

**Using benthic infauna abundance and genetic markers
in oysters as indicators of hypoxia downstream from a
NITREX permeable reactive barrier**

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ABSTRACT

Permeable reactive barriers like the one installed in Waquoit Bay, Massachusetts may be useful in removing nitrate from groundwater, but one disadvantage of these installations is that they lead to anoxic waters. To date, the effect of anoxic or hypoxic conditions on communities downstream from these systems has not been well assessed. This study sought to examine differences between regions downstream from a permeable reactive barrier and a control site at Waquoit Bay in three ways: (1) chemical analysis of the sediment, (2) abundance of benthic infauna, and (3) oyster response to hypoxia using genetic markers. By looking at chemical and biological differences between regions downstream from the barrier and a control site, we can better understand the impact of the barrier and whether observed differences change as a result of distance from the barrier.

Eh data collected at the control and barrier sites revealed that there are significant differences in the redox potential of the sediments between these locations, with the most pronounced differences occurring in the middle region sampled (6 meters from the edge of the barrier). Eh values were much lower for the barrier regions, suggesting the presence of sulfides and anoxic sediments. CHN analysis indicated that the barrier locations typically contained higher percent carbon, presumably due to leaching of dissolved organic carbon as a result of the woodchip medium that is the predominant material in the barrier. Sulfur data obtained by using a combustion technique shows that sediments downstream from the barrier also have higher total sulfur content.

The barrier and the control site differed in the number and type of benthic infauna found, with much greater abundance of organisms occurring in the control regions. Groups of the eastern oyster *Crassostrea virginica* were placed in cages at varying distances from the barrier, and a hypoxia-related gene sequence was targeted to analyze response in these oysters. No significant difference in genetic expression of oyster response to hypoxia was found, suggesting that the effect of the barrier may not permeate up to higher trophic levels. The barrier was seen to have a localized effect in regard to each parameter of this study, with the most dramatic changes seen in the middle region. By comparing differences in sediment chemistry, benthic infauna abundance, and genetic expression of a hypoxia-related gene in oysters, this study examined the effect of barriers on downstream communities, and lends insight towards determining whether permeable reactive barriers are practical solutions to treating nitrogen-loading in coastal ecosystems.

Key words: anoxia, benthic infauna, *Crassostrea virginica*, gene expression, heat shock protein, hypoxia, nitrate-removal, permeable reactive barrier, Waquoit Bay

INTRODUCTION

Permeable reactive barriers may be an effective means of removing nitrate from septic runoff (Robertson et al. 2005). By providing a carbon source, such barriers promote denitrification of nitrate to nitrogen gas. In doing this, however, the reactive environment becomes anoxic. In August 2005, a permeable reactive barrier was installed in Waquoit Bay, MA. At this site, it has been shown that the environment becomes anoxic both within and around the barrier (Figure 1). Previous studies have looked at nitrogen dynamics within the barrier and the influence of the barrier on groundwater flow paths. However, the effect of anoxic waters from the barrier on the community downstream has not been well assessed. If such permeable reactive barriers are to be implemented on coastal communities on a large scale, it is important to understand their effect on the surrounding waters. The effects of anoxic waters coming from the barrier on downstream communities can be 'detected' by looking at differences between these systems. Three regions were designated "upper"(3 m), "middle" (6 m), and "lower" (9 m) to compare changes as a result of distance from the barrier (Figure 1). An overall goal of this study was to compare differences between communities downstream from the barrier and a nearby control site, as well as to evaluate the potential for such barriers to be a safe, advantageous, and applicable means of treating wastewater in coastal systems.

Sediment Chemistry

One way to measure differences in sediments is by determining the Eh, or reducing potential of these areas. Eh is an intensity measure of the reducing or oxidizing conditions in a system and reflects the tendency of ions or molecules in a solution to donate or accept electrons. Eh data can be used to indicate the progress of a system towards reduction or oxidation, delineate redox gradients in a stratified system, and assist in further determination of redox reactions (Whitfield 1969). Because Eh values are a useful means of characterizing sediments, this study aimed to use this parameter as a means to show differences between the barrier and control sites.

In addition, a profile of total sulfur content can offer a portrayal of differences that exist between sediments. Hydrogen sulfides are most likely being released from the permeable reactive barrier, and are making their way downstream. These sulfides are often toxic to animals. By analyzing sulfur differences between sites, we can better characterize the differences that exist as a result of the barrier.

The barrier's woodchip medium may also have a noticeable effect on the sediments: this can be confirmed by CHN analysis on the carbon content of the sands downstream. This study sought to determine whether leaching of dissolved organic carbon (DOC) was detectable in the sediments, and to determine the role that this carbon enrichment may have on the sediments and the organisms that live there.

