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The killer within: Endogenous bacteria accelerate oyster mortality during sustained anoxia

Michael R.S. Coffin[1*], Jeff C. Clements[1], Luc A. Comeau[1], Thomas Guyondet[1], Michelle Maillet[1], Laura Steeves[2], Keryn Winterburn[2], José M.F. Babarro[3], Martin A. Mallet[4], Rémy Haché[5], Luke A. Poirier[1], Saswati Deb[1], Ramon Filgueira[2]

[1] Fisheries and Oceans Canada, Gulf Fisheries Centre, 343 Avenue Université, Moncton, NB E1B 9B6, Canada

[2] Marine Affairs Program, Dalhousie University, 1355 Oxford Street, Halifax, NS B3H 4R2, Canada

[3] Instituto de Investigaciones Marinas, IIM-CSIC, Eduardo Cabello 6, 36208, Vigo, Pontevedra, Spain

[4] L'Étang Ruisseau Bar Ltée., 432 Chemin des Huîtres, Shippagan, NB E8S 2N7, Canada

[5] Department of Agriculture, Aquaculture and Fisheries, Government of New Brunswick, 100 Rue de l'Aquarium, Shippagan, NB E8S 1H9, Canada

Abstract

Sustained periods of anoxia, driven by eutrophication, threaten coastal marine systems and can lead to mass mortalities of even resilient animals such as bivalves. While mortality rates under anoxia are well-studied, the specific mechanism(s) of mortality are less clear. We used a suite of complementary techniques (LT50, histology, 16S rRNA amplicon sequencing, and valvometry) to show that the proliferation of anaerobic bacteria within eastern oysters (*Crassostrea virginica*) accelerates mortality rate under anoxic conditions. Manipulative laboratory experiments revealed that oyster survival under anoxic conditions was halved when bacteria were present compared to when they were excluded by the broad-spectrum antibiotic chloramphenicol. Histological assessments supported this mechanism and showed infiltration of bacteria in oysters that were not treated with antibiotics compared to a general lack of bacteria when oysters were treated with antibiotics. 16S rRNA amplicon sequencing failed to identify any particular genera of bacteria responsible for mortality, rather a diversity of endogenous anaerobic and/or sulfate-reducing bacteria were common among oysters. In addition, monitoring of oyster valve gaping behaviour in the field revealed that oysters showed remarkable valve closure synchrony when first exposed to anoxia. However, oysters periodically opened throughout anoxia/hypoxia in both the lab and field, suggesting that the infiltration of exogenous bacteria from the environment may also influence mortality rates under natural settings. Coupled with previous studies, we posit that mass mortality events in a wide range of coastal bivalves are likely the result of co-morbidity from asphyxiation and bacterial processes.

***Corresponding author:** Dr. Michael R.S. Coffin, PhD; Fisheries and Oceans Canada, Gulf Fisheries Centre, 343 Avenue Université, Moncton, NB E1B 9B6, Canada; Michael.Coffin@dfo-mpo.gc.ca

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Introduction

Over the past century, increasing human populations and associated activities, such as intensive agriculture and shoreline development, have significantly increased nutrient loading to the coastal environment (Erisman et al. 2013; Beusen et al. 2016; Desmit et al. 2018). This increased nutrient input can result in adverse effects on ecosystems, starting with the proliferation of primary producers and culminating in the depletion of dissolved oxygen through increased biological oxygen demand – a process known as eutrophication (Breitburg et al. 2018). Research surveys have identified over 400 marine areas worldwide that are experiencing symptoms of eutrophication, highlighting the global scale of the problem (Díaz & Breitburg 2011). Shallow systems are particularly susceptible to adverse nutrient effects, especially when circulation is poor and organic matter accumulates (Valiela et al. 1997; Coffin et al. 2018). Furthermore, it is expected that climate change will exacerbate symptoms of eutrophication, as higher temperatures will increase water column stratification, decrease O_2 solubility, and stimulate biological processes such as respiration (Rabalais et al. 2009). The most acute symptoms of eutrophication, hypoxia and/or anoxia are particularly challenging for sessile marine animals and those with limited mobility that are unable to avoid exposure (Vaquer-Sunyer & Duarte 2008). Consequently, eutrophication and associated periods of hypoxia/anoxia could have direct consequences for marine ecosystem stability.

Against this backdrop of deteriorating coastal habitats, wild and cultured bivalves remain conspicuous ecosystem engineers that enhance environmental quality (Newell & Koch 2004). These animals promote biodiversity by creating three-dimensional structures (Marenghi & Ozbay 2010; Ysebaert et al. 2019) and can help mitigate eutrophication by grazing down excessive phytoplankton biomass and sequestering nutrients (Humphries et al. 2016; Clements & Comeau 2019; van der Schatte Olivier et al. 2020). Under experimental settings, bivalves exhibit a remarkable capacity to cope with hypoxic/anoxic conditions by closing their valves (Porter & Breitburg 2016), reducing their metabolic rate (Stickle et al. 1989), and shifting to anaerobic metabolic pathways over extended periods of time (Brooks et al. 1991; de Zwaan et al. 1993; Bumett & Stickle 2001). Despite these adaptive mechanisms to deal with hypoxia/anoxia, there have been reports of mass mortalities in natural bivalve populations associated with anoxic events (Steimle & Sindermann 1978; Lenihan & Peterson 1998; Lim et al. 2006; Higano et al. 2010). Given the resilience of bivalves to hypoxia/anoxia under laboratory conditions, these field observations suggest that the observed mortality is attributable to the synergistic effects of multiple disturbances, as opposed to oxygen depletion alone.

There are numerous reasons to suggest that high temperatures can exacerbate the effects of anoxia. Summer temperatures not only increase physiological rates and induce energetically demanding processes such as gonad development and spawning, but also stimulate both planktonic (Wright & Coffin 1983; Joint & Smale 2016) and benthic (Hicks et al. 2018) microbial activity, which may pose a threat to coastal animals under stress. Under low oxygen conditions, anaerobic bacteria (facultative and/or obligate) can thrive and may accelerate shellfish mortality (Babarro & de Zwaan 2001, 2002, 2008). Furthermore, respiration increases with temperature, which results in a concomitant increase

in oxygen demand that stimulates microbial activity and waste production, and the deleterious consequences thereof (Calder-Potts et al. 2018). Despite the potential negative ramifications of increased bacterial levels for coastal animals, and the ubiquity of bacteria during summer under low oxygen, this mechanism of mortality remains understudied from an ecological perspective. While there is increasing evidence that bacteria are an important factor affecting bivalve mortality (e.g., Babarro & de Zwaan 2001, 2002, 2008; Yamada et al. 2016), there are many uncertainties surrounding whether specific bacteria are responsible for bivalve death or if changes in behaviour may limit exposure to stressful environmental conditions and/or bacteria. Given the paucity of information on this subject, it remains unclear what role bacteria play in anoxia-related mortality, or how bivalves (and coastal organisms in general) respond to this additional stressor under hypoxia/anoxia. Establishing a mechanistic understanding of mass mortality events observed in the field would not only enhance biological knowledge but would complement existing calls for action to reduce nutrient loading, as its consequences may be more severe than currently thought.

In August 2016, mass mortality of cultured eastern oysters (*Crassostrea virginica*) was observed within a lagoon-type system in Tracadie Bay, northern New Brunswick, Canada (47.524 °N, 64.909 °W). This mortality event was later linked to sustained low oxygen through anecdotal reports from industry partners and direct observations by provincial biologists and veterinarians (disease and parasites were ruled out, pers. comm. with Pascale Nerette, a veterinarian working for the Government of New Brunswick, Canada. Given that *C. virginica* are hypoxia-tolerant and facultative anaerobes (Bayne 2017), the abrupt wide-scale mortality was surprising. As such, the mass mortality event prompted concern from industry and a subsequent collaborative investigation into the cause of death was initiated. The overarching objectives for this study were to determine if sustained anoxia was a plausible explanation for the observed field mortality, and to investigate what other factors, if any, affect mortality rate. To meet these objectives, we conducted three experiments: 1. field conditions, high temperature and anoxic seawater, were mimicked in the laboratory to assess whether mortality rate was comparable to anecdotal reports; 2. a fully-crossed experiment to test for effects of flow-type and bacteria (controlled through their exclusion by the broad-spectrum antibiotic chloramphenicol) on oyster mortality; and 3. a fully-crossed experiment to assess the effect of bacteria (as above), oyster density (individual *vs.* group exposures), and oxygen level (normoxia *vs.* anoxia) on oyster mortality. As a complement to the manipulative experiments we used state-of-the-art techniques, in both the laboratory and field, to monitor fine-scale behavioral responses (valve movements) and explored both the time and cause of death of *C. virginica* via bacterial community analysis (metabarcoding and histology).

