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Testing microbial pathogens as a cause of early juvenile mortality in wild populations of benthic invertebrates

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ABSTRACT: Microbial pathogens such as bacteria, viruses, fungi, and protozoa are potentially important causes of mortality during the critical first days of juvenile life in benthic invertebrates, but their effect on early juveniles in natural habitats remains unexplored. We therefore placed newly-settled mussels (*Mytilus trossulus*) and barnacles (*Balanus glandula, Chthamalus dalli*), and newly-hatched snails (*Nucella ostrina*) in screened cages submerged in the field, exposing them to ambient microbial organisms while isolating them from most non-microbial mortality factors. Mortality over a 5 d period was only 0–3% in *M. trossulus* and *N. ostrina* and 0–10% in *B. glandula* and *C. dalli*. Mortality was consistent among replicate trials in the same summer and between 2 different years, and was much lower than previously reported natural early juvenile mortality in these species. We then examined whether bacterial infection caused the few juvenile deaths in our field experiment. Dosage testing in the laboratory with 5 antibiotics resulted in an antibiotic cocktail providing broad-spectrum antibacterial protection without affecting the health of juveniles. In a second field experiment, early juvenile *M. trossulus* and *N. ostrina* exposed to the antibiotic cocktail 3 times daily did not experience reduced mortality relative to controls. Our findings suggest that, at least in some species, microbial pathogens are not a direct cause of juvenile mortality in the field, thus narrowing the list of potential causes of early juvenile mortality to the non-microbial factors excluded from our experiments.

KEY WORDS: Early benthic phase · Natural mortality · Bacteria · Viruses · Antibiotics · Immune response · Survivorship · Mussels · Barnacle cyprids · Snails

INTRODUCTION

Cohorts of benthic marine invertebrates experience a substantial bottleneck at the beginning of independent benthic life, a period known as the early benthic phase (EBP) that includes the early juvenile period and sometimes also the end of the larval period (Jenewein & Gosselin 2013a, de Bruyn & Gosselin 2014). As much as 90 to 100% of individuals die during the first few weeks after either settling from the plankton, emerging as a juvenile from an egg capsule, or following release by a brooding parent (Gosselin & Qian 1997, Pedersen et al. 2008). In fact, mortality is usually highest during the first few days of the EBP (Gosselin & Qian 1997, Gosselin & Jones 2010, Torres et al. 2016). Several factors are known to contribute to this high mortality rate, including predation (Lefcheck et al. 2014, Carroll et al. 2015), temperature (Nasrolahi et al. 2013), desiccation (Gosselin & Chia 1995, Jenewein & Gosselin 2013b), wave action (Naylor & McShane 2001), ultraviolet radiation (Gosselin & Jones 2010), and low energy reserves at the time of the transition to independent benthic life (Pechenik et al. 1998, Torres et al. 2016). In addition to these factors, microbial pathogens such as bacteria, viruses, fungi, and protozoa are potentially im-

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portant causes of EBP mortality in natural habitats (Gosselin & Qian 1997); however, the role of microbial organisms as causes of EBP mortality in natural habitats remains unexplored and thus hypothetical.

Microbial pathogens do cause mortality of adult marine invertebrates in natural habitats, as observed during mass-mortality events. For instance, the sea-star wasting disease that swept the west coast of North America in 2014 was linked to a densovirus infection (Hewson et al. 2014). Bacterial infections also propagate through wild invertebrate populations, causing substantial adult mortality (reviewed by Fey et al. 2015), such as the 1999 and 2003 mass mortalities of ascidians, bryozoans, cnidarians, mollusks, and sponges in the Mediterranean Sea, caused by a *Vibrio* spp. bacterium (Cerrano et al. 2000, Perez et al. 2000, Bonhomme et al. 2003). Reports of mortality during such events, however, only refer to adult and late juvenile animals. In addition, the effect of microbial organisms on mortality during non-outbreak periods is poorly understood for all life phases, including the adult phase.

Microbial infections can affect young juveniles, as evidenced by significant health problems in invertebrates reared in aquaculture facilities (Wilkenfeld 1992, Paillard et al. 2004) or in aquaculture-reared juveniles outplanted to field sites during viral outbreaks (Dégremont 2013, Keeling et al. 2014). For instance, pathogenic bacteria cause 4 diseases specifically lethal to juvenile bivalves reared in aquaculture: juvenile oyster disease (*Proteobacteria*), hinge ligament erosion disease (*Cytophaga*), chronic abscess syndrome (*Vibrio*), and an event referred to as summer oyster mortality (bacterial species not determined) (Paillard et al. 2004). Aquaculture stocks affected by these diseases may experience up to 100% juvenile mortality (Goulletquer et al. 1998, Paillard et al. 2004). In addition to bivalves, bacterial diseases also affect juveniles of other species reared in aquaculture, including sea cucumbers, crabs, and shrimp (Muroga et al. 1989, Lightner & Redman 1998, Wang et al. 2004). Microbial pathogens therefore cause some EBP mortality in aquaculture facilities, but it is yet to be determined whether the high infection and mortality rates observed in aquaculture settings result from the artificial environment and high densities of animals (Spaargaren 1998), or whether these mortalities reflect a susceptibility of EBP individuals to microbial pathogens in all environments, including natural habitats.