Benthic Infauna Abundance

Hypoxia is defined as dissolved oxygen less than 2.8 mg O₂ per liter (Wu 2002). Benthic communities are the most sensitive regions of coastal ecosystems to hypoxia, which in most aquatic systems, is a result of eutrophication. In contrast, this study sought to analyze the effect of hypoxia as a result of the nitrate-removal system installed in Waquoit Bay that was actually designed to decrease the effects of eutrophication. The

intent of this study was to survey the types and number of benthic infauna present between the barrier and a control location, and to evaluate differences in results as distance from the barrier (the anoxic source) increased. Previous studies have shown that the tolerance of stress by benthic species can influence their distribution and abundance. Polychaetes, bivalves, platyhelminths and cnidarians are cited as having relatively high tolerance to hypoxia, while crustaceans and vertebrates have relatively low tolerance to this type of stress (Sagasti 2001). In this way, the abundance of benthic infauna can tell us a great deal about the conditions that exist downstream from the barrier. This assessment of benthic infauna can be coupled with response to anoxic stress in an organism belonging to a higher level.

Oysters as Indicators

Hypoxic stress can be “detected” using oysters, which are an effective indicator species of water quality and for which relevant genetic markers for this type of environmental stress are known. Molecular methods such as quantitative real time PCR (RT-PCR) can be used to quantify the expression of specific stress-related genes in oysters. Traditional PCR reactions enzymatically replicate DNA, but this type of reaction is strictly qualitative; it can only offer information about the end products of a reaction. On the other hand, quantification of gene expression can be determined using real-time PCR, specifically by using a method that relies on double-stranded DNA binding chemistry. This method employs a fluorescent probe (SYBR Green I) to measure starting amounts of cDNA by binding to dsDNA. A fluorogenic minor groove binding dye, SYBR Green exhibits little fluorescence when in solution but emits a strong fluorescent signal upon binding to double-stranded DNA. In this way, quantification can be analyzed as the reaction proceeds, or in “real time”. Melting point analysis is used in conjunction with this technique to specifically identify amplicons. David et al discovered that the expression of one gene in particular, heat shock protein 70, was expressed at much higher levels in Pacific oysters (*Crassostrea gigas*) exposed to hypoxia than those belonging to a control group. This study compared differences in oyster (*Crassostrea virginica*) response between oysters that were placed downstream from the barrier and in a control region, and to see whether that response changes as a function of distance from the barrier.

The three approaches outlined above (sediment chemistry, benthic infauna abundance, and oyster response) each offer a unique means to assess differences between the barrier and control site. By using array of chemical parameters to analyze the sediment, as well as biological indicator organisms to understand the implication of sediment differences to community structure, this study provides a clearer picture of the effect of the barrier and to what extent that effect is seen.

METHODS

Chemical Analyses

(a) Redox Potential (Eh)

I used an Eh probe (Orion Research Model SA 210) to determine the redox potential of the sediments at both the barrier and control sites. The Eh probe consisted of a platinum electrode and a standard hydrogen electrode (SHE). Before going out into the field, I made up two standard solutions of hydroquinone to check to make sure the readings were within an accepted range: one solution in pH 7 read 43 mV; the other solution in pH 4 read approximately 220 mV. These correlated well with published values. I took Eh readings at 12 locations (Figure 4). I obtained Eh measurements at the lowest tide possible (-0.1m) for the time of this experiment. An Eh reading was taken at every centimeter up to 10 cm (where possible) at each of the 12 sites. I took three to four replicate readings. I rinsed the tip of the electrode with DI between readings. The redox potential was obtained by adding the appropriate value for the reference electrode potential (247.75, in this case) to correct for liquid junction effects.

(b) CHN and Sulfur Analysis

I took two replicate sediment cores (6.5 cm in diameter) at each of the six sites (upper, middle, and lower regions of both the barrier and the control locations = 12 sediment cores total). I used rubber stoppers (size 13 on top, 12 on bottom) to close off the cores and bring them back to the lab for sectioning. In the lab, I used a wooden dowel with a rubber stopper attached (drilled in with a screw) to extrude the cores into a PVC pipe of the same size. I sectioned the top 10 cm of core material as follows: 0-2 cm, 2-6 cm, and 6-10 cm. Thirty-six samples (3 regions, 3 depths, 2 replicates, 2 locations) were created from this sectioning procedure. I placed the wet sediment on small aluminum foil weigh boats and dried them in a 60 ° C drying oven. After drying, I ground the samples as finely and uniformly as possible using a mortar and pestle. I placed subsamples of the sediment (36; 1 for each sample) into acid-washed 2 oz. Qiorpak jars. I used the same subsample for both sulfur and CHN analysis (only a small amount was needed for CHN; a larger amount was required for sulfur combustion).