Materials and Methods

Design of laboratory experiments

Laboratory work was conducted at the Aquatron at Dalhousie University, Halifax, Nova Scotia, Canada (https://www.dal.ca/dept/aquatron.html) starting Winter 2018. The experiments were conducted under the following settings: 1. temperature-controlled water-bath tanks (n = 4), each containing four 4.3 L experimental chambers (n = 16 chambers experiment^{-1}) with six oysters chamber^{-}

^{1}; and 2. temperature-controlled 1 L jars (n = 84 jars) with a single oyster each. Water supply (sand-filtered and UV-sterilized) to the chambers was either flow-through or static, depending on the experiment (see descriptions for individual experiments in following sections), whereas jars contained only static water. The flow-through design was selected to better mimic field conditions and to reduce the build-up of secondary metabolites, while static conditions represent a no water exchange scenario. No oysters were fed in any of the experiments, thus controlling for the influence of starvation on mortality rate (Dunphy et al. 2006, Knowles et al. 2014). For all experiments, control oysters (n = 24; unfed) from the same experimental cohort were kept in normoxic, flow-through conditions at the same temperature as the ongoing experiment. No mortality was observed in any of these control groups (i.e., control mortality rate was always zero).

Anoxic seawater was created via continuous bubbling of nitrogen gas through UV-sterilized seawater in a header tank until anoxia ($[O_2]$ = 0.0 mg L^{-1}) was reached. This anoxic water was then transferred, via peristaltic pump, into the 4.3 L flow-through chambers at a constant rate of ≈13 L $chamber^{-1}$ day^{-1} (≈3 exchanges day^{-1}). The same process was used to fill chambers and jars under static-flow conditions. Anoxia in static settings was maintained by sealing each chamber/jar with an air-tight removable lid fitted with a rubber gasket to prevent atmospheric oxygen from entering. Normoxia, for the treatments requiring it, was achieved by leaving the container open to enable oxygen diffusion and constant aeration of the water using magnetic stirrers. An optical dissolved oxygen probe (Fibox 3 trace v3, PreSens, Regensburg, Germany) was used to confirm that dissolved oxygen was 0.0 mg L^{-1} in anoxic treatments before the addition of experimental oysters, and dissolved oxygen was measured daily thereafter. These optical probes measure dissolved oxygen through the transparent chamber wall via luminescence quenching spots (SP-PSt3-SA23-D5-OIW, PreSens, Regensburg, Germany) on the inside of each chamber. For jar experiments, luminescence quenching spots were randomly allocated to 1/3 of the jars for each treatment. For both chamber and jar treatments where anoxic conditions were required, antibiotics and/or oysters could be added or removed according to the experimental protocol.

For all experiments, adult oysters (≈60 mm shell height) were sourced from Sober Island Oysters Ltd (Sheet Harbour, Nova Scotia, Canada). Oysters were brought to the lab and immediately placed in a holding tank with flow-through seawater (≈5 °C, 32 PSU, pH 7.9) without food. Water temperature was then raised by 2 °C daily until reaching the target temperature at which the oysters were maintained until the experiment commenced (<2 weeks). Temperature and dissolved oxygen concentration were checked daily, during the temperature acclimation period and throughout the course of each experiment, whereas pH, ammonia and hydrogen sulfide were checked weekly. The latter three parameters were checked using test strips on water extracted from the experimental units using a syringe; pH (≈8) did not change throughout the experiment and both hydrogen sulfide and ammonia were either below or near the limit of detection until mortality was observed. Throughout the laboratory experiments, oyster mortality was assessed daily by lightly tapping the chamber or jar. If the oyster was gaping and failed to close in response to this stimulus (potentially dead or moribund), the lid was removed so that the oyster valves could be closed manually with metal tweezers (note that removing the lid did not lead to an influx in dissolved oxygen through atmospheric exchange as this process took only a few seconds to complete). If the oyster opened again within 15 seconds it was considered dead and was immediately removed (thus preventing the continued decomposition of tissue and therefore buildup of secondary metabolites). This protocol

ensured a standardized, albeit conservative, approach for assessing mortality. Each experiment concluded upon death of the final oyster under anoxic conditions.

Experiment 1: Anoxia-related mortality under flow-through conditions

The goal of this experiment was to test whether mortality rate in the laboratory was similar to that observed in the field. Further, results would represent a baseline of mortality under flow-through conditions without accumulation of toxic by-products. Oysters were exposed to anoxic conditions in chambers as described above (four tanks, each with four chambers; six oysters chamber^{-1}; n = 96 individuals). Temperature at the time of mass mortality was not recorded, therefore we selected the maximum water temperature observed the subsequent year (28 °C) to mimic high stress field conditions (M. Coffin unpub. data). Flow rate, mortality and environmental conditions were monitored as above.

Experiment 2: Effects of antibiotics and water flow on anoxia-related mortality

A second experiment with the 4.3 L chambers comparing flow-types (flow-through *vs.* static) under anoxic conditions was conducted to test whether the build-up of secondary metabolites (hydrogen sulfide, ammonia, and changes to pH) in the static setting would affect mortality rate. For this, a 2×2 factorial design was used with flow-type (flow-through *vs.* static) and antibiotics (presence/absence) as fixed factors. A systematic randomized design was used to allocate treatment levels to a specific chamber in each tank, thus each treatment level was contained in each of the 4 tanks (n = 4 chambers treatment level^{-1}; n = 6 oyster chamber^{-1}). Chambers were filled with anoxic water from the header tank via peristaltic pump for both flow-types. For flow-through treatments, flow rate was maintained with a peristaltic pump as in Experiment 1, whereas static treatments had no water exchange for the duration of the experiment. Half of the flow-through and static chambers were treated with antibiotics (chloramphenicol), by delivering a dose of 10 mgL^{-1} in a UV-sterilized anoxic seawater solution into the water column via syringe through the removable lid. Mortality and environmental variables (temperature, dissolved oxygen, and secondary metabolites) were checked as above, and temperature was maintained via water bath at ≈20 °C (temperature represents the approximate average over growing seasons in the area).

Experiment 3: Effects of antibiotics and oyster density on anoxia-related mortality

The goal of this experiment was to test the role of bacteria on oyster mortality via their exclusion, treating the environment with antibiotics, oyster density (one individual 1 L jar^{-1} *vs.* a population of 6 individuals 4.3 L chamber^{-1}), and under different oxygen conditions (anoxia *vs.* normoxia), representing a 2×2×2 factorial design. This experiment was conducted under static water conditions in all treatment levels, with a selected water temperature of 20 ^{o}C, representing the average temperature from June-August in Tracadie Bay, NB (M. Coffin unpub. data). For chambers, a systematic randomized design was used to allocate treatment levels to a specific chamber in each tank, thus each treatment level was contained in each of the 4 tanks (n = 4 chambers treatment level^{-1}; n = 6 oyster chamber^{-1}). Chambers and jars were both filled with anoxic water from the header tank via peristaltic pump. Anoxic and normoxic conditions, respectively, were maintained as described above. Half of the anoxic and normoxic treatments were administered antibiotics (chloramphenicol),

by delivering a dose of 10 mg L^{-1} in a UV-sterilized anoxic seawater solution into the water column via syringe through the removable lid. Mortality and environmental variables were checked as described above.

Histology and 16S rRNA amplicon sequencing (Experiments 2 and 3 only)

For the Experiments 2 and 3, dead oysters were immediately removed from the experimental chamber or jar and preserved either for histological assessment of oyster tissues, or genetic analysis of bacterial communities. For histology, an arbitrarily assigned subset of these dead oysters (n = 5 treatment^{-1}) were refrigerated (4 ^{o}C) prior to being sent to the Gulf Fisheries Centre in Moncton, New Brunswick for histological processing. Likewise, for genetic analysis, arbitrarily assigned oysters (n = 5 treatment^{-1}) were preserved in 99% ethanol.

