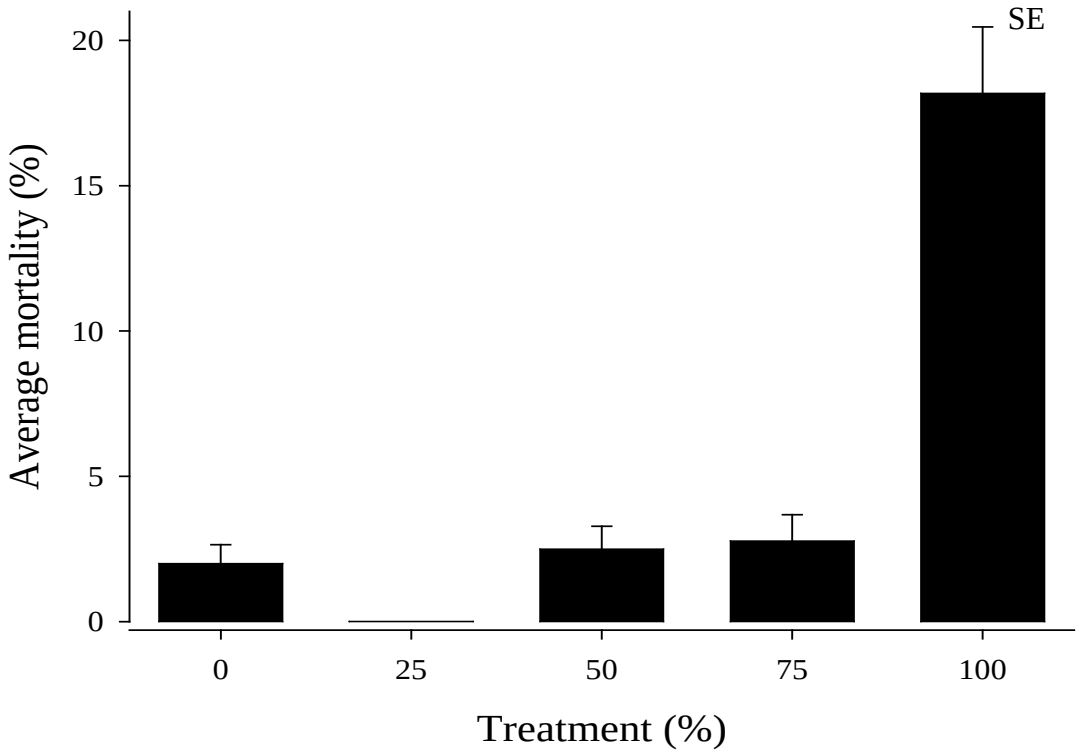

Figure 4: Mortality of *M. trossulus* in the combined antibiotic dosage trial. Each value represents the average of four replicates of 10 individuals. The full (100%) antibiotic cocktail used in this dosage trial consisted of oxytetracycline at 400 mg/L, kanamycin sulfate at 20 mg/L, a 1:10 ratio of trimethoprim:sulfamethoxazole at 22.5:225 mg/L, and chloramphenicol at 80 mg/L. These antibiotics were mixed at 100% and then diluted to 75%, 50%, 25%, and 0% of full strength for the other treatments.