We therefore tested the hypothesis that microbial pathogens cause EBP mortality in wild populations of benthic marine invertebrates, with particular attention to the role of naturally occurring bacteria. Our specific objectives were to (1) determine the combined effect of all naturally occurring microbial pathogens on mortality in the field during the critical first 5 d of the EBP in 4 species of benthic invertebrates; and (2) assess the specific contribution of bacteria to EBP mortality by determining whether mortality in a natural setting decreases when early juveniles are periodically exposed to a broad-spectrum antibiotic cocktail.

MATERIALS AND METHODS

Study site and organisms

This study was conducted during the summers of 2014 and 2015 at the Bamfield Marine Sciences Centre (BMSC) and at 2 nearby field sites in Barkley Sound, on the west coast of Vancouver Island, Canada. Four species were examined: a bivalve, the Pacific blue mussel *Mytilus trossulus*; a gastropod, the northern striped dogwinkle *Nucella ostrina*; and 2 crustaceans, the acorn barnacles *Balanus glandula* and *Chthamalus dalli*. These highly abundant species occupy mid-intertidal rocky shores along the west coast of North America, from California to Alaska (Palmer et al. 1990, Rawson & Hilbish 1995), and past studies have documented high levels of mortality during the first few days of independent benthic life in 3 of these species: *M. trossulus* (Phillips 2002, 2004), *N. ostrina* (Moran & Emlet 2001), and *B. glandula* (Gosselin & Qian 1996, Gosselin & Jones 2010, Jenewein & Gosselin 2013a).

EBP specimens of *M. trossulus* and *N. ostrina* were collected from Prasiola Point (48° 81′ 76″ N, 125° 16′ 84″ W). *M. trossulus* juveniles were extracted from the filamentous alga *Cladophora columbiana*, and the smallest (≤0.75 mm shell length) of those individuals were set aside for use in experiments. *N. ostrina* juveniles were obtained by collecting ripe egg capsules (unplugged capsules containing fully developed juveniles that have not yet emerged), placing these capsules in screened cages in seawater in the laboratory for 24 h, as described by Gosselin & Chia (1995), and then collecting all individuals that hatched during those 24 h for immediate use in experiments. *B. glandula* and *C. dalli* were collected from Wizard Islet (48° 51′ 28″ N, 125° 09′ 34″ W). For each trial with *B. glandula* and *C. dalli*, we collected, from the mid-intertidal zone, 25 stones (5–10 cm in diameter) that were already colonized by at least a few barnacles. On each stone we marked the perime-

ter of a selected area 4–6 cm in diameter using small spots of nail polish and then removed all cyprids as well as juvenile barnacles ≤4 mm diameter under a dissecting microscope. The stones were returned to the intertidal zone at Wizard Islet for 20 h to allow new settlers of both species to attach, and then the stones were recovered and returned to the laboratory. Upon recovery, most stones had been colonized by new settlers of both species, some still in the cyprid stage and some already metamorphosed into juveniles. This finding is consistent with a previous report that *B. glandula* cyprid larvae metamorphose into juveniles within 48 h after attaching to a surface (Gosselin & Jones 2010); although no published data exist regarding the timeline for metamorphosis in *C. dalli*, our observations suggest it is likely similar to that of *B. glandula*. Finally, cyprids (Arnsberg 2001) and newly-metamorphosed juveniles (L. A. Gosselin pers. obs.) of these 2 species differ morphologically and could readily be distinguished under a microscope or magnifying lens; *C. dalli* metamorphs are smaller in size, have lateral shell plates that are more inwardly curved near the operculum, and are darker in color than *B. glandula* metamorphs.

