Behavioral effects of low dissolved oxygen on the bivalve *Macoma balthica*

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Abstract

Hypoxia, a dissolved oxygen concentration (DO) below 2 mg l\(^{-1}\), is a significant stressor in many estuarine ecosystems. Many sedentary organisms, unable to move to avoid hypoxic areas, have metabolic and behavioral adaptations to hypoxic stress. We tested the effects of hypoxia on the behavior and mortality of the clam *Macoma balthica*, using four levels of dissolved oxygen in flow-through tanks. We used five replicates of each of four treatments: (1) Hypoxic (DO mean±SE=1.1±0.06 mg O\(_2\) l\(^{-1}\)), (2) Moderately hypoxic (DO 2.6±0.05 mg O\(_2\) l\(^{-1}\)), (3) Nearly normoxic (DO 3.2±0.04 mg O\(_2\) l\(^{-1}\)), (4) Normoxic (DO=4.9±0.13 mg O\(_2\) l\(^{-1}\)). We lowered the dissolved oxygen with a novel fluidized mud-bed, designed to mimic field conditions more closely than the common practice of solely bubbling nitrogen or other gasses. This method for lowering the DO concentrations for a laboratory setup was effective, producing 1.4 l min\(^{-1}\) of water with a DO of 0.8 mg O\(_2\) l\(^{-1}\) throughout the experiment. The setup greatly reduced the use of compressed nitrogen and could easily be scaled up to produce more low-DO water if necessary. The lethal concentration for 50% of the *M. balthica* population (LC\(_{50}\)) was 1.7 mg O\(_2\) l\(^{-1}\) for the 28-day experimental period. *M. balthica* decreased its burial depth under hypoxic and moderately hypoxic (~2.5 mg O\(_2\) l\(^{-1}\)) conditions within 72 hours of the onset of hypoxia. By the sixth day of hypoxia the burial depth had been reduced by 26 mm in the hypoxic tanks and 10 mm in the moderately hypoxic tanks. Because reduced burial depth makes the clams more vulnerable to predators, these results indicate that the sub-lethal effects of hypoxia could change the rate of predation on *M. balthica* in the field.

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1. Introduction

1.1. Hypoxia

Hypoxia, or dissolved oxygen concentrations (DO) below 2.0 mg l\(^{-1}\), is often one of the consequences of cultural eutrophication in aquatic systems (Diaz and Rosenberg, 1995; Cloern, 2001; Gray et al., 2002; Kemp et al., 2005). It is associated with a decrease in the density of motile animals that flee hypoxic waters (Pihl et al., 1991; Das and Stickle, 1994; Bell and Eggleston, 2005; Powers et al., 2005) and a decrease in the abundance, biomass, and diversity of the sessile benthic community (Rosenberg, 1977; Gaston, 1985; Dauer et al., 1992; Diaz et al., 1992; Llansó, 1992; Powers et al., 2005).

Benthic organisms have physiological and behavioral adaptations to hypoxia. Organisms respond by decreasing metabolism and thus oxygen consumption (Stickle et al., 1989; Wu, 2002). This, in turn, can lead to diminished growth and reproduction (Das and Stickle, 1993; Nilsson, 1999; Condon et al., 2001; Grove and Breitburg, 2005). Organisms change their behavior to either increase oxygen supply or decrease oxygen demand. For example, bivalves and polychaetes extend siphons or palps, decrease burial depth, or even float up above the benthic boundary layer into higher DO waters (Brafield, 1963; Rosenberg et al., 1991; Taylor and Eggleston, 2000; Seitz et al., 2003). Some polychaetes and anemones will elongate, increasing their surface-area-to-volume ratio to facilitate oxygen diffusion. Organisms also lower their oxygen demand by reducing feeding and movement (Sagasti et al., 2001).

These sub-lethal responses may have an effect on the food web and trophic transfer if they increase an organism’s vulnerability to predation. For example, the extension of siphons and palps farther into the water column and decrease of burial...
depth make detection by predators more likely for species that use burial depth to obtain a refuge from predation, such as the clam, *Macoma balthica* (Piersma et al., 1995; De Goeij et al., 2001). Whether predators are capable of taking advantage of these stressed prey is a matter of debate, as predators may leave hypoxic areas (e.g., Pihl et al., 1991; Bell and Eggleston, 2005). Predation on tethered *Glycera* in field experiments was DO dependent with predation occurring at low rates under hypoxic conditions (Nestlerode and Diaz, 1998). In laboratory experiments, hypoxia changed the predator-prey relationship, likely due to a change in both predator and prey behavior (Breitburg et al., 1994; Breitburg et al., 1997; Taylor and Eggleston, 2000; Sagasti et al., 2001; Seitz et al., 2003).