For histological assessments, macroscopic observations of the dead oysters were noted during dissection, which was done within 24 hours of receiving the sample. Oyster tissues were excised, fixed in Davidson's fixative solution and then embedded in wax and cut into 6 µm cross-sections using a microtome for subsequent evaluation via microscopy (Howard et al. 2004). Two slides were created from adjacent sections: the first section was stained using Hematoxylin-Eosin (H&E) for detection of pathogens and characterization of general health status (McGladdery et al. 1993); the second section was stained using a McDonald's Gram stain kit to help distinguish bacteria from the rest of the tissue. Microscope slides were subsequently observed at 50x, 100x, 200x, 400x and 1000x, using a Leica DM 1000 LED microscope. All slides were observed blindly without the observer being privy to information related to treatment type.

To document the effect of bacteria on oyster tissue via histology two general metrics were recorded: (a) the presence/absence of bacteria under the highest magnification, 1000x, and (b) the presence/absence of tissue necrosis of the observed tissue. The presence of bacteria was noted in the H&E-stained slides and then confirmed with the McDonald's Gram-stained slides. Given that these results are (i) semi-quantitative, (ii) only a small section of each oyster was examined, and (iii) tissue necrosis was never severe (<25% of the tissue), we elected to forego statistical analysis because interpretation of these observations do not require formal analysis.

For 16S rRNA amplicon sequencing, each oyster sample was placed in a whirl-pack bag, homogenized by hand, and ≈25 mg of coarse homogenate was removed for DNA extraction using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). The V3-V4 region of the bacterial 16S rRNA gene was amplified in 35 µL reaction volumes with 0.4 µM forward (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG[CCTACGG GNGGCWGCAG]) and reverse (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG[GACTACHVGGGTATCT AATCC]) primers (Illumina Inc., San Diego, CA, USA) and 17.5 µL AccuStart II PCR Toughmix (Quantabio, Beverly, MS, USA) using the following amplification protocol: 94 ^{o}C for 10 minutes; followed by 25 cycles of 94 ^{o}C for 30 seconds, 55 ^{o}C for 30 seconds, 72 ^{o}C for 30 seconds; then a final 72 ^{o}C incubation for 5 minutes. PCR products were cleaned using Ampure XP beads (Beckman Coulter, Brea, California, USA) followed by another round of PCR using Nextera XT dual Index Primers, v2, Set A and Set B (Illumina, Inc., San Diego, CA, USA) and 2X KAPA HiFi HotStart Ready Mix (Roche, Pleasanton, CA, USA) using 5 µL of the cleaned round 1 PCR product as template with the following cycling protocol: 94 ^{o}C for 4 minutes; 8 cycles of 94 ^{o}C for 15 seconds, 55 ^{o}C for 30 seconds; then

followed by a final 72 ^{o}C incubation for 5 minutes. Resultant PCR products were again cleaned using Ampure XP beads (Beckman Coulter, Brea, California, USA). PCR products were quantified using the Quant-iT PicoGreen dsDNA Assay kit (Life Technologies Corporation, Eugene, OR, USA) and standardized to the same concentration (4 nM) then pooled in equimolar amounts. A final pooled library of 12 pM supplemented with 20% PhiX was then sequenced using the MiSeq Reagent Kit V2 (500 cycles) on the Illumina MiSeq platform (Illumina, Inc., San Diego, CA, USA). DNA sequences were processed using the 16S Metagenomics workflow in the MiSeq Reporter analysis software version 2.5.1. Quality-filtered indexed reads were demultiplexed for generation of individual FASTQ files. Taxonomic classification of reads was performed to the kingdom, phylum, class, order, family, genus, and species levels using the Illumina-curated version of the Greengenes v13.5 (May 2013) database, though only genus level taxonomy data were analyzed.

Valve gaping behaviour under anoxia (Experiment 3 and additional field observations)

Behavioral responses to anoxic conditions were assessed in Experiment 3 by wiring oysters to a non-invasive valvometry system described in Nagai et al. (2006). A detailed description of the operating principle and sensor attachment is provided in Clements et al. (2020) and Comeau et al. (2019).

In Experiment 3 wired oysters (n = 12) were equally distributed within each treatment level at the individual oyster density level (1 L jars, Experiment 3), therefore, allocating three individuals for each oxygen × antibiotics treatment combination. For each oyster and for the entire duration of the experiment, the valvometry system continuously recorded the extent of valve opening at a frequency of one measurement second^{-1}.

In a separate field mission, valvometry was used to monitor oyster behaviour in a eutrophic estuary (Mill River, Prince Edward Island; N46.7466, W64.1599) to document oyster behaviour under natural periods of anoxia/hypoxia. In July 2017, prior to the typical onset of anoxic conditions in this system (Coffin et al. 2018), oysters (n = 3) were wired in the laboratory and then deployed from a buoyant raft equipped with a battery pack and a solar panel, similar to the setup described in Comeau et al. (2018). The oysters were hung from the raft on a flexible Vexar® sheet at 0.5 m depth to simulate suspended bag aquaculture practiced in the area (Comeau 2013). Valve gaping was recorded every second. A dissolved oxygen logger (Onset Hobo optical Dissolved Oxygen logger U-26; accuracy of ± 0.2 mg L^{-1}) was deployed adjacent to the oysters and set to record dissolved oxygen concentration (mg L^{-1}) at 30-minute intervals.

It should be noted here that because oysters were sampled post-mortem bacterial communities may have been influenced by increased bacterial proliferation and, consequently, tissue necrosis. The immediate preservation (i.e., within 24 hours of death; either on ice for histological analysis or in alcohol for 16S rRNA amplicon sequencing) of dead oysters was done to minimize the influence of post-mortem histological artefacts. The potential presence of post-mortem artefacts, however, precludes us from drawing any linkages between bacterial communities (identified via 16S rRNA amplicon sequencing) and the rates or causes of oyster death.

Data analysis

All analyses for calculating LT50s and survival analyses were conducted using R version 3.6.3 (R Core Team, 2020). For each of the experiments, LT50 (i.e., time taken for 50% of population to die) and associated fiducial confidence limits were estimated for each treatment with Probit models (Finney 1971; Wheeler et al. 2006; Robertson et al. 2007) using the LT probit() function from the ecotox package (Hlina in Review).

Survival analysis was used to test for differences in survival rates between chambers in Experiment 1, and between treatments in Experiments 2 and 3 (i.e., flow type × antibiotics for Experiment 2; density × antibiotics for Experiment 3). Kaplan-Meier survival estimations were first computed using the Surv() function in the survival package (Therneau & Grambsch 2000). Mixed effects Cox proportional hazards models were then constructed using the coxme() function from the coxme package (Therneau 2020). For Experiment 1, "chamber" was included as a fixed categorical variable and tank as a random categorical factor (tanks are the water baths in which four chambers are located). For Experiments 2 and 3, treatment was a fixed categorical factor with tank as a random categorical variable. For Experiment 3 (which had chambers and jars), the random effect of "tank" was dropped for the analysis for "jar" data (because jars were not housed in tanks as chambers were) and a non-mixed Cox model was built using the coxph() function. Significance for the treatment effect was then computed using the Anova() function from the car package (Fox & Weisberg 2018), specifying Type 3 Sum of Squares and using a significance threshold of $p < 0.05$. Where a significant effect of treatment was observed, pairwise comparisons were obtained with Tukey HSD tests using the glht() function from the multcomp package (Hothorn et al. 2008).

All taxonomic data obtained via 16S rRNA amplicon sequencing were explored using Plymouth Routines in Multivariate Ecological Research package version 7.0.13 (Clarke and Gorley 2006; Anderson et al. 2008). Bacterial community data, Bray-Curtis dissimilarity resemblance matrices were created and then explored visually using Principal coordinate analysis (PCoA) ordinations with treatment identified by shape and colour, throughout this manuscript the colour blue denotes anoxic conditions and the colour red denotes anoxic conditions with antibiotics treatment. PERMANOVAs were then conducted on the same Bray-Curtis dissimilarity matrices to test between main effects, which were all fixed factors. Similarity Percentages (SIMPER) were also calculated to determine which taxa contributed most to the aforementioned Bray-Curtis dissimilarity matrices for each treatment type and the overall dissimilarity between them (Clarke and Gorley 2006).