Effects of microbial pathogens on EBP mortality in the field

To determine the combined effects of all microbial pathogens (bacterial, viral, fungal, and protozoan) on EBP mortality in the field, EBP individuals of each of the 4 species were placed in cages suspended on weighted ropes off the dock of the BMSC for 5 d. Cage mesh sizes were as follows: *M. trossulus*, 102 µm; *N. ostrina*, 660 µm; *B. glandula* and *C. dalli*, 200 µm. These mesh sizes were small enough to prevent the escape of motile juveniles and the entry of predators and debris, while allowing the flow of water, phytoplankton, and microbial organisms. The screens also prevented larvae in the water column from entering and settling in the cages. In addition, the cages were constantly submerged at depths of 0.75–1.25 m during the 5 d trials, thus isolating the EBP individuals from desiccation, heat stress, ultraviolet radiation exposure, and rapid salinity changes that occur in the intertidal zone during rainfall events. Thus, EBP individuals were fully exposed to ambient microbes but were isolated from most non-microbial causes of mortality.

M. trossulus and *N. ostrina* were placed in cages made of microcentrifuge tubes, with 10 juveniles of 1 species per cage. For each *B. glandula* and *C. dalli* trial, 14 stones bearing the most cyprids and newly-metamorphosed juveniles were placed in 400 ml cages, 1 stone per cage; these cages thus held varying numbers of newly-settled cyprids and juveniles, depending on the numbers settling on the stones during the previous 20 h settlement period. For *C. dalli*, at the start of Trial 1, the numbers of cyprids (11 to 29) and juveniles (5 to 22) per stone were sufficiently high to calculate percent mortality for each stone and thus calculate an average and a standard error among replicate stones. In the second *C. dalli* trial and in both *B. glandula* trials, the numbers of cyprids and of juveniles were ≤4 on several stones; in these cases, data from all 14 stones in the trial were pooled to obtain a single overall mortality value, precluding the calculation of a standard error.

Five replicate 5 d trials with *M. trossulus* were started on 25 July, 8 August, 10 August and 12 August 2014, and on 6 August 2015; 5 *N. ostrina* trials were also carried out on those same dates. Two joint trials with *B. glandula* and *C. dalli* were started on 5 July and 14 July 2014. In all trials, the fate of each individual was determined at the end of the 5 d period by careful microscopic examination.

Role of bacterial infection in early juvenile mortality

Antibiotics have proven effective at protecting marine invertebrate larvae and juveniles against bacterial infection and thus can be useful in experiments aiming to quantify the effects of bacterial infection on mortality in natural populations. They are commonly used to curb the appearance and spread of bacterial infection in invertebrate aquaculture operations (Holmstrom et al. 2003, Thuy et al. 2011, de la Cruz et al. 2014, Wang et al. 2014), and thus to increase yields without apparent adverse effects to animal health (Bray et al. 2006). Invertebrate researchers also commonly use antibiotics 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). We therefore used antibiotics in 3 sets of experiments, involving *M. trossulus* and *N. ostrina*, to examine the role of bacteria as causes of early juvenile mortality: (1) dosage testing, to determine concentrations of antibiotics that could be used without causing mortality of early juvenile *M. trossulus* and *N. ostrina*; (2) efficiency testing, to verify the effectiveness of the final antibiotic cocktail against bacteria occurring in local sur-

face seawater; and (3) field experiments examining whether repeated exposure of early juveniles to an antibiotic cocktail reduces EBP mortality in the natural habitat. Dosage and efficiency testing experiments were performed in the laboratory, whereas field experiments took place off the BMSC docks.

Selection of antibiotics

Five 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 and thus create an effective broad-spectrum antibacterial treatment: oxytetracycline hydrochloride (OTC), kanamycin sulfate (KS), chloramphenicol (CM), and a 1:10 mixture of trimethoprim and sulfamethoxazole (TMP-SMX). These antibiotics were also selected based on previous studies indicating effectiveness in protecting marine invertebrates (OTC: Paillard et al. 2004, Banerjee et al. 2007, Azam & Narayan 2013; KS: Forsythe et al. 1990; CM: Sutton & Garrick 1993, Joyner et al. 2003; TMP-SMX: Boettcher et al. 1999, Liu et al. 2012, de la Cruz et al. 2014). Stock antibiotic solutions were prepared by placing antibiotics in 0.2 µm filtered and autoclaved seawater and then continuously stirring for 1 to 2 h at room temperature.

Dosage trials: individual antibiotic solutions

Dosage testing, used to determine the concentration of each antibiotic to include in an antibiotic cocktail, was carried out in June and July 2014 with *M. trossulus*. Juvenile *M. trossulus* were immersed in antibiotic solutions for 30 min, 3 times a day, for 5 d, and then assessed for mortality. Dosage trials involved testing 5 treatment concentrations for each antibiotic: OTC at 0, 100, 200, 300, and 400 mg l^{-1}; KS at 0, 5, 10, 15, and 20 mg l^{-1}; CM at 0, 20, 40, 60, and 80 mg l^{-1}; and TMP-SMX at 0:0, 7.5:75, 15:150, 22.5:225, and 30:300 mg l^{-1}. Each treatment concentration was tested on 4 replicates of 10 individual *M. tossulus*. The highest dosages were based on the Merck Veterinary Manual (Aiello & Moses 2010), previously published literature, or home marine aquarium dosage guidelines. Periodic observations during the dosage trials revealed that *M. trossulus* opened their valves during the trials, thus directly exposing them to the antibiotic solutions.