1.2. Study species and objectives

*M. balthica* is a small, thin-shelled clam that is common in estuarine systems on the East Coast of the U.S. north of South Carolina. Shell lengths are typically <40 mm. In Chesapeake Bay, this species comprises over 85% of the infaunal biomass in many areas in mesohaline muddy habitats, (Holland et al., 1977; Hagy, 2002). It is an important species in the food-web, as it is preyed upon by numerous fish and crustaceans, comprising ~50% of the diet of the blue crab, *Callinectes sapidus* (Hines et al., 1990). *M. balthica* is an excellent model organism for studying the effects of hypoxia, because it is common in areas that experience hypoxia and is easier to manipulate than other benthic species, such as crustaceans and polychaetes. Tolerant of hypoxia, it can survive for an average of 15 days under near anoxic (0 mg O₂ l⁻¹) conditions (Brafield, 1963) and extends its siphons farther into the water column in response to hypoxia (Seitz et al., 2003). This clam avoids predation by burying deep in the sediment, down to 35 cm (Hines and Comtois, 1985), which makes it a good organism for examining the effect of hypoxia on burial depth.

The objectives of this study were to examine the effect of low DO on *M. balthica* burial depth and mortality and to test the effectiveness of a novel mechanism for the supply of low-DO water. Prior experiments on the effects of hypoxia on *M. balthica* used two levels of DO, hypoxic and normoxic (e.g., Seitz et al., 2003). We designed this experiment to determine the effects over a range of DO concentrations observed in the field, including moderate hypoxia (2-3 mg O₂ l⁻¹) and near normoxia (3–4 mg O₂ l⁻¹). We also designed a system that uses natural sediment oxygen demand (SOD) to reduce DO and that generates a continuous supply of low-DO water, allowing test organisms to be held in a flow-through system. The goal was to remove confounding factors of toxicity inherent in recirculating water systems and to generate low-DO water with a chemical composition more typical of field conditions during hypoxic events. In most previous experiments the DO was reduced by bubbling gases, such as nitrogen (e.g. Seitz et al. 2003), or hydrogen sulfide (e.g., Brafield, 1963) through the water.

2. Materials and methods

2.1. Fluidized-mud reactor

To produce and distribute low-DO water for experiments, we designed a system that minimized our reliance on nitrogen and allowed us to conduct the experiment in a flow-through system. DO was lowered with two large tanks connected in series that acted as fluidized-mud reactors (described in detail below; Fig. 1). At the bottom of each tank, we embedded a perforated array of poly-vinyl chloride (PVC) pipe in ~15 cm of crushed oyster shell. Above this, we layered ~50 cm of mud collected from shallow coves in the York River, Chesapeake Bay, enriched with a small quantity (approximately 50 grams) of Osmocote slow-release fertilizer to stimulate microbial growth. Unfiltered seawater from the York River was dispensed into the first tank, where it was distributed by the pipe array and flowed up through the mud, where SOD reduced the DO. The water drained by gravity from the first reactor into a second identical

![Fig. 1. Illustration of fluidized-mud reactor and experimental setup. Darker water indicates lower dissolved oxygen and arrows indicate direction of water flow. Four experimental tanks are shown on the bottom level, however, multiple replicate tanks for each treatment were used, and treatments were randomly interspersed. On the top level, the two tanks on the right are reactor tanks, and the two on the left are distribution tanks.](image-url)
reactor, and from there into a 400-l distribution tank covered with a sheet of plastic to reduce air exchange. When we ran the system at maximum flow and allowed it to come to equilibrium, the DO in the distribution tank stabilized at \( \sim 0.8 \text{ mg \ O}_2 \text{ l}^{-1} \) (measured with a DO probe, YSI Model 85, Yellow Springs Instruments, Dayton, Ohio, USA). The flow from the reactors, about 1.4 l min\(^{-1}\), was slightly less than what was needed for the experiment, so we supplemented it with additional unfiltered river water (about 70% of the low DO water came from the reactors, and the remainder was the supplement). This necessitated bubbling a small amount of nitrogen though the water to maintain hypoxic conditions; however, the amount used was approximately an order of magnitude less than what was necessary in previous experiments without the addition of the reactors. Slowly bubbled nitrogen had the added benefit of filling the headspace and reducing the diffusion of oxygen into the tank. The reactor could have been the only means of lowering the DO if we had built second reactor or if we had reduced the number of replicates. However, we did not have adequate space for a second reactor, and we did not want to reduce the statistical power of the study by reducing the number of replicates. We filled second distribution tank, for normoxic water, with unfiltered seawater and bubbled air through it.