(1) Sulfur Combustion

I determined total percent sulfur by using a LECO SC-32 Sulfur Analyzer, which combusts samples at 2500 degrees Celsius. I used sulfur in coal (1.67%) as a standard for calibration purposes. I ran four standards to calibrate the machine before attempting to measure sulfur in the experimental samples. A dessicant tube (filled with perchlorate) and halide-removal tube (with antimony metal) were also prepared and installed before using the sulfur analyzer. I combusted twenty-four sediment samples to determine the total sulfur content present in each. The percent sulfur in the upper region for both the barrier and control regions was too low to be detected for each of the three core depths (0-2, 2-6, 6-10). I placed 4-5 grams of each sample in a ceramic combustion boat and combusted the sediment for several minutes before obtaining a percent sulfur reading.

(2) CHN Analysis

In order to estimate the carbon and nitrogen content of the sediment, I prepared the samples for CHN analysis. In order to remove carbonates from the marine sediment, I acidified all the samples. To do this, I placed the remaining sediment from each of the Qiorpak jars into glass scintillation vials, moistened them, and stored them in a dessicator over full-strength HCl (25 mL) for 3 days. After acidification, I dried the samples and

ground them again. I carefully weighed out a small amount of sample (20-30 mg), which I then packed into tin vials for CHN analysis, along with blanks and standards for correction purposes (CHN Lab Handout).

Abundance of Benthic Infauna

(a) Collection

I took twelve sediment cores (6.5cm in diameter) from both the barrier and control sites (see Sampling Protocol, Figure 2). A similar method to that used to extrude the cores for chemical analysis was employed for benthic infauna collection. I sectioned off the top 10 cm of the cores and sieved the material into three size fractions: small (250-590 μ l), medium (590-2mm), and large (>2mm). I washed the material caught in the sieve into clean 36 oz. canning jars. I preserved the samples (36 jars---12 cores, 3 size fractions each) in 37% formalin with Rose-Bengal solution for several days. (I added 2mL Rose-Bengal dye to stain the organisms per every 2 liters of formalin). I continually monitored and shook the jars to ensure that the formalin and dye solution remained mixed throughout. Sediment composed approximately half of the samples; this posed a problem in retrieving the worms.

(b) Separation

While the worms could have been separated by washing and sieving, the use of silica sols such as Ludox© has been found to be a superior technique in separating meiofauna from sediments (Burgess 2001). Retrieval rates are much higher with this method. I poured the formalin from each jar over the finest sieve into an appropriate chemical waste container under a fume hood. I then washed the sieve several times with water to remove the formalin and to collect the organisms on one small portion of the sieve. I then washed the organisms with water into a tall graduated cylinder. The infauna and sediment were elutriated with water five times (I capped them with parafilm and shook them). The sample was allowed to settle and was then sieved again. After each elutriation, I washed the recovered organisms on the sieve into a funnel back into their jars. After these five elutriations with water, the remaining sediment and unrecovered organisms were left in the graduated cylinders. I filled each of the graduated cylinders to the top with Ludox© and allowed the material to settle for 1-2 hours. Some organisms were retrieved in this final washing, but most organisms had actually been separated by the elutriations with water. The Ludox© solution helped to disturb some of the heavier organisms (gastropods, large worms) that remained in the sediment.

After the settling time, one final elutriation with Ludox© recovered the rest of the organisms. I collected these organisms one last time on the finest sieve, washed the infauna into their appropriate jars, and then re-filled the jars with formalin.

(c) Identification

I poured off the formalin from benthic infauna samples that I was going to identify that day and replaced the formalin with water. I poured the sample out onto a gridded glass Petri dish and placed the dish under a dissecting microscope. Moving one square at a time, I used several published keys to identify organisms (Weiss 1995 and Pollock 1998). Worms and other benthic infauna were carefully removed from the gridded Petri dish using a Pasteur pipette. I placed the organisms into small round dishes designated for each of the benthic infauna types: polychaetes, oligochaetes, and

nematodes. Other organisms such as ostracods, foraminifera, and “unknowns” were placed in their own separate dishes.