Dosage trials: combined antibiotic solutions

In this second set of dosage trials, the highest dose of each antibiotic that had been found to cause no mortality in the individual trials were combined into a single solution, and then this antibiotic cocktail was tested with *M. trossulus* and *N. ostrina* to ensure no lethal effects of the combined antibiotics. Dosage trials for the 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 consisted of: OTC at 400 mg l^{-1}, KS at 20 mg l^{-1}, CM at 80 mg l^{-1}, and TMP-SMX at 22.5:225 mg l^{-1}. Each treatment concentration was tested on 4 replicates of 10 *M. trossulus* and on 7 replicates of 10 *N. ostrina*. As in the earlier trials with individual antibiotics, juveniles were exposed to their antibiotic cocktail treatment for 30 min, 3 times daily for 5 d, and then assessed for mortality.

Effectiveness of the antibiotic cocktail against ambient marine bacteria

This experiment examined whether the antibiotic cocktail effectively prevents growth of ambient marine bacteria. First, a stock culture of marine bacteria was obtained by inoculating 10 ml of Difco™ marine broth with 2% (vol/vol) unfiltered surface seawater, and then incubating at 35°C for 24 h. Then 3 sets of 10 replicate vials were prepared for the experiment, providing each vial with a combined solution totaling 10 ml. One set of 10 vials (Broth + Antibiotics + Bacteria treatment) received sterile marine broth, the antibiotic cocktail to a final concentration of 75% of full strength, and 100 µl of the bacterial culture. A second set of 10 vials (Broth + Antibiotics [Sterile] treatment) received sterile marine broth and the antibiotic cocktail to a final concentration of 75% of full strength, but no bacteria. The third set of 10 vials (Broth + Bacteria treatment) received sterile marine broth and 100 µl of the bacterial culture, but no antibiotics. The optical density (OD) of each vial was measured after 0, 24, 48, and 70 h on an Ultrospec 2100 pro UV/visible spectrophotometer at 600 nm.

Field experiment: bacteria-induced mortality

This experiment, carried out with *M. trossulus* and *N. ostrina*, involved 4 replicate 5 d field trials for each species, from 25 July to 17 August 2014 off the BMSC docks. Both species were tested at the same time but

in different sets of cages, using a different set of animals from one trial to the next. Replicate cages, each containing 10 early juveniles of a single species, were hung on ropes at depths of 0.75–1.25 m. Cages were assigned to 1 of 2 treatments: exposure to antibiotics or control (no antibiotics). For both treatments, the cages were lifted out of the ocean 3 times each day, at 8 h intervals, and placed in 500 ml of either filtered autoclaved seawater (control treatment) or antibiotic cocktail (antibiotic treatment) for 30 min. For the antibiotic treatment, *M. trossulus* were exposed to a 75% concentration of the cocktail solution, and *N. ostrina* were exposed to a 100% antibiotic cocktail solution, as determined by the previous dosage testing. In the first field trial, the pH of the antibiotic solution was not corrected, resulting in low pH values due to the acidifying effect of the antibiotics; the 100% concentration treatment had a pH of 6.0 and the 75% concentration treatment had a pH of 6.5. In the 3 subsequent trials, the pH of the antibiotic solution was adjusted, by adding NaOH, to match that of surface seawater in Barkley Sound at the time of the trial, which ranged from 8.2 to 8.4. The results of the control treatment (no antibiotics) were also included as part of the other field experiment, described above ('Effects of microbial pathogens on EBP mortality in the field').

Data analysis

Data from 2 of the experiments were analyzed using nonparametric analyses due to non-normal distributions or heterogeneous variances: the effects of individual antibiotics and combined antibiotics on early juvenile mortality in the dosage experiments were compared using Kruskal-Wallis tests, and mortality in the control and antibiotic treatments of the field antibiotic experiment were compared using Friedman's nonparametric randomized block test, the separate trials being used as blocks. The OD measurements taken 24 h after the start of the experiment in the antibiotic cocktail effectiveness experiment were analyzed using a 1-way ANOVA, followed by a Tukey HSD multiple comparisons test.