2.2. Experimental setup

We conducted the experiment in 40-l, transparent, plastic tanks. We filled each tank with 20 cm of mud collected from shallow coves in the York River where Macoma balthica are abundant. We sieved the mud through a 2-mm mesh sieve to remove larger bivalves and other macrofauna. Twenty tanks were randomly assigned five replicates of each of four nominal treatments: (1) Hypoxic (DO \( <2.0 \text{ mg \ O}_2 \text{ l}^{-1} \)), (2) Moderately hypoxic (DO \( 2.5 \pm 0.5 \text{ mg \ O}_2 \text{ l}^{-1} \)), (3) Nearly normoxic (DO \( 3.5 \pm 0.5 \text{ mg \ O}_2 \text{ l}^{-1} \)), and (4) Normoxic (DO \( >4.0 \text{ mg \ O}_2 \text{ l}^{-1} \)). We maintained DO levels by manually adjusting the relative amounts of hypoxic and normoxic water from the distribution tanks. Holes drilled in the lids of the tanks and sealed with corks allowed us to monitor the DO and temperature without fully opening the tank lids.

We collected M. balthica with shell lengths >20 mm from the York River via suction sampling (Eggleston et al., 1992), transported the clams back to the lab, and allowed them to recover for at least 24 hours in a flow-through aquarium prior to use. We transplanted 20 healthy clams with whole shells and a quick siphon-withdrawal reflex to each tank. We attached a monofilament line to the center of each clam shell with cyanoacrylate glue and ran the line through a pinhole in the lid of the tank. We made a mark on the line with permanent ink 35 cm above the clam, and after the clams acclimated, we estimated the relative burial depth by measuring the distance from the top of the lid to the mark and adjusted it for the distance from the lid to the sediment surface to calculate the absolute depth. Due to the curvature of the lids, topology of the sediment surface, and the fact that the clams were not always directly under their holes, our estimate of absolute burial depth was only precise to \( \pm 3 \text{ cm} \), and we tended to overestimate. However, because we calculated the change in burial depth as the difference between the distance from the lid to the mark on two different days, thus eliminating the other sources of error, it was precise (\( \pm 2 \text{ mm} \)). Clams acclimated in the tanks under normoxic conditions for at least 24 hours to allow them to recover from handling and to bury to natural burial depths. We replaced clams that died or did not bury before the experiment. The day we started the experimental treatment, we removed any dead or unburied clams without replacement. Before the experiment, four of the tanks did not receive adequate water flow and had high clam mortalities. These were tanks at the end of the flow lines where water-pressure was lowest, and we prevented additional mortalities by increasing flow to the distribution tanks; these tanks were excluded from analyses.

After the acclimation period, we adjusted the DO in each experimental tank to its nominal treatment range. This was achieved by increasing the flow of hypoxic water and decreasing the flow of normoxic water. The Hypoxic tanks received only hypoxic water, the Normoxic tanks received only normoxic water, and the Moderately hypoxic and Nearly normoxic tanks received a mixture of hypoxic and normoxic water. We monitored the DO and temperature in each experimental tank daily, and adjusted the flow of the hypoxic or normoxic water at the same time if necessary to maintain the DO within the nominal range. We monitored the burial depth of each clam daily for the first week and, after that, every two-three days. Dead clams were identified if they gaped and continued to do so when handled; they were removed. We kept the DO within the nominal experimental ranges for 28 days before returning it to normoxia. At the end of the experiment, we sieved mud from each tank and counted and measured the clams that were still alive. Proportional survival was calculated for each tank.