With valvometry, in both the laboratory and field, the main goal was to provide a descriptive account of oyster behavior during anoxia, specifically with respect to whether they interacted with the adverse environment or completely isolated themselves from it by keeping their valves closed. A relative valve-gaping metric for each oyster was computed as percent of the maximum recorded opening amplitude during the lengthy monitoring period (> 1 month). This computational step assigned to each consecutive measuring point (1 second) a relative gaping value ranging between 0% (fully closed) to 100% (fully opened). A simple metric was subsequently computed as the maximal gaping observed for each 3600 seconds (1 hour) interval. This maximal gaping metric was plotted over time to assess whether oysters interacted with their surrounding environment.

Results

Oyster survival under anoxic conditions

Since all oysters eventually died in anoxic treatments and none died under normoxic conditions, we interpret oyster survival rates between normoxic and anoxic conditions to be different and formal statistical comparisons were only made between treatment groups for oysters under anoxia.

Experiment 1: Anoxia-related mortality under flow-through conditions

The first experiment on the effect of anoxia on oyster survival at 28 ^{o}C under flow-through conditions in 16 flow-through chambers grouped into 4 independent tanks, LT50 survival estimates (days [lower, upper 95% confidence bounds]) were 7.9 [7.3, 8.4], 7.1 [6.5, 7.5], 6.9 [6.4, 7.3], and 8.6 [8.1, 9.1] in the four tanks. The Cox proportional hazards model indicated a significant overall tank effect ($\chi_3 = 8.7$, $p = 0.033$); however, Tukey HSD indicated no significant pairwise differences. Coupled with the fact that LT50 differences were within approximately a day of each other, which is within the resolution of observations, we concluded that tank effects on survival are negligible, and the average LT50 was 7.6 days.

Experiment 2: Effects of antibiotics and water flow on anoxia-related mortality

The Cox proportional hazards model revealed an overall main effect of treatment on oyster survival ($\chi 3 = 38.3$, $p < 0.001$; Figure 1a). Oysters not treated with antibiotics had similar LT50 estimates in the static (10.1 [9.5, 10.6]) and flow-through (10.3 [9.8, 10.8]) treatments (Tukey HSD $p = 0.998$; Figure 1a), indicating that build-up of metabolic by-products in the static chamber did not contribute to mortality rate, thus enabling comparisons between flow types. In contrast, LT50 estimates in oysters treated with antibiotics were significantly higher in the static treatment (23.5 [22.9, 24.1]) as compared to the flow-through treatment (14.9 [14.0, 15.7]) (Tukey HSD $p = 0.027$; Figure 1a), which was perhaps due to antibiotics being steadily diluted under flow-through conditions. Regardless of flow treatment, oysters treated with antibiotics had significantly higher LT50 estimates than those not treated with antibiotics (Static-flow, Tukey HSD $p < 0.001$; Flow-through, Tukey HSD $p = 0.001$; Figure 1a).

Experiment 3: Effects of antibiotics and oyster density on anoxia-related mortality

For the third experiment on the effects of anoxia, antibiotics, and oyster density (one oyster in jars *vs.* six oysters in chambers), there was a significant overall effect of treatment (Cox proportional hazards model $\chi_3 = 108.7$, $p < 0.001$; Figure 1b). When not treated with antibiotics, LT50s of oysters were similar in jars (one oyster; 13.7 [13.3, 14.2]) and chambers (six oysters; 13.0 [12.4, 13.6]) (Tukey HSD $p = 0.961$; Figure 1b). When treated with antibiotics, LT50s for oysters in jars (33.6 [32.7, 34.5]) were similar to those in chambers (27.5 [26.8, 28.2]), although this outcome was only marginally non-significant **(**Tukey HSD $p = 0.052$**)** as survival probabilities started diverging in the latter part of the series (Figure 1b). Specifically, the maximum survival time for oysters under anoxic conditions with antibiotics was 56 days in jars compared to 38 days in chambers. The LT50s for oysters treated with antibiotics were statistically higher than those not treated with antibiotics (Tukey HSD $p < 0.001$ for both jars and chambers; Figure 1b). Mortality rates under anoxia without antibiotics were faster in Experiment 1 at

28 °C (LT50 = ≈8 days) compared to those in Experiments 2 and 3 at 20 °C (≈10 and13 days, respectively).

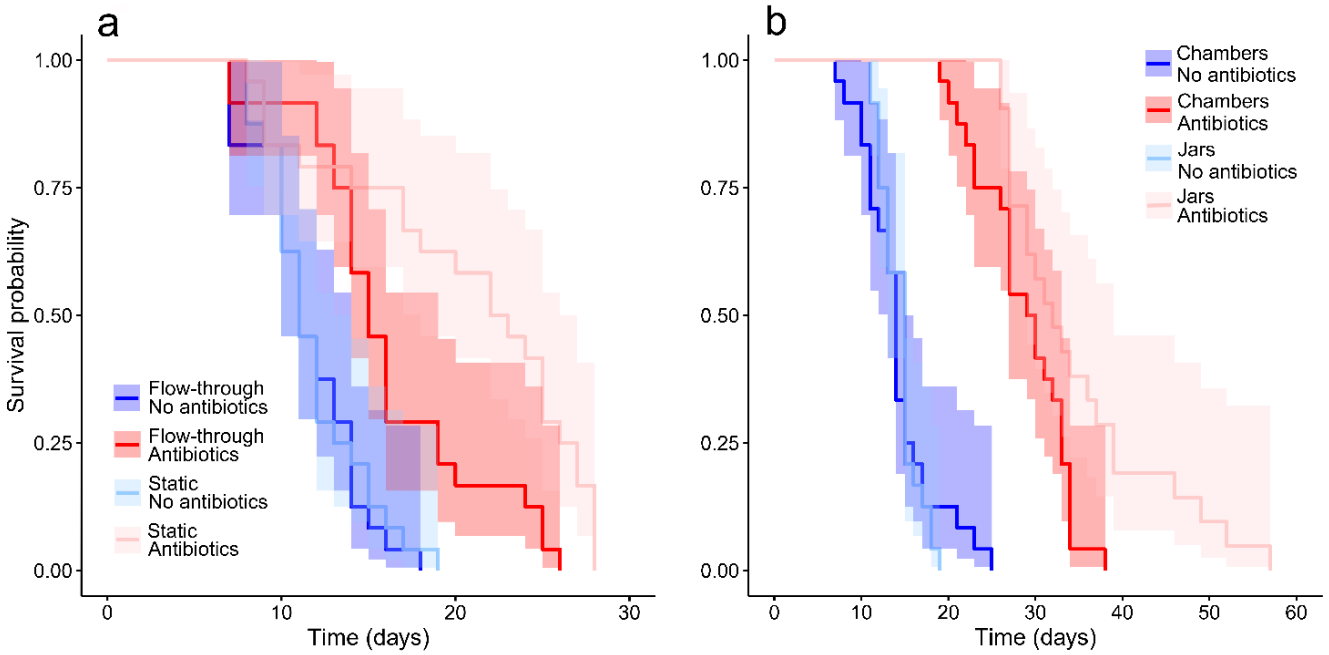

Figure 1. a) Experiment 2 (effects of flow-type and antibiotics) – survival curves under anoxia for eastern oysters, *Crassostrea virginica*, under either static or flow-through conditions and with or without antibiotics (chloramphenicol). All treatments were conducted in 4.3L chambers with six oyster chamber^{-1} **b**) Experiment 3 (effects of oyster density and antibiotics) – survival curves under anoxia for eastern oysters held in chambers (six oysters chamber^{-1}) or jars (one oyster jar^{-1}) and with or without antibiotics (chloramphenicol). All treatments were conducted under static-flow conditions.