(d) Enumeration

After the entire sample on the Petri dish had been sorted, I counted up the number of worms in each of the smaller dishes with a clicker counter. I then transferred the worms from the round dishes into small glass vials. The glass vials were filled with 75% ethanol and capped. I covered these caps with Parafilm to prevent evaporation of ethanol and to prevent the tops from popping off.

Oyster Response to Hypoxia Exposure

Oyster Exposure and Treatment

Approximately 80 oysters native to Waquoit Bay were obtained from Waquoit Bay National Estuarine Research Reserve. Sixty oysters were put out in the field; 20 oysters were brought back to the lab for other experiments (see description of hypoxia experiment below). The oysters in the field were housed in large cages (approximately 0.75 m x 0.75 m x 0.325 m). I lined the oyster trays with black plastic lining to prevent the oysters from falling out the sides and bottom. I cut metal hardware cloth to cover the top of the trays. I placed 10 oysters in each of the oyster cages (6 trays x 10 oysters each= 60 oysters) and then tied the cages shut with plastic zip ties. Metal stakes were driven into the sediment with a rubber mallet to ensure that the cages stayed in place. I deployed the oyster cages in six locations: three were placed downstream from the barrier and three were placed the same distance away from the shore at a control location nearby (Figure 2). Each of the trays was placed at varying distances from the barrier, at the areas designated upper, middle and lower (Figures 1 and 2).

Hypoxia Experiment

I subjected 16 oysters to 4 different hypoxic conditions (4 oysters per condition) for varying time durations (0 hours, 3 hours, 6 hours, 12 hours) to serve as a positive control. Seawater was bubbled with helium gas until oxygen concentrations reached less than 1.9 mg per liter. I removed oysters from the hypoxia experiment following each of the time exposures and removed 25mg of gill tissue using sterile technique (see *Oyster Retrieval and Gill Tissue Removal*, below).

Gene Targeting

The gene I targeted in this study was heat shock protein 70 (Hsp70). Heat shock proteins are constitutively expressed in cells and are involved in protein folding, assembly, and degradation (Piano 2004). They act as molecular chaperones. Previous studies have determined that Hsp 70 is involved in response to hypoxia in oysters, and it has been suggested that there is a time-dependent response in these organisms.

Oyster Retrieval and Gill Tissue Removal

After 10 days of exposure, I retrieved the oysters from the field and placed them in a bucket of seawater collected from Waquoit Bay. I retrieved 59 out of the original 60 oysters from the experimental and control sites; one oyster from the upper tray in the barrier region could not be found. Upon returning to the lab, I shucked the oysters and cut

25 mg of tissue from the fluttery gill (contains the most hemocytes) using sterile technique. I placed the samples in 500 µl TRI reagent in microcentrifuge tubes on ice. I used TRI Reagent, a combination of phenol and guanidine thiocyanate in a mono-phase solution, to facilitate the immediate and most effective inhibition of RNase activity (RNases are harmful because they degrade RNA). After placing the gill tissue samples into the TRI reagent, I homogenized them using a sonicator (amplitude=30%). I rinsed the sonicating device with deionized water between each sample. I froze the samples at -80°C until the following day.

RNA Extraction

The homogenate was removed from the refrigerator and stored at room temperature for 5 minutes. Under the hood, I added 100 µl of chloroform to each sample in order to separate the homogenate into aqueous and organic phases. Then, I vortexed the samples for 15 seconds and stored them at room temperature for 10 minutes before placing them into a centrifuge at 12,000 g for 15 minutes at 4 °C.

Following centrifugation, RNA remained exclusively in the aqueous phase (clear layer); DNA in the interphase and proteins in the organic phase (white layers). The aqueous phase (approximately 50% of the volume of TRI reagent used for homogenization) was carefully transferred to a new tube. RNA was precipitated from the aqueous phase by adding 250 µl of isopropanol. The samples were then stored at room temperature for 5-10 minutes and centrifuged at 12,000 g for 8 minutes at 4 °C. The RNA precipitate (often invisible before centrifugation) formed a gel-like white pellet on the bottom of the tube. I removed the supernatant and washed the RNA pellet by vortexing with 500µl of ethanol. The samples were subsequently vortexed at 12,000 g for 5 minutes at 4 °C.

I removed the ethanol wash and briefly air-dried the RNA pellet under the fume hood for 3-5 minutes. I spot-spun any ethanol that remained in the tube and then carefully pipetted this liquid out. This was done because ethanol can negatively affect PCR reactions. I carefully monitored the samples as they dried until they began to turn invisible before resuspending them in water; if RNA pellets are completely dried, they lose their solubility. Smaller pellets were solubilized in 10 µl of nuclease-free water; larger pellets were solubilized in 20 µl of nuclease-free water. Samples that did not solubilize completely at room temperature were heated at 55 °C for five minutes on a heating block. Ten RNA extractions typically took 2 hours to complete; the full set of 40 extractions was completed over a series of 3 days.