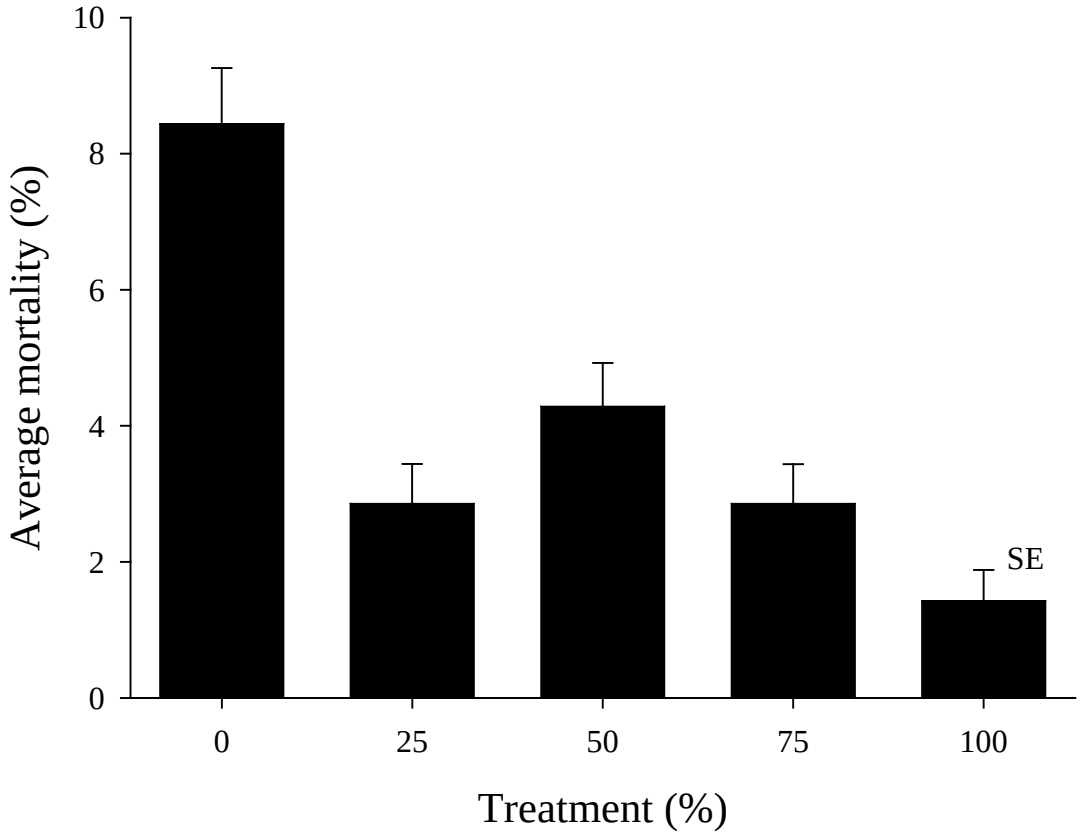

Figure 5: Mortality of *N. ostrina* in the combined antibiotic dosage trial. Each value represents the average of seven replicates of 10 individuals. The full (100%) antibiotic cocktail used in this dosage trial consisted of oxytetracycline at 400 mg/L, kanamycin sulfate at 20 mg/L, a 1:10 ratio of trimethoprim:sulfamethoxazole at 22.5:225 mg/L, and chloramphenicol at 80 mg/L. These antibiotics were mixed at 100% and then diluted to 75%, 50%, 25%, and 0% of full strength for the other treatments.

Effects of antibiotics on mortality in the field

A first analysis compared mortality in the first trial (not pH balanced) with mortality in the three subsequent trials. This analysis found there was no significant difference in mortality between the pH-corrected and non-pH-corrected trials (Kruskal-Wallis test; *M. trossulus*: $H_{8,3,5,3}$=0.99, df=1, p=0.321; *N. ostrina*: $H_{9,19,5,5}$=0.04, df=1, p=0.837). A second analysis using each of the four field trials as blocked replicates compared mortality in the antibiotic

treatment with that in the control treatment. This analysis found that there was no significant difference in mortality between the control and antibiotic treatment for either *M. trossulus* or *N. ostrina* (Friedman nonparametric randomized block test; *M. trossulus:* X^2_r>0.00, df=1, p>0.99; *N. ostrina*: X^2_r=0.33, df=1, p=0.564).