Histology and genetic analysis (Experiments 2 and 3 only)

In general, oysters that were treated with antibiotics had no or low prevalence of bacteria and a lower prevalence of tissue necrosis when compared to untreated oysters as observed at 1000x. Although tissue necrosis was regularly observed in untreated oysters, it was never severe, possibly because dead oysters were removed within 24 hours of their death. Representative images contrasting treatments are contained in Figure 2 for reference.

For Experiment 2, on flow-type and antibiotic application under anoxic conditions, there was a significant difference in bacterial community composition (Table 1, Figure 3a) for each of the fixed factors: flow-type (Pseudo-$F_{1, 16}$ = 4.5327, p = 0.003) and antibiotics (Pseudo-$F_{1, 16}$= 13.341, p = 0.001). Although there was a significant interaction between flow-type and antibiotics (Pseudo-$F_{1, 16}$ = 3.8739, p = 0.002), this test is for the generality of whether flow-type varies with antibiotic application and does not affect interpretation of the main effects (Anderson et al., 2008). In Experiment 3, bacterial communities were again more similar within treatments (n = 5 for each treatment) than between them when Bray-Curtis dissimilarities were analyzed with PERMANOVA (Figure 3b). Both oyster density

(Pseudo-$F_{1, 16}$ = 2.1363, p = 0.037) and antibiotics (Pseudo-$F_{1, 16}$ = 3.8264, p = 0.002), significantly affected the bacterial community, whereas the interaction term was nonsignificant (Table 1).

When comparing the genera that contributed most to the dissimilarity between treatments, using SIMPER analysis, we observed that most abundant genera were shared for both Experiment 2 and 3, and were predominantly anaerobic, gram-negative bacteria and/or sulfate-reducing (Table 2). In total there were over 1800 genera of bacteria identified using NGS, which comprised ≈90% of reads for Experiment 2 and ≈75% of reads for Experiment 3; the other ≈10 and 25% for Experiment 2 and 3, respectively, were read as "unclassified" genera. These results were not surprising given that oysters were all from the same commercial oyster farm and all experiments were conducted using sand-filtered and UV-sterilized water, which likely prevented exogenous bacteria from entering the experimental system. Though there is an implicit alternate hypothesis that antibiotics might promote or eliminate certain genera of bacteria, which is seemingly supported by the PERMANOVA analyses and accompanying ordinations (Figure 3a,b, Table 1), the SIMPER results indicate that most differences are the result of changes to relative abundance and not presence/absence of a bacteria. Altogether, the Bray-Curtis Similarity indices (Figure 3a,b) and the SIMPER analyses (Table 2) support the results from the PERMANOVA analyses, that there are differences among bacterial communities between treatment types.

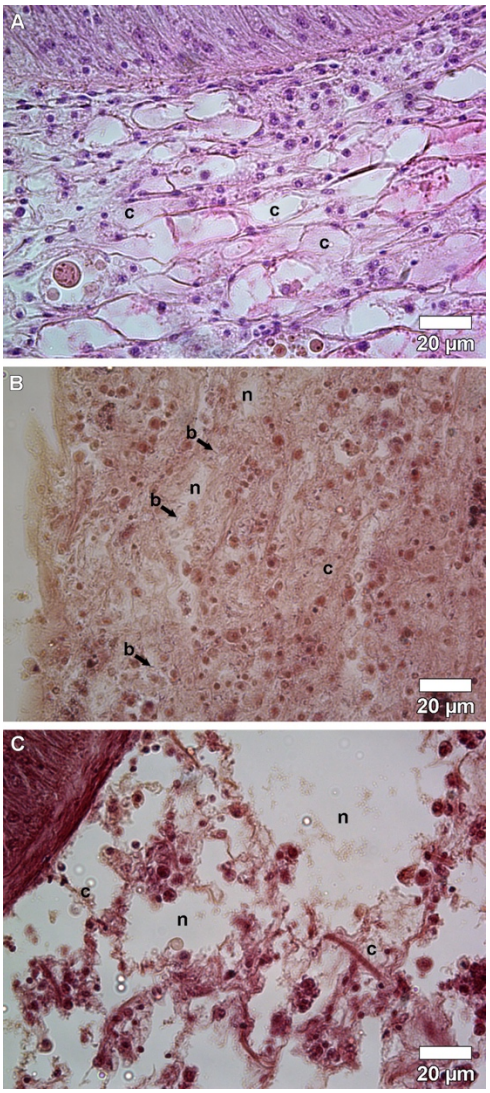

Figure 2. Histology images of oyster, *Crassostrea virginica*, connective tissue at 40 x. Tissue in panel **a** was taken from a healthy (i.e., live) oyster that was not part of this experiment, while tissues in panels **b** and **c** were taken from dead oysters immediately upon confirmation of death (see Methods). In panel **a,** note that the connective tissue (c) is intact and there are no signs of necrosis (n) and bacteria (b) are not present. Panel **b** is a representative slide of tissue necrosis from oysters that were under anoxic conditions without antibiotic treatment (chloramphenicol). Note tissue necrosis (n) is presented as "gaps" in tissue as it breaks down and metaplasia occurs and that bacteria (b) are evident in the necrotic tissue. Panel **c** is a representative slide of tissue necrosis of oysters under anoxic conditions with antibiotic treatment (chloramphenicol). As in panel **b** tissue necrosis (n) is evident, and there are advanced necrotic cells, but bacteria (b) are not present. All oysters were starved throughout these experiments.

Table 1. Results from PERMANOVA tests of fixed factors for Experiment 2 (flow type [flow through *vs.* static] and with or without antibiotics [chloramphenicol]) and Experiment 3 (density [six oysters in chambers *vs.* one oyster in jars] and with or without antibiotics [chloramphenicol]) on community composition of bacteria (relative abundance), at the genus level, contained within whole oysters, *Crassostrea virginica,* after death from sustained exposure to anoxia. Columns headed by the abbreviations df, SS and MS are for degrees of freedom, Sums of Squares and Mean Square, respectively. Statistically significant differences, $p \leq 0.05$, are in bold.

Source of variation	df	SS	MS	Pseudo-F	P(perm)	Variance components estimation
Experiment 2						
Flow Type	1	4869.5	4869.5	4.5327	**0.003**	19.481
Antibiotics	1	14332	14332	13.341	**0.001**	36.411

Flow Type × Antibiotics	1	4161.6	4161.6	3.8739	**0.002**	24.849
Residuals	16	17189	1074.3			32.776
Total	19	40551				
Experiment 3						
Density	1	2512.8	2512.8	2.1363	**0.037**	11.561
Antibiotics	1	4500.9	4500.9	3.8264	**0.002**	18.234
Density × Antibiotics	1	2077.8	2077.8	1.7664	0.071	13.428
Residuals	16	18820	1176.3			34.297
Total	19	27912				