RESULTS

Age and size of EBP individuals used in the experiments

This study included individual *Nucella ostrina*, *Balanus glandula*, and *Chthamalus dalli* either hatched from egg capsules or settled from the plankton within 20 to 24 h before use in experiments, thus ensuring that the responses of individuals were documented during their first days of independent benthic life. For *Mytilus trossulus*, the exact time of settlement of individuals could not be determined. Consequently, only individuals from the smallest size classes observed in the field during the summer were used (Fig. A1 in the Appendix). These sizes were very close to the average size at settlement in this species (330 µm shell length, Martel et al. 2000); however, *M. trossulus* settlers were not inspected for the presence or absence of a velum, indicative of metamorphosis. Almost all (98%) *M. trossulus* used in our study fell within the 250–750 µm size range at the end of the 5 d trials; individuals would have been even smaller at the start of the trials. We are therefore confident that *M. trossulus* juveniles in this study had transitioned from pelagic to benthic habitats very shortly before use in experiments.

Effects of microbial pathogens on EBP mortality in the field

When submerged in the field in a screened cage for 5 d, juveniles of all 4 species experienced very low mortality (Fig. 1). In addition, mortality remained consistent among trials in each species, and between the years 2014 and 2015 in *M. trossulus* and *N. ostrina*. Juvenile mortality for the 5 d period across all trials averaged (± SD) 2.55 ± 2.30% in *M. trossulus*, 1.89 ± 1.18% in *N. ostrina*, 8.60 ± 8.60% in *B. glandula*, and 9.30 ± 1.30% in *C. dalli*. Mortality of attached barnacle cyprids, however, was much higher in both barnacle species; the proportion of attached cyprids that failed to metamorphose into juveniles ranged from 28 to 74% (Fig. 1C,D).

Role of bacterial pathogens in early juvenile mortality

Dosage trials: individual and combined antibiotics

Mortality of *M. trossulus* was close to 0 and not significantly different among dosage treatments, including the control (no antibiotics), in each of the individual antibiotic trials (Kruskal-Wallis tests; OTC: $H_{4,4,4,4,4} = 3.34$, $p = 0.502$; KS: $H_{4,4,4,4,4} = 3.68$, $p = 0.451$; CM: $H_{4,4,4,4,4} = 5.74$, $p = 0.219$; TMP-SMX: $H_{4,4,4,4,4} = 3.00$, $p = 0.558$). Consequently, the

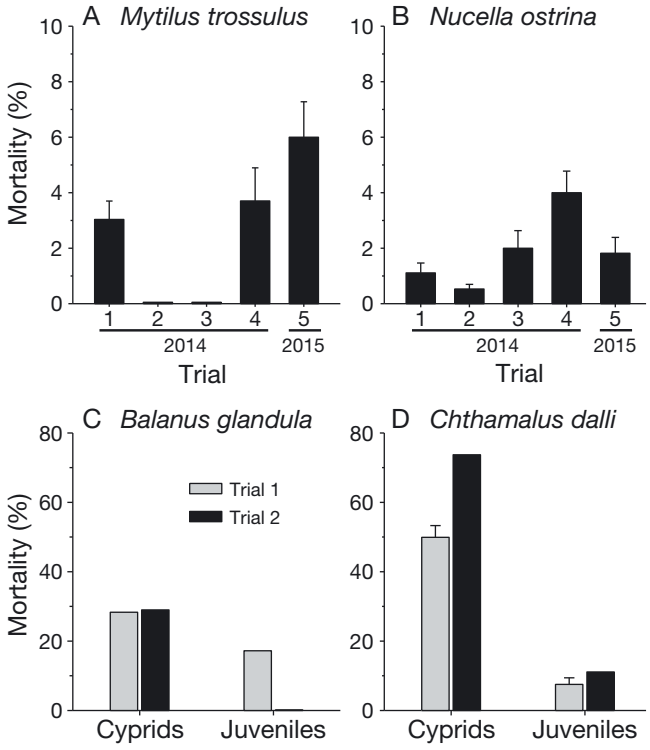

Fig. 1. Percent mortality of early benthic phase (A) *Mytilus trossulus*, (B) *Nucella ostrina*, (C) *Balanus glandula*, and (D) *Chthamalus dalli* after 5 d in the field. From left to right, each value in (A) is based on 8, 3, 5, 3, and 5 replicate cages; values in (B) are based on 9, 19, 5, 5, and 5 replicate cages; values in (C) are based on pooled data from 14 replicate cages; and values in (D) for Trial 1 are based on 14 replicates and values for Trial 2 are based on pooled data from 14 cages. Error bars represent SE

highest concentration of each antibiotic used in the individual dosage trials was used to produce an antibiotic 'cocktail', with the exception of TMP-SMX, for which the second highest concentration was selected because this value corresponds to the maximum solubility of sulfamethoxazole in an aqueous solution with a slightly basic pH (Dahlan et al. 1987).