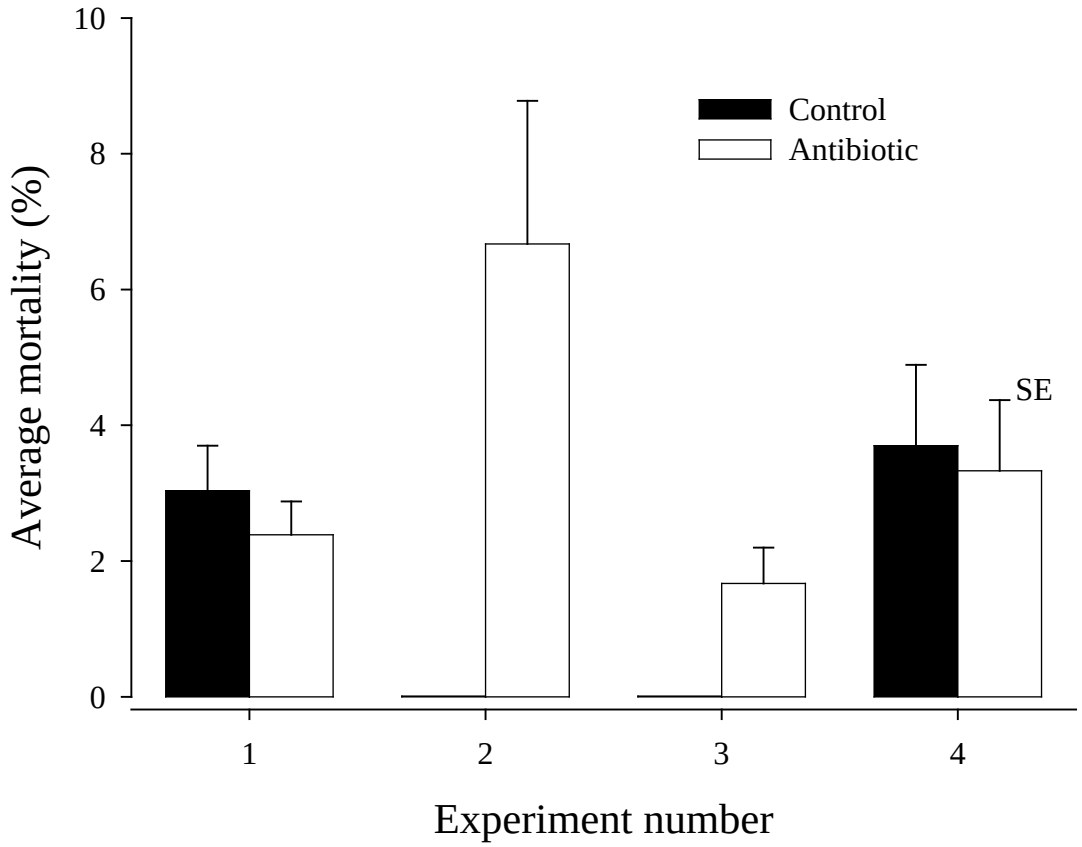

Figure 6: Mortality of *M. trossulus* in field trials 1-4. The 75% antibiotic cocktail used in these field trials consisted of oxytetracycline at 300 mg/L, kanamycin sulfate at 15 mg/L, a 1:10 ratio of trimethoprim:sulfamethoxazole at 16.88:168.75 mg/L, and chloramphenicol at 60 mg/L. Values of trials 1-4 in the control treatments are based on 8, 4, 5, and 3 replicates of 10 individuals, while values in the antibiotic treatments are based on 8, 3, 6, and 3 replicates of 10 individuals, respectively.