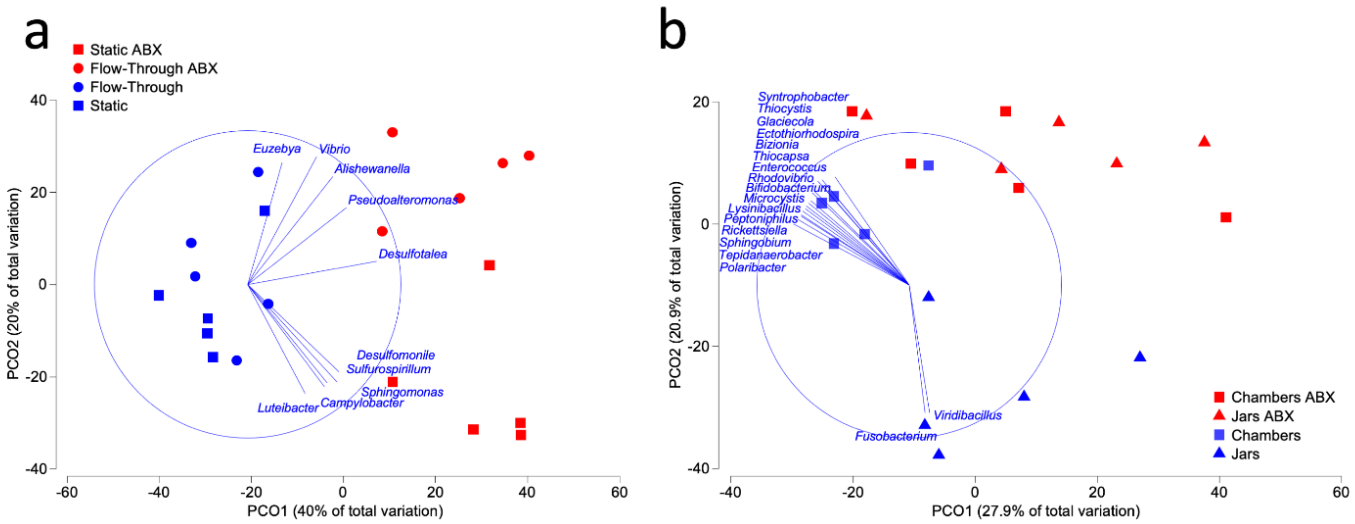

Figure 3. a) Experiment 2 (effects of flow-type and antibiotics) – Principal Coordinates Analysis using Bray-Curtis Similarity indices on the relative abundance of genera of bacteria contained within whole oysters, *Crassostrea virginica,* after death from sustained exposure to anoxia. Symbols are indicative of flow-type (flow-through by circles and static by squares) and colour which denotes the application of the antibiotic chloramphenicol (red with antibiotics and blue without). **b**) Experiment 3 (effects of oyster density and antibiotics) – Principal Coordinates Analysis using Bray-Curtis Similarity indices on the relative abundance of genera of bacteria contained within whole oysters after death from sustained exposure to anoxia. Symbols are indicative of experimental vessel, with six oysters in chambers *vs.* one oyster in jars (by squares and triangles, respectively). Vector length corresponds to a Pearson's correlation of $r < 0.85$. Colour again denotes the application of the antibiotic chloramphenicol (red with antibiotics and blue without).

Table 2. Results of SIMPER analysis describing the top genera contributing to the dissimilarity between oysters treated with antibiotics or not (No Antibiotics) for each treatment in Experiments 2 and 3. The cutoff of thirteen genera of bacteria that contributed most to the dissimilarity between antibiotics and no antibiotics for each treatment was arbitrarily selected to be included in this table.

		Average relative abundance		Contrasting Taxa	
Experiment /treatment	**Genera**	**Antibiotics**	**No Antibiotics**	**Average Dissimilarity**	**Contribution %**
Experiment 2 Flow-Through	*Vibrio*	36.79	7.17	14.83	22.01
	Desulfovibrio	1.80	29.84	14.02	20.81
	Desulfotalea	24.51	1.26	11.78	17.49
	Alkaliphilus	9.49	13.78	4.53	6.73
	Sphaerochaeta	0.12	8.17	4.06	6.03
	Flavobacterium	4.40	6.54	2.64	3.92
	Photobacterium	0.01	4.04	2.02	2.99
	Unclassified	8.03	10.05	1.63	2.42
	Bacteroides	0.36	3.44	1.54	2.28
	Desulfofaba	2.48	0.17	1.17	1.74
	Tindallia	1.11	0.40	0.48	0.72
	Desulfuromusa	1.00	0.73	0.43	0.64
	Fusobacterium	0.04	0.89	0.42	0.63
Static	*Alkaliphilus*	0.32	32.63	16.15	20.66
	Desulfotalea	18.17	0.14	9.01	11.53
	Arcobacter	11.88	0.28	5.81	7.43
	Slackia	11.06	0.18	5.44	6.96
	Desulfovibrio	1.47	11.19	5.14	6.57
	Desulfofaba	9.84	0.69	4.57	5.85
	Vibrio	5.68	5.26	3.59	4.59
	Sphaerochaeta	1.21	6.23	2.97	3.80
	Flavobacterium	5.23	0.77	2.56	3.27
	Unclassified	10.23	11.86	2.40	3.07
	Fusobacterium	0.63	5.02	2.28	2.91
	Bacteroides	1.77	5.92	2.26	2.89
	Cetobacterium	0.64	4.36	1.97	2.51
Experiment 3 Chambers (6 oysters)	*Desulfotalea*	14.64	0.74	6.96	14.85
	Unclassified	25.10	33.72	5.52	11.77
	Mycoplasma	9.65	2.82	4.67	9.95
	Bacteroides	7.27	1.52	3.36	7.16
	Sphaerochaeta	0.37	5.25	2.46	5.25
	Desulfovibrio	0.61	4.77	2.08	4.44
	Arcobacter	4.48	1.69	1.63	3.49
	Desulfobacter	0.51	2.66	1.18	2.52
	Slackia	2.29	0.18	1.07	2.29
	Alkaliphilus	0.10	1.85	0.88	1.87
	Flavobacterium	0.49	1.71	0.61	1.30
	Fusobacterium	0.58	1.31	0.59	1.25

	Cetobacterium	0.84	1.12	0.58	1.23
Jars (1 oyster)	*Bacteroides*	9.45	11.67	6.15	10.01
	Arcobacter	13.56	2.07	6.02	9.79
	Unclassified	25.45	18.86	5.32	8.65
	Sphaerochaeta	0.04	8.72	4.35	7.07
	Fusobacterium	0.02	6.80	3.39	5.51

Table 2 continued

	Desuflovibrio	0.57	6.68	3.05	4.97
	Mycoplasma	6.34	2.34	2.95	4.81
	Cetobacterium	0.03	5.70	2.84	4.62
	Flavobacterium	0.41	4.60	2.11	3.44
	Vibrio	2.01	3.70	1.75	2.85
	Amphritea	3.39	0.04	1.67	2.72
	Desulfuromusa	0.41	2.34	1.10	1.79
	Desulforhopalus	1.66	1.93	1.09	1.77

Valve gaping behaviour under anoxia (Experiment 3 and additional field observations)

In Experiment 3, individuals subjected to anoxia without antibiotics were generally closed with occasional brief re-openings that increased in frequency in the days preceding death (Figure 4). Those subjected to anoxia with antibiotics displayed similar behaviors, although with an extended morbidity and apparent weakening phase prior to death. By comparison, oysters under normoxia were frequently open, regardless of antibiotics.