2.3. Data analyses

The DO measurements were analyzed with a nested Analysis of Variance (ANOVA; tank nested within DO treatment). The data were square-root transformed to reduce heteroscedasticity. Change in burial depth (initial burial depth minus burial depth on each subsequent day) was analyzed for each day up through day 10 with a nested ANOVA (tank nested within DO treatment). Burial depth after day 10 was not analyzed, as the number of clams remaining in the lowest DO treatments was too low. Where there was a significant effect of treatment, a Tukey’s pair-wise multiple comparison test was performed. We plotted survival in each tank against the average DO, and analyzed it with a sigmoid least-squares regression and determined the LC\(_{50}\) (relative to the Normoxic controls) in SigmaPlot (Version 8.02, SPSS Science, Chicago, IL; Haendel et al., 2004).

3. Results

Our setup allowed us to keep each experimental tank within its nominal DO range. There was a significant difference in dissolved oxygen among the treatments (nested ANOVA; \( F_{3,656}=799.42; p<0.0005 \)), and the mean for each treatment
fell within the nominal range and differed from the other means (Tukey’s test, p<0.0005; Fig. 2).

Low DO conditions caused *M. balthica* to reduce its burial depth. On average on day 1, the overall burial depths were 126 mm for all treatments. By day 6, average burial depth decreased (i.e., clams moved up toward the sediment-water interface) by approximately 26 mm in the Hypoxic treatment (<2.0 mg O₂ l⁻¹) and by 10 mm in the Moderately hypoxic treatment (~2.5 mg O₂ l⁻¹), while increasing slightly (i.e., clams moving away from the sediment–water interface) in the Nearly normoxic and Normoxic treatments. There was a significant effect of DO treatment on change in burial depth on days 4 through 10 (nested ANOVAs; p<0.05; Fig. 3), and a significant effect of tank (nested within DO treatment) on days 2 and 4. In both Hypoxic (DO<2.0 mg O₂ l⁻¹) and Moderately hypoxic (2.5±0.5 mg O₂ l⁻¹) treatments, the burial depth decreased beginning almost immediately (on day 2) and reached an asymptote at around day 6. The Hypoxic and Moderately hypoxic treatments both differed from the Normoxic treatment (DO>4.0 mg O₂ l⁻¹) on days 5 through 10 (Tukey’s test).

Low DO was associated with higher clam mortality after 28 days. Clam survival decreased in a non-linear fashion with Fig. 2. Dissolved Oxygen (DO) observations for each of the four treatment levels over the course of the experiment. Nominal treatment levels were (1) Hypoxic (DO<2.0 mg O₂ l⁻¹), (2) Moderately hypoxic (DO 2.5±0.5 mg O₂ l⁻¹), (3) Nearly normoxic (DO 3.5±0.5 mg O₂ l⁻¹), and (4) Normoxic (DO>4.0 mg O₂ l⁻¹). Points are the mean DO (+1 SE) of the tanks within each treatment. The first observation and last three observations are before and after the experimental period, respectively, when all tanks were normoxic.

Fig. 3. Mean (±1 SE) change in burial for each DO treatment. Hypoxic treatments were initiated on day one. Positive numbers indicate movement towards the water-sediment interface. Asterisks indicate a significant effect of DO treatment on burial depth. Treatments with different letters next to them differ at the 0.05 level (Tukey’s test; shown for day 10 only). Dashed line indicates no net vertical migration.

Fig. 4. Effect of DO on *M. balthica* survivorship. Proportional survival at the end of the 28-day experiment is plotted against average DO for each tank. Trend line is from best-fit equation: y = \frac{0.76}{1+e^{-x}}, (R^2 = 0.55, p = 0.0021).
decreasing DO, and we estimated a lethal concentration for 50% of the population (LC50) of 1.7±0.5 (SE) mg O2 l\(^{-1}\) for the 28-day experiment (Fig. 4).