Reverse Transcription

Oyster gill RNA was reverse-transcribed into complementary DNA (cDNA). I thawed 5µl of RNA, incubated it at 75°C for 5 minutes, and then put it on ice for 5 minutes. I made up a master mix for 40 samples. Each sample well contained 5 µl of RNA, 8 µl DNTPs, 1µl of water, 1 µl of primers, and 10 u/ml AMV. All 40 samples were run on one PCR plate (clear type), along with 3 NTCs (no template controls). Oyster gill RNA was reverse transcribed into complementary DNA using a thermal cycler set to the Conventional RT 1 Program. The reaction proceeded as follows: 23 °C for 10 minutes, 37 °C for one hour, 95 °C for 3 minutes. The plate was then spot-spun and stored at -20 °C until later use.

Quantitative Real-Time PCR

To check the concentration of cDNA, I ran a 1:4 dilution with 2µl of cDNA and 6µl of water. Three samples were chosen from RNA extractions performed on different days (1 from 1st day extraction, 1 from 2nd, 1 from 3rd). The reaction mixture for each well contained the following: 10.3 µl of water, 12.5µl of 2X Mix, 0.1µl of forward primer, 0.1µl of reverse primer, and 2µl of the 1:4 diluted cDNA. I then ran the reaction on SYBR Green cDNA Melt at 55 °C (2 reads) for approximately 1.5 hours.

After checking the concentration of cDNA, the final experimental reactions (40 samples with 18S, 40 samples with Hsp 70, and 3 NTCs for each of the 2 primers=86 wells) were carried out on one clear PCR plate. Each assay was placed in an individual well of a 96 well-plate with a reaction cocktail containing 10.3 µl of water, 12.5µl of 2X Mix, 0.1µl of forward *C.virginica* 18S or Hsp primer, 0.1µl of *C.virginica* 18S or Hsp reverse primer, and 2µl 1:4 diluted cDNA from each of the barrier, control, and hypoxia samples (a 25µl reaction total for each well).

The reaction was then run on SYBR Green cDNA Melt at 55 °C (2 reads) for approximately 1.5 hours using quantitative real-time RT-PCR (Thermoscript One-Step System, Invitrogen). This procedure was considered two-step real-time RT-PCR because reverse transcription and PCR were performed separately in different tubes.

Data analysis

Each fluorescence curve was checked with its corresponding melting curve to ensure that the product was real (SYBR Green does not distinguish against non-specific amplification). Next, the C(t), or the cycle at threshold, was determined. This is the cycle number at which the fluorescence emission exceeded a fixed threshold (pre-set significantly above the baseline by the operator). At the threshold cycle, the system begins to detect the increase in the signal associated with the exponential growth of PCR product during the log-linear phase of the reaction. This phase provides the most useful information about the reaction (more important than the end-point). The slope of the log-linear phase is a reflection of the average efficiency, where the efficiency of the reaction can be calculated as follows:

$$\text{Eff} = 10^{(-1/\text{slope})} - 1$$

The relative amount of each gene was calculated using the following equation:

$$\text{Relative amount of gene} = (1/1 + \text{average efficiency})^{-C(t)},$$

where C(t) is the cycle at threshold.

The raw data from the Opticon 2 Continuous Fluorescence Detection System (MJ Research) was then exported to the Data Miner program. This software program uses a four-parameter logistic model to fit the raw fluorescence data as a function of PCR cycles to identify the exponential phase of the reaction (Sheng 2005). A three-parameter simple exponent model then fits the exponential phase using an iterative non-linear regression algorithm. Within the exponential portion of the curve, the technique identifies candidate

regression values using the P-value of regression and then uses a weighted average to compute a final efficiency for quantification.

Results for this oyster study were analyzed using a relative gene expression approach, where the expression of the Hsp70 (the target gene) was normalized to 18S RNA, a non-regulated reference gene that is often used as a control in such studies.

Statistical Analysis

Statistical tests on the relative expression of Hsp70 between the barrier and control, as well as between the barrier, control and the hypoxia experiment found that there were no significant differences between any of the treatments. Relative Expression Software Tool (REST) analysis was used, which compares two or more treatment groups with up to 100 data points in a sample or control group for multiple reference genes and up to 15 target genes (Pfaffl 2002). The mathematical model used is based on the correction for exact PCR efficiencies and the mean crossing point deviation between sample group(s) and control group(s). The expression ratio results of the investigated transcripts are then tested for significance by a Pair Wise Fixed Reallocation Randomisation Test © and plotted using standard error (SE) estimation via a complex Taylor algorithm.