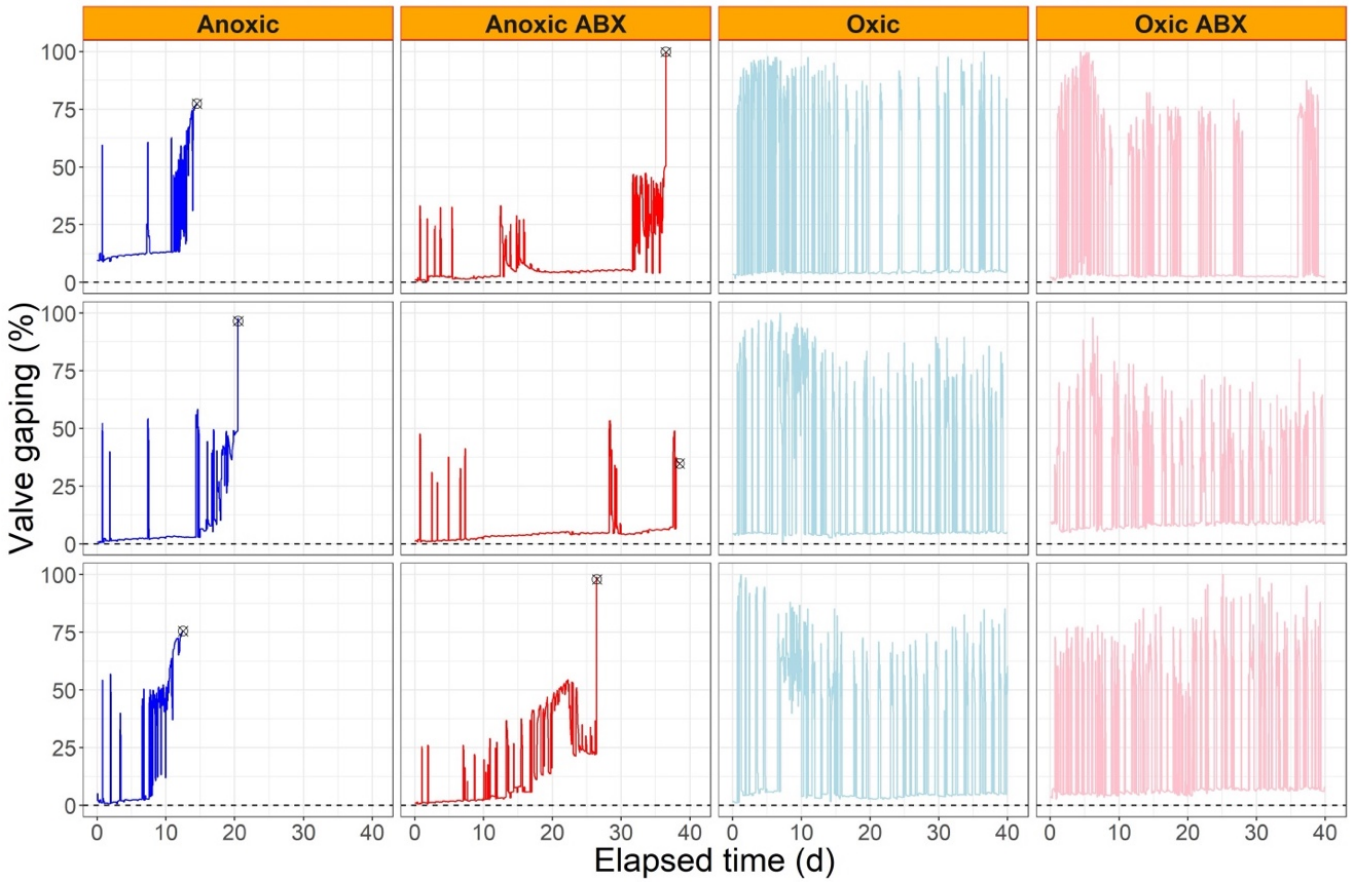

Figure 4. Valve gaping of eastern oysters, *Crassostrea virginica*, plotted against time for each of the four treatments in 1 L jars. Each panel represents one oyster and its maximal gaping over 1-h intervals; black circles denote death confirmation by lab observer; black dotted horizontal lines provide reference with respect to a state of complete valve closure

Oysters subjected to sustained anoxia in Mill River under field conditions exhibited similar behaviors compared to those under laboratory conditions in Experiment 3. For additional field observations, the dissolved oxygen time series from Mill River revealed a sudden and pronounced anoxic event during the summer of 2017 (Figure 5). In the early evening of July 26, dissolved oxygen concentration dropped from 21.0 to 0.0 mg L^{-1} within a time span of only 1.5 hours. Anoxia persisted over a period of 43 hours during which time there were intermittent although brief (< 2 hours) episodes of hypoxia (< 4 mg L^{-1}). Oysters wired to the valvometry system displayed a remarkable synchrony at the onset of anoxia, as their valve gape fell from 79% to 9% of maximal opening (means of three individuals) during the initial drop in oxygen level (Figure 5). Throughout the duration of the anoxic event oysters intermittently re-opened their valves to nearly their full extent (Figure 5). These re-openings were not synchronized amongst individuals and not necessarily concurrent with the brief hypoxic episodes. Nonetheless, the violin plots (Figure 5) highlight a population tendency towards valve closure during anoxia, intermediate valve opening during hypoxia, and full valve opening during normoxia.

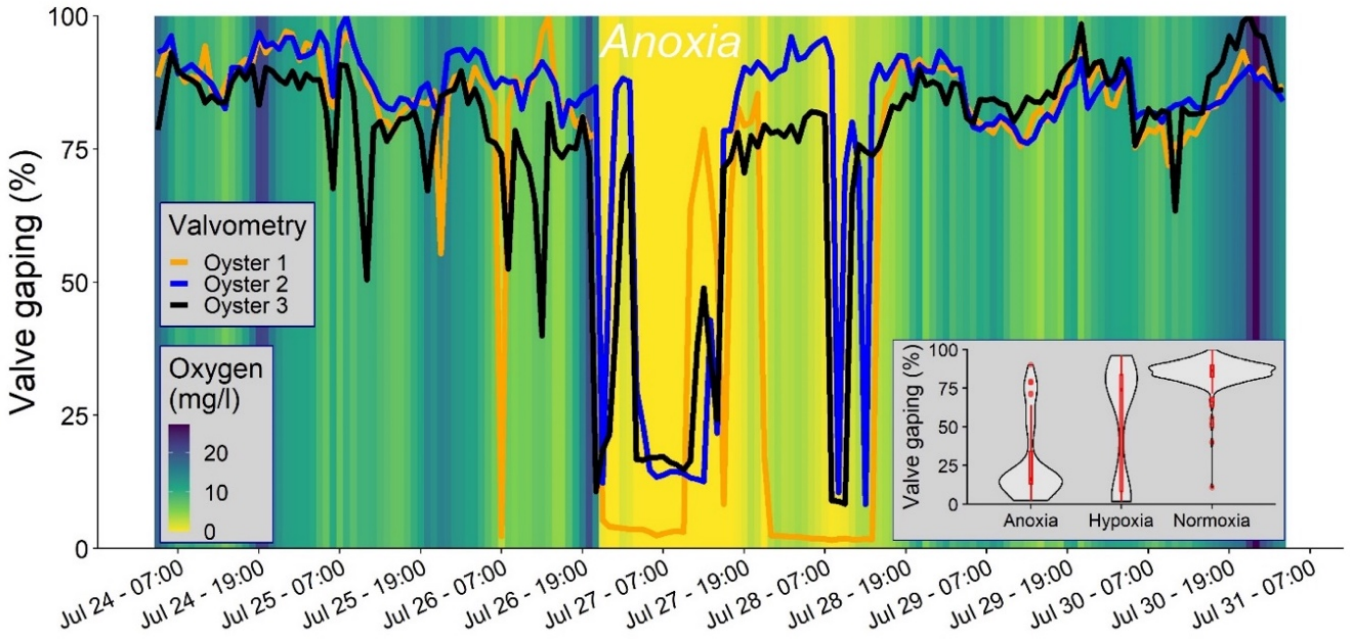

Figure 5. Valve gaping behaviour of three oysters (*Crassostrea virginica*) against the backdrop of dissolved oxygen in Mill River, PEI, in 2017. Though several months of valvometry data were collected, we present only a week's worth of data surrounding a sustained anoxic event. Data represents the maximal gaping and average oxygen concentration over 1-h intervals. Inset: Violin plots showing the full distribution of gaping for the three oysters during episodes of anoxia (0 mg $O_2 L^{-1}$, indicated by a yellow background), hypoxia (0-4 mg $O_2 L^{-1}$), and normoxia (> 4 mg $O_2 L^{-1}$).

Discussion

Eutrophication events and associated periods of anoxia/hypoxia are increasing in both frequency and severity worldwide (Breitburg et al. 2018). A mechanistic approach to understanding how such conditions affect individual species is necessary for predicting and mitigating effects on populations and biological communities. We found that around half of our experimental oysters were able to endure anoxic conditions for 1-2 weeks at summer temperatures, corroborating anecdotal mortality rates observed in the field. However, when we controlled for bacteria by treating oysters with antibiotics, oyster survivorship roughly doubled. This mechanism of mortality was corroborated by the absence of bacteria in oyster tissue when treated with antibiotics, indicating that mortality in antibiotic-treated oysters may have been the result of exhaustion or depletion of energy reserves, or some other unknown factor (Babarro & de Zwaan 2001). This is in contrast with oysters that were not treated with antibiotics, where the presence of bacteria and tissue necrosis were always evident and mortality rates were accelerated. In terms of behaviour under field conditions, we observed that while oysters synchronously closed in response to anoxia, they reopened their valves throughout the anoxic episode without any indication of synchrony or response to changes in dissolved oxygen concentration. This intermittent opening is perhaps explicable as a means for oysters to test whether water quality conditions have improved or to release toxic by-products of anaerobic metabolism, though inherent to this behaviour is a potential further exposure to anoxia. While coastal animals are

resilient to intermittent bouts of anoxia/hypoxia, our results provide compelling evidence that when anoxia is sustained, bacteria – not anoxia directly – drives mortality. Given that even the most resilient animals are susceptible to the consequences of eutrophication, ecosystem stability under global climate change may be more precarious than is generally thought. Furthermore, the implications for bivalve aquaculture are ominous, particularly given its importance to global food security.