4. Discussion

Our method for lowering the DO concentrations for a laboratory setup was effective and has several benefits. As desired, it produced a continuous supply of flowing, hypoxic water (DO 0.8 mg l\(^{-1}\)) for the full duration of the experiment. Although the flow rate of 1.4 l min\(^{-1}\) was lower than was needed, it could easily be scaled up by either using larger tanks as reactors, or by making additional reactors. The mechanism for lowering the DO is similar to that in the field; DO concentrations are reduced by sediment oxygen demand (SOD). In previous laboratory experiments, the DO was reduced by displacement through bubbling a gas or mixture of gases, usually nitrogen (e.g., de Zwaan and Babarro, 2001) or hydrogen sulfide (Brafield, 1963), through the water. This difference between field and laboratory DO development in experiments using bubbling gas for development of hypoxia may confound the results. As our design used a flow-through system, it avoided the use of stagnant or re-circulated water (e.g., Modig and Ólafsson, 1998; Grove and Breitburg 2005) and the resulting high abundance of bacteria known to lower survival times in lab experiments (de Zwaan and Babarro, 2001; de Zwaan et al., 2001). This design was cost effective, as it substantially reduced the amount of gas necessary for maintaining hypoxic conditions.

A 28-day LC50 for *M. balthica* of 1.7 mg O2 l\(^{-1}\) is comparable to values presented elsewhere in the literature; Borsuk et al. (2002) modeled the survival of *M. balthica* using a compilation of literature values and estimated a 21-day LC50 of 1.5 mg O2 l\(^{-1}\). Thus, *M. balthica* is apparently quite tolerant of hypoxia in the lab; however, *M. balthica* populations suffer heavy mortality in the field under moderate to severely hypoxic conditions, comparable to the range used in this experiment (e.g., Llansó, 1992; Buzzelli et al., 2002; Powers et al., 2005). This indicates that another form of mortality is driving the pattern observed in the field.

These results, combined with those of prior experiments, indicate that sub-lethal exposure to hypoxic conditions may result in increased predation risk. *M. balthica* responds to hypoxia by vertically migrating upward in the sediment. Clams in both the Hypoxic (DO<2.0 mg O2 l\(^{-1}\)) and the Moderately hypoxic (DO~2.5 mg O2 l\(^{-1}\)) treatments moved towards the sediment surface by about 26 and 10 mm, respectively. Although no vertical migration was observed in previous similar experiments (Seitz et al., 2003), this is likely due to differences in sediment type; Seitz et al. (2003) used sand and we used mud. *M. balthica* buries deeper in mud (its preferred habitat), down to 35 cm, than in sand, down to 20 cm (Hines and Comtois, 1985), allowing substantial scope for change in mud. Changes in *M. balthica*'s burial depth of as little as 2 cm, the change observed in the Hypoxic tanks, can decrease predator handling time by 66% (Piersma et al., 1995). In addition, this vertical migration may be increased in field situations by siphon cropping. *M. balthica* extends its siphons into the water column in response to hypoxia (Seitz et al., 2003), which can lead to higher rates of siphon cropping (Peterson and Skilleter, 1994; Skilleter and Peterson, 1994) and decreased burial depth (De Goeij et al., 2001), thus, further increasing the vulnerability of clams to predation.

The time scale of the behavioral changes in *M. balthica* is much shorter than that for lethal effects. Siphon extension occurs within a day (Seitz et al., 2003) and burial depth reduction begins within two days and decreases substantially within four days (this study), whereas significant mortality under moderately hypoxic conditions (1–2 mg O2 l\(^{-1}\)) does not occur for one to two weeks (Borsuk et al., 2002), or longer (Seitz et al., 2003). In many systems where hypoxia is episodic, such as the York River (Diaz et al., 1992), the Neuse River (Powers et al., 2005), and parts of the Baltic Sea (Modig and Ólafsson, 1998), hypoxic conditions seldom last for more than a week at a time before returning to normoxia. As *M. balthica* is highly tolerant of episodic hypoxia (Modig and Ólafsson, 1998), we suggest that predators taking advantage of stressed prey may be a major source of mortality in these systems.

Predator behavior in systems that suffer from hypoxia could lead to an increase in foraging after a hypoxic episode. Motile predators can rapidly reinvade areas after relaxation of hypoxic conditions (e.g., Pihl et al., 1991; Nestlerode and Diaz, 1998; Bell and Eggleston, 2005). They may take advantage of stressed benthic organisms following hypoxia, as their diet shifts to include a higher percentage of deeper-burying organisms, including *M. balthica* (Pihl et al., 1992; Long and Seitz, 2008). These results indicate that this increase in predator foraging may be due to the demonstrated vertical migration of *M. balthica* in response to hypoxia in systems where this species is common.

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