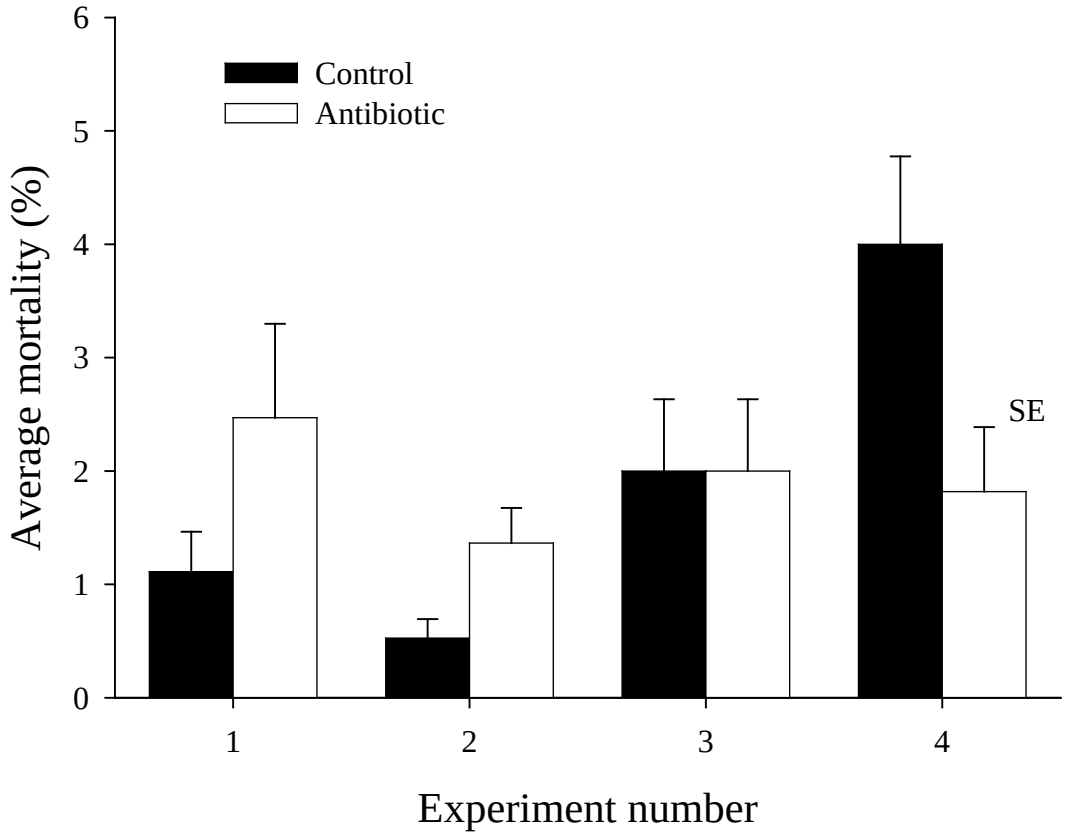

Figure 7: Mortality of *N. ostrina* in field trials 1-4. The full strength (100%) antibiotic cocktail used in these field trials consisted of oxytetracycline at 400 mg/L, kanamycin sulfate at 20 mg/L, a 1:10 ratio of trimethoprim:sulfamethoxazole at 22.5:225 mg/L, and chloramphenicol at 80 mg/L. Values for trials 1-4 in the control treatments are based on 9, 19, 5, and 5 replicates of 10 individuals, while values in the antibiotic treatments are based on 9, 20, 5, and 5 replicates of 10 individuals, respectively.

DISCUSSION

Size range of *M. trossulus* used in the experiments

The juvenile *M. trossulus* used in this study were of the smallest size classes available in the field, most being between 0.250 and 0.750 mm. This size is consistent with the findings of Martel et al. (2000), who determined the mean settling size of *M. trossulus* to be 0.330 mm and the early juveniles to range in size up to about 1 mm.

Extent of *M. trossulus* exposure to the antibiotic solutions

Most *M. trossulus* (71%) were observed to open at some point during the 30 minutes of exposure to the antibiotic cocktail. The actual proportion opening when placed in an antibiotic solution was probably even higher, as mussels reflexively close when disturbed and these observations required near constant disturbance of the mussels.

Dosage trials

Appropriate dosages of the antibiotics were determined experimentally prior to use in field experiments, as very little dosage information is available for juvenile marine invertebrates. The highest dosages of the individual antibiotics used in this experiment found to cause no mortality of juveniles were: 400 mg/L of oxytetracycline (OTC), 20 mg/L of kanamycin sulfate (KS), 80 mg/L of chloramphenicol (CM), and 30:300 mg/L of trimethoprim:sulfamethoxazole (TxS), although 22.5:225 mg/L TxS was used in the antibiotic cocktail as this concentration approaches the upper limit of solubility of sulfamethoxazole (Dahlan et al. 2011). These findings are in line with previous studies using these antibiotics, which exposed invertebrates to lower dosages than the maximum dosages used in this study's 100% antibiotic cocktail. However exposed invertebrates to antibiotics for longer periods of time than were used in this study, and those longer exposure times did not cause mortality of the invertebrates (Azam & Narayan, 2013).

In the present study, the above antibiotics were then combined into one 'cocktail' using the aforementioned dosages which was administered to both *M. trossulus* and *N. ostrina* at various concentrations, and no difference in mortality was observed between the control groups and treatment groups. A dose of 75% was selected for the field experiments with *M.*

trossulus, however, as mortality was slightly higher in the 100% concentration treatment and the use of small sample sizes may have precluded the statistical analysis from being significant.