RESULTS

Sediment Chemistry

The barrier and control sediments were considerably different both visually and chemically. Sediments downstream from the barrier were much blacker in color than the control sediments (Figure 3). The stark visual differences seen between the control and barrier sediments were supported by strong chemical differences as well.

a. Reducing Potential

In terms of reducing potential, the barrier sediments had much lower average Eh values than the control sediments (for all three regions: upper, middle, and lower). The greatest difference in average reducing potential between the barrier and control regions occurred in the middle region (Figure 4). For all regions of both the control and barrier sediments, Eh values decreased with increasing depth. In Figure 5, the individual readings for each of the series of replicates show that the same trend occurred in each of the replicates. In addition, there was a much greater range of Eh values found in the barrier sediments over the various depths than in the control sediments. The Eh readings remained much more constant in the control regions as depth increased.

b. Total Sulfur Content

Total percent sulfur was higher in the barrier sediments than in the control sediments for both the middle and lower regions (Figure 6). Sulfur readings for the upper region could not be obtained; they were not detectable by the methods used in this study. The greatest differences in total sulfur content between the barrier and control locations for both the upper and middle regions were found at the 2-6cm depth. The least amount of sulfur was found in the 0-2 cm depth for both the barrier and control locations.

c. *CHN Analysis*

In general, percent carbon was greater in the barrier sediments than in the control sediments (Figure 7). Greater percent carbon tended to occur with increasing depth. The most apparent differences in carbon content between the barrier and the control existed in the middle region. For this region at the 0-2cm depth, the control had higher percent carbon, but from 2-6cm and 2-10cm, the barrier had higher percent carbon. (Readings for sulfur and CHN were low; more sediment could have been packed per vial for each sample run).

Benthic Infauna Abundance

Several species of benthic infauna were found in the sediment cores, primarily oligochaete, polychaete and nematode worms (Figure 8). Small clams, foraminifera, snails, and ostracods were also found. Large differences between the barrier and control locations were found. For all three regions, the barrier locations had significantly lower abundance of benthic infauna. The greatest differences were seen in the middle region (control in this region had highest overall; barrier had lowest overall). In the middle control region, nematodes were in the greatest abundance and all four types of organisms were found to be present. There were few polychaetes or “other” organisms found in the middle barrier region.

In the upper region, in the control, polychaetes were found in the greatest abundance; in the barrier, nematodes were found in the greatest abundance (Figure 10). In the middle region, the control location was comprised primarily of nematodes; in the barrier oligochaetes and other were dominant. In the lower region, the control location was comprised primarily of nematodes, in the barrier; polychaetes were dominant. The main types of polychaetes found were several *Nereis* species and *Syllidae* worms. Nematodes and oligochaetes could not be identified.

Oyster Response to Hypoxic Stress

No mortality was observed in the oysters subjected to any of the three treatments: barrier, control, and hypoxia. The PCR reactions ran smoothly; only one PCR reaction well failed (Barrier Middle Sample 4), most likely due to DNA contamination (the 18S peak for this sample came too early).

Differences in expression of the Hsp gene were seen between the barrier and control location across each of the regions (Figure 11). The middle region showed the highest expression of the Hsp gene. The barrier showed slightly higher expression than the control at this region. In the lower region, Hsp expression was slightly higher in the barrier than in the control. However, differences between the barrier and the control locations for all three regions were not found to be statistically significant. Oysters that were exposed to the hypoxia experiment (12 hours) exhibited greater expression than the oysters placed in the field at the upper and lower regions (both barrier and control), but lower expression than both the barrier and control in the middle region.