In the antibiotic cocktail dosage trials, mortality was again very low and not significantly different among cocktail dosages in *M. trossulus* (Kruskal-Wallis test: $H_{4,4,4,4,4} = 5.38$, p = 0.251) and in *N. ostrina* ($H_{7,7,7,7,7} = 5.32$, p = 0.256). For the subsequent field experiment with *M. trossulus*, we nevertheless chose to use the 75% strength cocktail as a precaution, because mortality in the 100% cocktail dosage (14.4 ± 7.5%, average ± SE) appeared somewhat higher, though not significantly, than in the control (2.0 ± 2.2%). The 100% cocktail concentration was used in the *N. ostrina* field trials.

Effectiveness of the antibiotic cocktail against ambient marine bacteria

The antibiotic cocktail at 75% of full concentration proved highly effective against bacterial growth in marine broth. Optical density (OD) values (Fig. 2) differed significantly among treatments after only 24 h (ANOVA, arcsine-transformed data: $F_{2,27} = 240.95$, n = 30, p < 0.001). The OD of the Broth + Bacteria treatment was significantly higher than in the 2 other treatments (Tukey HSD multiple comparisons test), whereas the OD of the Broth + Antibiotics + Bacteria treatment and of the Broth + Antibiotics treatment did not differ significantly. Only marine broth inoculated with seawater (Broth + Bacteria treatment) increased in OD over time; the unchanging OD in the Broth + Antibiotics + Bacteria treatment and in the sterile Broth + Antibiotics treatment indicated no detectable bacterial growth.

Field experiment: bacteria-induced mortality

Mortality in the first trial, in which the pH of the antibiotic cocktail was not balanced to match that of ambient seawater, was not significantly different from mortality in the subsequent 3 trials (Fig. 3) with balanced pH (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). Thus, for each species, the results of all 4 replicate trials were included in a randomized-block analysis comparing average mortality

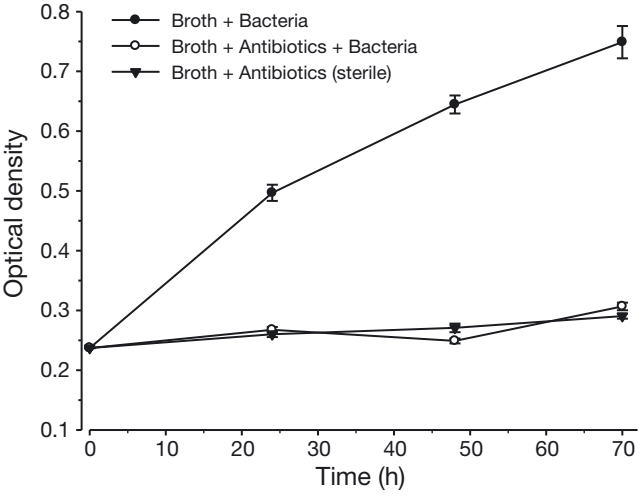

Fig. 2. Optical density (mean ± SE), at 600 nm wavelength, of solutions in 3 treatments over a 70 h period. Treatments consisted of combinations of Difco™ marine broth, a 75% concentration of the full antibiotic cocktail, and bacterial inoculation (natural seawater)