Effects of antibiotics on mortality in the field

The design of this study involved using small cages to house the juvenile invertebrates and expose them to antibiotics, thereby (1) isolating the juveniles from non-infectious factors that are known to cause juvenile mortality while leaving them exposed to infectious diseases, and (2) evaluating if the remaining mortality could be decreased by protecting the juveniles from bacterial infection.

Levels of mortality of both *M. trossulus* and *N. ostrina* in the control treatments in the field experiments were much lower than natural field mortality rates reported in other studies of juveniles over similar time frames. Mortality of *M. trossulus* in this study was 0 – 6.7% over five days. In contrast, Phillips (2002 and 2004) found mortality levels of 77-97% and 69-99% in *M. trossulus* juveniles after two weeks in the field. Meden (2012) reported 54 and 64% mortality of *Perna perna* mussels within two days of settlement over two different sampling cycles.

Mortality of *N. ostrina* in the present study was 0.5 - 4.0% over a five day period. This is far below the mortality rates reported by other studies with juvenile *N. ostrina*. Moran and Emlet (2001) found 35-60% mortality of *N. ostrina* juveniles after nine days in the field.

The low levels of juvenile mortality observed in this study are likely because this is the first study that used an enclosed cage design, which maintains the juveniles in a protected and submerged state, removing many common causes of juvenile marine invertebrate mortality in

the intertidal zone. These include predation, temperature stress, desiccation stress, UV radiation stress, and major fluctuations in pH and salinity (reviewed in Gosselin & Qian, 1997). The cage design also allowed complete recovery of all of the juveniles as opposed to the open 'settling pad' or walled arena designs normally used and which necessitate use of absence as a proxy for mortality. The present findings therefore reveal that infectious agents cause very little (≤6%) or no juvenile mortality.

The low levels of mortality that did occur in the present field trials were not the result of bacterial infection. Statistical analyses revealed no difference in mortality between the controls and the antibiotic treatments, indicating that bacterial infection did not cause juvenile mortality in these two species. The few individuals that did die during the trials may have been killed by fungal or viral infections, handling stress, yolk depletion, or developmental and genetic failure, all of which remained as potential stressors in the experimental design.

It should be noted that this study did not take into account the potential interactive effects of elevated temperature and desiccation stress on an animal's susceptibility to infectious stress. Knowing which factors cause mortality of juveniles is important because the amount and timing of juvenile mortality can affect other factors relevant to the species, such as abundance and distribution. Knowledge of the selective pressures that are in place early in juvenile life can also help to elucidate the evolution of adaptive traits, help focus conservation efforts on factors limiting population recovery, and help obtain estimates of invertebrate recruitment for fisheries.

Implications and future directions

Low levels of mortality when exposed to the natural microbial community were likely a consequence of the nature of the invertebrate immune system. Like any marine organism, marine invertebrates are constantly surrounded by potential pathogens. There are anywhere from 10^3 - 10^6 bacteria and 10^7 viruses per millilitre of sea water (Austin, 1988; Børsheim et al., 1990). As such, marine animals have developed a myriad of ways of coping with these stressors. For example, recent research on invertebrate immune systems indicates that although they do not possess an adaptive immune system, their innate immune system may have ways of responding to subsequent infections more strongly than on the first exposure, a model which is being referred to as 'trained immunity'(Cong et al., 2008; Ng et al., 2014; Quintin et al., 2014).

Invertebrates have also developed ways to pass their immunity on to their offspring, before the offspring can protect themselves. Egg masses of many invertebrates, such as polychaetes (Benkendorff et al., 2001), cephalopods (Atkinson, 1973), and many species of molluscs (Benkendorff et al., 2001; Lim et al., 2007; Hathaway et al., 2010; Peters et al., 2012) have been shown to possess antibacterial activity, often in the form of antimicrobial peptides passed down from the parent to the egg mass. This includes the leathery egg capsules of snail species in the same family as *N. ostrina* (Benkendorff et al., 2001). The antibacterial activity of these egg capsules shows a general trend of being highest when the capsules are freshly laid and decreasing as the embryos develop into juveniles, indicating this characteristic is a parental investment (Benkendorff et al., 2001). Further, the immune system of marine snails appears to become competent only after metamorphosis, the juveniles having relatively high

survivorship in a laboratory setting whereas the veligers dying of bacterial infection in a short time frame after being removed from the egg capsule (Pechenik et al., 1984; Lord, 1986).