DISCUSSION

Sediment Chemistry

The darker color of the barrier sediments is most likely due to the presence of iron-sulfides. These sulfides have often been linked to anoxic conditions in aquatic systems. The barrier locations possess greater reducing potential due to the presence of various electron acceptors. Hydrogen sulfides as a result of sulfate reduction occurring within and around the barrier are most likely being released and are making their way downstream. The lower Eh values that were found in all the barrier locations are typical of marine sediments that have high sulfide activity (Whitfield 1969). This is supported by the idea the sulfur results obtained from this study, in which there were higher concentrations of sulfur with increasing depth, especially in the barrier location. The lower Eh values with increasing depth may be due to the accumulation and settling of sulfides or the fact that the barrier itself is closer to these deeper depths. The greatest effect of the barrier is seen in the middle region, suggesting that there is a localized effect. In these areas, the sediment may act as a trap for electron acceptors in the overlying water, and these areas are linked to oxygen depletion. This oxygen depletion is often followed by nitrate reduction and the release of toxic sulfur compounds into the water (Whitfield 1969). At the upper location, for example, it may be too close to the barrier to have an effect. At the lower region, however, the effect may be diluted this far out from the barrier. The same trends were seen in carbon content; this is presumably due to the leaching of DOC from the barrier as a result of the woodchip medium. The barrier's woodchip medium has a noticeable effect on the sediments. The "legacy" of the barrier was detected in this way, and the buildup of phenolics and other compounds may be disadvantageous to animals. Coupled with anoxic sediment and high sulfide content, the barrier locations seem to be less ideal as a habitat for organisms.

Benthic Infauna Abundance

The fact that there were far fewer organisms found in the barrier locations at each of the three regions suggest that the barrier is having a drastic effect on the community structure present at these sites. Oligochaetes were found in all of the sites, suggesting that these organisms are highly tolerant to many conditions. In other studies, polychaetes have been shown to be one of the most tolerant of the marine organisms to hypoxia (Wu 2002). Previous studies on benthic worms have identified polychaetes to be most tolerant to stress associated with organic loading and low oxygen, and are therefore indicative of hypoxia or organic pollution (Levin 2000). Polychaetes thrived in the lower barrier region. In this way, the polychaetes may have a competitive advantage over the other types of worms.

The barrier seems to have a localized effect on the abundance and distribution of benthic infauna; nematode worms are able to thrive in the control location at the middle region, but have a hard time coping with the effects of the barrier at this location. Differences in abundance between each of the sites may be due to the effects of hypoxia as well as other accompanying stresses. The barrier seems to have a pronounced effect on benthic communities downstream by creating a less hospitable environment, affecting both the type and number of species that can live there.

Oyster Response to Hypoxic Stress

The oyster response results are less clear in depicting whether the barrier is having a harmful effect on the health of these organisms. Oysters downstream from the barrier displayed slightly higher expression of hypoxic stress. However, no significant difference in genetic expression of oyster response to hypoxia was found, suggesting that the effect of the barrier may not permeate up to higher trophic levels. The middle region displayed higher expression of the Hsp gene for both the barrier and the control, suggesting that something about this region is particularly stressful to oysters. Here, the greatest effects of hypoxia, sulfides, and poor mixing may be seen.

The results from the hypoxia-induced oysters suggest that these organisms may display a time-dependent response. A future experiment might extend the time of exposure to hypoxia to equal the amount of time that the oysters were placed out in the field (7-10 days). Oysters may need to be subjected to longer periods of hypoxia before a strong genetic response is seen. Marine mollusks may be able to withstand hypoxic conditions up to a point before a molecular response is observed. In other words, organisms such as oysters may possess a threshold level of hypoxia that they can be exposed to before genetic expression is altered. While gene expression can change in minutes, the triggering of such a response may take more time.

In addition, the primers designed for this experiment were based on sequences published for the Pacific oyster, *Crassostrea gigas*. While it is presumed that *Crassostrea virginica* behaves in the same way, this is still an assumption that needs to be explored further. The hypoxia-inducible factor (HIF 1 alpha) may be a more effective indicator of hypoxic stress, and has been found to be expressed at significantly higher levels in oysters exposed to this type of stress. For the purposes of this experiment, primers for this sequence could not be obtained, but results gleaned from a study targeting this gene may prove to yield clearer results. Other important target sequences for future study are metallothioneins, which are small, cysteine-rich heat-stable proteins involved in the cellular regulation of essential metals and in detoxification of heavy metals (David et al. 2005). A future study could look at the response of oysters to hypoxia in regard to metallothionein expression.

A Unified Look

The information gained from this study has important implications for aquatic communities, particularly in understanding the response to hypoxic conditions that occur as a result of permeable reactive barriers installed in these locations.

Hypoxic waters coming from the barrier do seem to have an effect that is detectable downstream in sediment chemistry, benthic organism abundance, and bioindicator response. Hypoxia can alter community structure and tends to decrease both species diversity and species richness in benthic communities (Wu 2002).