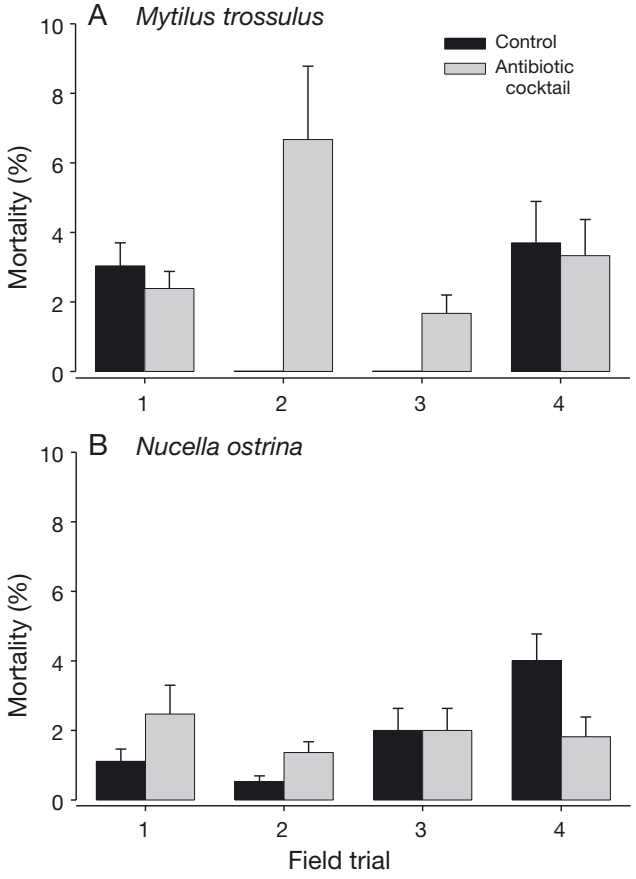

Fig. 3. Mortality (mean ± SE) of (A) *Mytilus trossulus* and (B) *Nucella ostrina* in field trials, July and August 2014. *M. trossulus* were periodically exposed to 75% concentration of the antibiotic cocktail; *N. ostrina* were exposed to 100% concentration of the antibiotic cocktail. The number of replicates per treatment in Trials 1 to 4, which depended on availability of juveniles, was 8, 3, 5, and 3 replicates of 10 individuals for *M. trossulus*, and 9, 19, 5, and 5 replicates of 10 individuals for *N. ostrina*

between control and antibiotic cocktail treatments, with each trial serving as a block. Mortality in the control and antibiotic treatments did not differ significantly for *M. trossulus* (Fig. 3A; Friedman nonparametric randomized-block test; $\chi^2_r = 0.00$, df = 1, p = 1.00) or *N. ostrina* (Fig. 3B; $\chi^2_r = 0.33$, df = 1, p = 0.564).

DISCUSSION

Knowledge of the factors responsible for EBP mortality can help elucidate the evolution of adaptive traits, help focus conservation efforts on factors limiting population recovery, and help obtain estimates of invertebrate recruitment for fisheries. The findings of this study unexpectedly suggest that microbial organisms may cause little or no mortality during the first few days of juvenile life, and possibly also for newly-settled larvae. When juveniles were placed in a field setting isolated from most known non-microbial mortality factors (predation, desiccation, temperature stress, low salinity, wave action, impacts by debris, ultraviolet radiation), but exposed to ambient microbial organisms, mortality was only 0–3% in *Mytilus trossulus* and *Nucella ostrina* and 0–10% in the barnacles *Balanus glandula* and *Chthamalus dalli* over a 5 d period. Results were consistent across juveniles of the 4 species, among replicate trials in the same summer, and also among trials carried out in 2 different years with *M. trossulus* and *N. ostrina*. These results sharply contrast natural mortality levels of unprotected juveniles of the same species in the intertidal zone over similar time frames. Phillips (2002, 2004) reported field mortality levels of 77–97% and 69–99% in *M. trossulus* juveniles over 2 wk periods; similarly, von der Meden et al. (2012) reported 54% and 64% juvenile mortality in the mussel *Perna perna* within 2 d of settlement. For newly-hatched *N. ostrina*, Moran & Emlet (2001) reported 35–60% mortality after 9 d in the field. Natural juvenile mortality of *B. glandula* recently recorded at the same field site (Wizard Islet) and for the same duration as in the present study (5 d) was 47–50% (Gosselin & Jones 2010) and 11–31% (Jenewein & Gosselin 2013a). More broadly, the low juvenile mortality levels recorded for these 4 species in our study contrast with the very high juvenile mortality reported in all benthic invertebrate species studied to date, often exceeding 30% and reaching as high as 100% mortality during the first days of juvenile life (Gosselin & Qian 1997).

The effect of microbial pathogens on cyprid larvae that had just transitioned to the benthic habitat, in contrast, was not as clear. Barnacle cyprid mortality (i.e. attached cyprids failing to complete metamorphosis) in this experiment was high: 28–29% in *B. glandula* and 50–74% in *C. dalli*. Those values fall within the range of mortality levels previously reported for unprotected *B. glandula* cyprids at Wizard Islet, ranging from 70 to 100% (Gosselin & Jones 2010) and from 25 to 79% (Jenewein & Gosselin 2013a). Our results therefore do not rule out microbial pathogens as a cause of cyprid mortality. Other studies, however, have attributed a large portion of barnacle cyprid mortality to low cyprid energy reserves at the time of settlement relative to the high energetic cost of metamorphosis (Jarrett 2003, Thiyagarajan et al. 2003, Tremblay et al. 2007). In the pres-

ent study, high cyprid mortality contrasted sharply with low mortality of newly metamorphosed juveniles of the same cohort, an outcome more consistent with low energy reserves preventing some cyprids from completing metamorphosis than with infection by microbial pathogens.

The second part of this study, designed to establish whether bacterial infection might have caused the few deaths in the first field experiment, provides further evidence that microbial pathogens were not a cause of early juvenile mortality. Although it cannot be confirmed whether the antibiotic cocktail provided universal protection from all pathogenic bacteria, the dosage and effectiveness experiments indicate that the antibiotic cocktail provided broad-spectrum protection, without affecting the short-term health of juveniles. Yet, treating juvenile *M. trossulus* and *N. ostrina* with this antibiotic cocktail 3 times a day did not reduce juvenile mortality relative to the control treatment in our field experiment.