In species that employ a swimming larval stage for dispersal (such as *M. trossulus*), the parents still protect their offspring from infection until the offspring become immune competent. For example, the mRNA expressed by *M. galloprovincialis* larvae pre- and post-metamorphosis are different: those expressed pre-metamorphosis are parental investments and those expressed post-metamorphosis are actively produced by the juvenile itself (Balseiro et al., 2013). Further, the mRNA expressed by the juveniles immediately post metamorphosis have a similar range and magnitude to the mRNA expressed by adult *M. galloprovincialis*, indicating that the juveniles have immune systems similar to that of the adults immediately after metamorphosis (Balseiro et al., 2013).

Emerging is a pattern of parental immune factors protecting the offspring until a certain point, usually metamorphosis, at which time the juveniles begin producing their own immune factors. That pattern could help explain the results found here: no mortality from bacterial infection was observed because after metamorphosis the juveniles had already developed a competent immune system. This result also suggests that bacterial outbreaks that impact adult populations would also affect juveniles and juvenile recruitment.

Given the findings of the present study, future research should examine the effectiveness of the antibiotics used in this study on the bacteria associated with juvenile *M. trossulus* and *N. ostrina*. This could be achieved by conducting bacterial assays testing (1) the effectiveness of the antibiotics themselves, and (2) the types of bacteria harboured by antibiotic-treated versus control invertebrates. This assay could be completed by homogenating small samples of *M. trossulus* and *N. ostrina*, plating the homogenate on marine agar, and observing the number

and types of bacterial colonies grown. A positive assay examining if bacteria can cause invertebrate mortality would also be useful and could be achieved by concentrating bacteria normally found in seawater, soaking the invertebrates in water with an increasing bacterial load, and examining when mortality begins to increase.