The results of this study serve as strong confirmation of a bacterial influence on the mortality of bivalves under environmental stress. Specifically relating to anoxia/hypoxia, Babarro and de Zwaan (2001, 2002, 2008) reported that controlling for bacteria resulted in higher bivalve survival rates under low oxygen conditions. The effects of other co-varying environmental parameters associated with anoxia/hypoxia (e.g., ammonia, hydrogen sulfide, pH) can influence mortality rate but occur because of bacterial proliferation and are therefore of no consequence when antibiotics are administered. Furthermore, bacteria appear to be important mediators of mortality rates of Pacific oyster (*Crassostrea gigas*) under other forms of stress as well, including disease and temperature (Wendling & Wegner 2013; Lorgeril et al. 2018; Green et al. 2019). For example, experiments conducted at high temperature under normoxic conditions found that bacteria from the genera *Vibrio* spp. were promoted and lead to Pacific oyster mortality (Wendling et al. 2013; Green et al. 2019). While our experiments were all conducted at relatively high temperatures (28 °C for the first experiment and 20 °C for the second; to mimic field conditions when anoxia occurs in this region, Coffin et al. 2018), we did not observe the promotion of any specific genera. It thus seems that under anoxic conditions, in a UV-sterilized environment, an array of endogenous anaerobic bacteria (facultative and/or opportunistic) can proliferate and drive oyster mortality. Similarly, Lorgeril et al. (2018) reported that elevated mortality rates of Pacific oysters in the field were associated with the proliferation of opportunistic bacteria when oysters were in an immunocompromised state due to viral infection. Cumulatively, these studies suggest that bivalve mortality under a range of stressors appears to be influenced by bacterial infiltration. Although bacteria were only assessed on oysters post-mortem, the relative difference in tissue necrosis between individuals treated with antibiotics and those without, indicates that bacterial proliferation post-mortem was likely negligible (Lokmer & Wegner 2015). Therefore, we do not suspect that there would be large differences in bacterial community or level of tissue necrosis if oysters had been sampled immediately prior to death. The universality of this mechanism across species with different life histories, and across a broader range of stressors, awaits further investigation.

A density dependent effect on mortality rate was not observed in this study, however the rapid removal of dead oysters may have prevented the buildup of secondary metabolites like hydrogen sulfide and ammonia which may have accelerated mortality rate (Vaquer-Sunyer & Duarte 2010). Some individual oysters in jars treated with antibiotics did live substantially longer than the populations of oysters within chambers, but this difference was not sufficient to affect the average mortality rate (LT50). While mortality rates, as previously discussed, were similar between chambers and jars, it was observed that bacterial communities were more similar within chambers compared to among jars. These results, together with the brief re-openings of the valves under persistent anoxia, hint that bacterial communities may be shared among individual oysters in each experimental chamber. In this study, all bacteria were endogenous to the animals as the seawater was UV-sterilized before its use in the experiment. Consequently, we believe that these endogenous bacteria were responsible for the tissue degradation that led to the accelerated mortality rate (hence, "the killer

within"). While bacteria endogenous to the animal can drive mortality, as indicated in the present study, it is likely that anaerobic bacteria from the external environment (e.g., bacteria from sediments, or from con- and hetero-specifics that are less resilient to, and perish under, anoxia) can further accelerate oyster mortality under anoxia (Winterburn, in preparation).

Across all treatments, there was substantial and unpredictable variation between relative abundances of different genera of bacteria. For example, in Experiment 3, *Desulfotalea* were almost 20× higher in the antibiotic treatment in chambers (i.e., six oysters) and were responsible for ≈15% of the contrast between the antibiotic treatments. In contrast, this genus contributed only 1% to the between-antibiotic contrast in jars (i.e., one oyster). Furthermore, bacteria from the genera *Bacteroides* had similar relative abundances between antibiotic treatments in jars, but abundances nearly 5× higher in the antibiotic treatment (compared to no antibiotics) in chambers. Given that the water was kept static in both jars and chambers for Experiment 3, coupled with previous reports of similar seawater bacterial communities on plastic and glass (Pinto et al. 2019), there is no reason to suspect differences between jars and chambers for Experiment 3. While differences between the various factors included in this study are evident, they do not manifest through changes to specific genera of bacteria (or at least these differences are not apparent). These heterogenous responses among genera may be attributed to differences in the duration that oysters were living under anaerobic conditions, which in turn could alter which genera are most prevalent. Between experiments variation in initial bacterial communities may also drive some of this variation given that oysters were collected at different times of year; however, all oysters were sourced from the same location and this explanation thus appears unlikely to drive differences within experiments. Ultimately, we can only conclude that the promotion of bacterial genera endogenous to the oysters is widely variable and depends on factors outside the scope of this study.

Our field monitoring of *C. virginica* at Mill River revealed a synchronized tendency towards valve closure at the onset of anoxia. This initial response is highly consistent with *C. virginica* responding to diel-cycling hypoxia in Maryland, USA (Porter & Breitburg 2016). Prior to this study, however, it remained unclear how this species behaves under chronic anoxia, especially since it is metabolically well-adapted to deal with such environmental disturbances (i.e., facultative anaerobes; Hammen 1969). Mill River oysters may have responded to anoxia by initially closing their valves, switching ATP production to anaerobic pathways, and subsequently re-opening their valves on an occasional basis to release acidic end-products of glycolysis and avoid self-poisoning (Bayne 2017). Once glycolysis starts, end-products such as succinate and propionate can accumulate quite rapidly (≈12 hours) in oyster tissues (Michaelidis et al. 2005), whereas the depletion of endogenous energy reserves may span over several months (Deslous-Paoli & Héral 1988; Ren & Schiel 2008; Whyte et al. 1990). Previous studies have suggested that infrequent and minimal shell openings allow the discharge of anaerobic metabolic wastes in overwintering *C. virginica* (Comeau et al. 2012; Comeau et al. 2017; Mayrand et al. 2017). In Mill River oysters facing anoxia, the remarkable extent of gaping amplitude that we observed over hours may have been motivated by other physiological processes. There is convincing evidence that bivalves can increase their ventilation (pumping) rates under hypoxic conditions in order to maintain oxygen partial pressure in the mantle cavity, and consequently oxygen diffusion rates into the hemolymph (Bayne 1971; Le Moullac et al. 2007). Our recording of wide gaping events in Mill River oysters may thus correspond to oxygen-ventilation drives meant to re-establish aerobic pathways. Regardless of the underlying physiological mechanism, the

intermittent re-opening of valves during an anoxic event, as was recorded in the present study, invariably exposes oysters to exogenous bacteria at a time when these bacteria are proliferating from the degradation of excess organic matter.

The eastern oyster, is a critical component of coastal systems along the Atlantic coast of North America and represents an important and sustainable source of protein for future food security. This species is among the most resilient to environmental stressors and thus any negative biological impacts likely have community level consequences. While anoxia has long been considered a stressor for this species (Lenihan & Peterson 1998), the influence of bacteria is not often considered as a mechanism for accelerating lethality. Indeed, despite examinations of bacterial influence on mortality rate occurring almost two decades ago (see Babarro & de Zwaan 2001, 2002, 2008), these findings have been largely ignored in both the literature and, perhaps more importantly, industry (fisheries and aquaculture). Thus, our results enhance the mechanistic understanding of how bivalve mass mortalities can occur under hypoxia, and thus be of benefit to decision-making processes, both for experimental protocols and for fisheries and aquaculture management . We posit that mass mortality events, not only in oysters but in a wide range of coastal fauna, may be the result of co-morbidity between asphyxiation and bacterial processes (Vaquer-Sunyer & Duarte 2010) rather than only a function of hypoxia (as suggested by Nakano et al. 2017). The implication, therefore, is that bacteria endogenous (internal) to the animal, and perhaps those within its environment, are strong drivers of mortality rate, particularly for animals considered tolerant to direct environmental stressors. The consequences of global climate change may thus be underestimated at present, and we strongly contend that future studies must incorporate the role of bacteria and how they are influenced by other stressors in shaping population and community-level responses to climate change and environmental stress.

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