The lack of microbial-induced EBP mortality during the summers of 2014 and 2015 could have been caused by an absence of microbial pathogens in coastal waters. While possible, this explanation seems unlikely given the broad diversity of potential pathogens (viral, bacterial, fungal, protozoan), the high abundance of juvenile invertebrates at the time of the study, and the high population abundance of each of the 4 species in our study area providing a substantial bank of potential pathogen carriers. An effective immune system constitutes a more likely explanation for low EBP mortality. Invertebrates possess an innate immune system that is fully developed early in life and is able to respond faster to infectious agents than adaptive immune systems (Song et al. 2010, Ng et al. 2014, Quintin et al. 2014). In marine invertebrates, an individual's immune system becomes competent late in larval development (Song et al. 2016) or at the time of metamorphosis (Balseiro et al. 2013), gradually taking over from maternally derived proteins and mRNA that confer initial immunoprotection (Yue et al. 2013, Wang et al. 2015). The very low juvenile mortality observed in the present study suggests that by the start of the juvenile period, individuals have already developed an effective immune system capable of warding off microbial pathogens present in coastal seawater.

The present study thus helps clarify the causes of natural mortality during the critical EBP. Although microbial pathogens cannot be dismissed as causes of mortality in newly-settled barnacle cyprid larvae, our findings do not support the hypothesis that microbial pathogens directly cause early juvenile mortality in intertidal invertebrates. A small proportion of early juveniles did die during our experiments, likely killed by depletion of energy reserves, developmental or genetic failure, or handling stress, all remaining as potential stressors in our experimental design. Most early juvenile mortality in natural populations is thus likely caused by factors excluded in our experiments: predation, desiccation, heat stress, reduced salinity, ultraviolet radiation, and damage or dislodgement by water-borne debris.

Whether intense but sub-lethal events such as low food availability, elevated temperature, desiccation, or reduced salinity increase juvenile susceptibility to microbial pathogens to the point where such pathogens periodically cause early juvenile mortality in natural habitats requires further work. Of particular interest is whether low tide conditions in the intertidal zone, such as increased temperature and desiccation stress, affect the susceptibility of EBP invertebrates to microbial pathogens. Finally, further work should also explore whether the findings of this study, which was carried out in a temperate region with relatively mild climate and seawater temperature, apply to warmer climates, where microbial pathogens may grow substantially faster.

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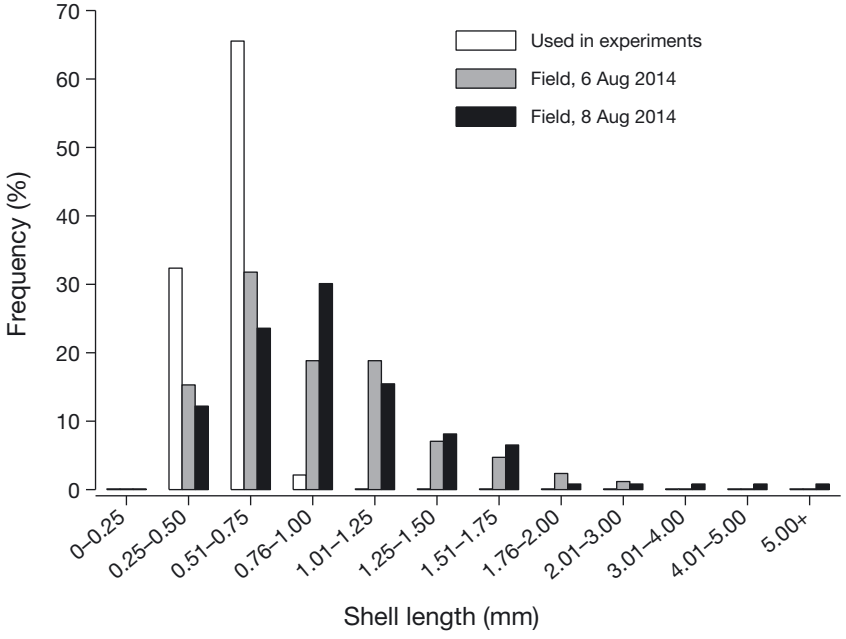
Appendix

Fig. A1. Size frequency distribution of all *Mytilus trossulus* extracted from *Cladophora columbiana* (n = 208) on 2 sampling dates in 2014, and of individuals used in field trials as measured at the end of the 5 d trials (n = 235). All *M. trossulus* were photographed and digitally measured using ImageJ version 1.48



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