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APPENDIX

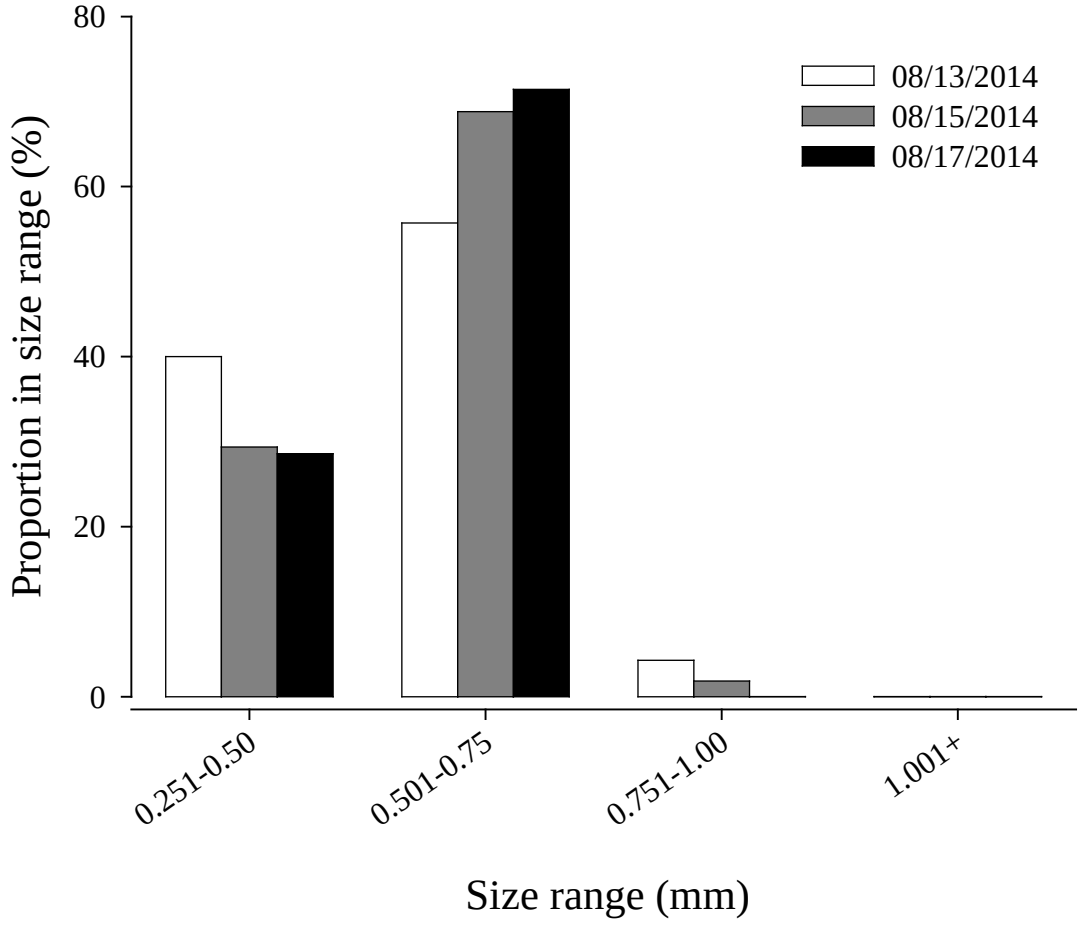

Figure 1: Total proportion of *Mytilus trossulus* used in experimentation displayed by size range and date of measurement. A total of 235 individuals were photographed and digitally measured, 70 from 8/13/2014, 109 from 8/15/2014, and 56 from 8/17/2014.

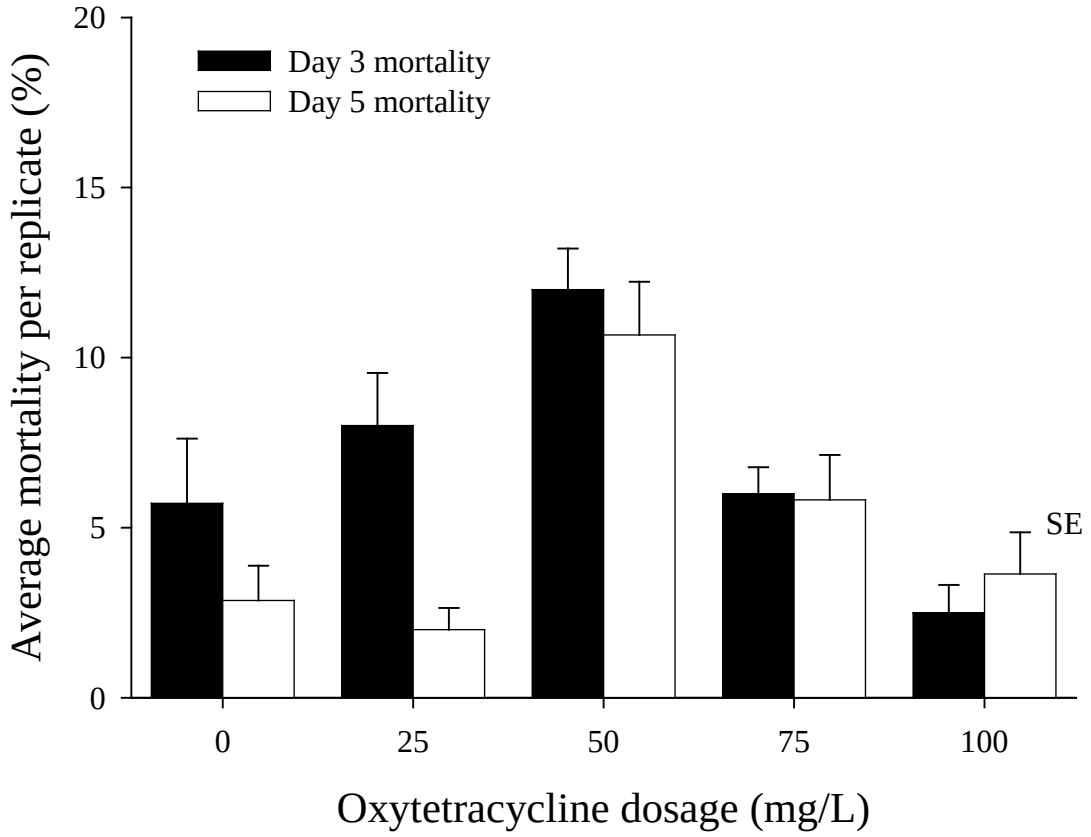

Figure 2: Mortality of *M. trossulus* in the oxytetracycline dosage experiment conducted from 21/06/2014-26/06/2014, mortality being counted on 24/06/2014 (day 3) and 26/06/2014 (day 5). Between counts some individuals were lost, resulting in some day 5 mortality being lower than day 3 mortality. Each value represents the average of five replicates of 10 individuals.

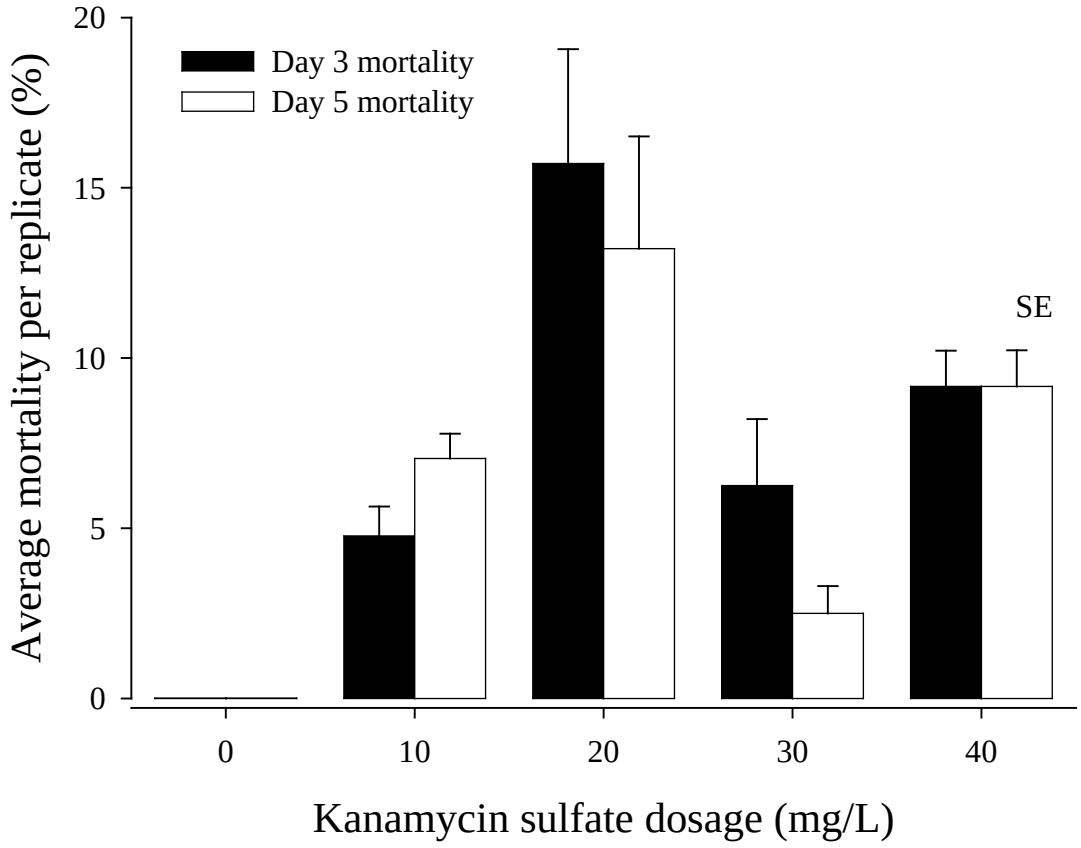

Figure 3: Mortality of *M. trossulus* in the kanamycin sulfate dosage experiment conducted from 24/06/2014-29/06/2014, mortality being counted on 27/06/2014 (day 3) and 29/06/2014 (day 5). Between counts some individuals were lost, resulting in some day 5 mortality being lower than day 3 mortality. Each value represents the average of four replicates of 10 individuals.