In addition to the organisms used to detect hypoxia in this study, other organisms may respond differently to hypoxia exposure. The results of this study could be combined with other efforts to better understand what might occur in other creatures belonging to a hypoxic barrier community. For example, during hypoxia, many benthic organisms such as polychaetes will leave their burrows and move to the sediment surface. In addition, many bivalves will extend their siphons into the water column above the sediment-water interface in order to find the more oxygenated water they need. Demersal fish may

change their feeding habitats because the diet they typically consume has become scarce, or they may change their feeding habits to feed upon stressed macrobenthic organisms that have made their way to the sediment surface. Hypoxia may cause animals to become more vulnerable to predation in their natural habitats. Hypoxia does not only affect community structure, but also has an effect on the trophodynamics of marine ecosystems.

Using oysters and other indicator species may help shed light on the extent to which animals can endure hypoxia. High mortality and detrimental effects on development and growth were found when oysters were exposed to <0.07 mg O_2 per liter for more than 24 hours. While conditions in Waquoit Bay would never reach these dramatically low levels of hypoxia due to the mixing of waters, such studies support the idea that hypoxic waters, of any kind, can have a detrimental effect on organisms that are exposed to such conditions. The effect of hypoxia seems to differ depending on organism, age of organisms, proximity to hypoxia, concentrations of oxygen, and exposure time.

CONCLUSIONS

The barrier had a significant effect on the chemistry of the sediment and the biological community downstream. In many ways, this effect was localized, with the most apparent differences taking place 6 meters away from the barrier. Both the biological and chemical parameters used in this study suggest that it is most likely hypoxic waters that are creating this observed difference. Hypoxia can heavily influence the structure of aquatic ecosystems and can thus have a critical impact on their health and future.

Molecular tools combined with biogeochemical techniques can prove useful in indicating the effect of this stress on benthic organism abundance and distribution. While permeable reactive barriers may be an effective means of removing nitrate, their benefit may come at a cost to benthic communities. Future studies should aim to continue to monitor the barrier and its effects to better understand how the results may change over time. Permeable reactive barriers should be implemented with caution; the results of this study suggest that further research needs to be conducted before applying these systems to coastal communities.

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LIST OF FIGURES

- Figure 1. Dissolved oxygen levels within and around the permeable reactive barrier.
- Figure 2. Field sampling schematic---oyster cage and sediment core locations.
- Figure 3. Sediment cores taken from the upper region
- Figure 4. Average redox potential (Eh) values for the 3 distance regions.
- Figure 5. Representative replicates: actual Eh values for readings at the middle region
- Figure 6. Total sulfur content of the middle and lower sediment regions
- Figure 7. Percent carbon composition of the upper, middle, and lower regions
- Figure 8. Diversity of benthic infauna found in sediment cores.
- Figure 9. Benthic infauna abundance (number) by region
- Figure 10. Benthic infauna abundance (percent) by sample
- Figure 11. Relative abundance of Hsp gene expression by region

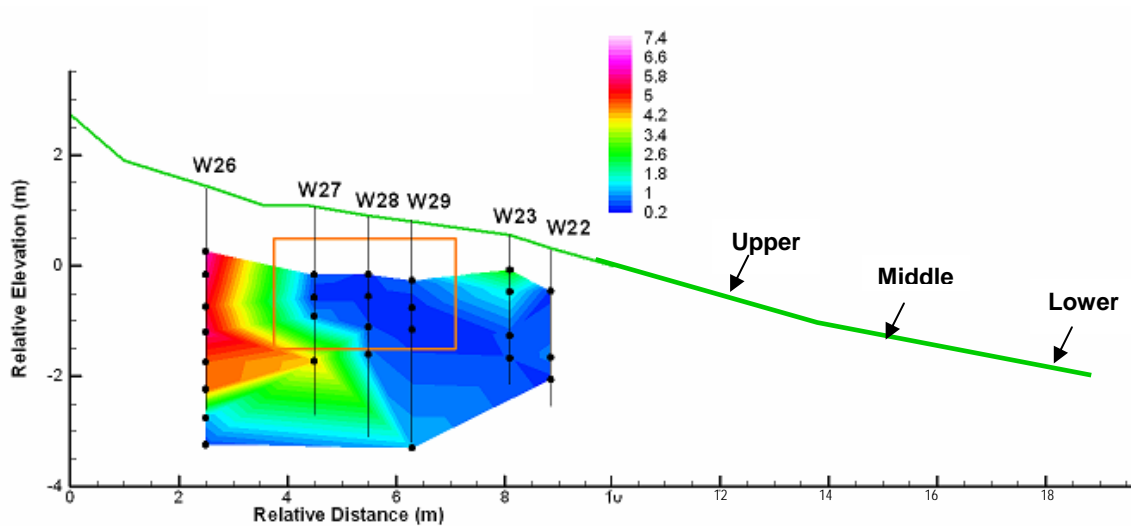


Figure 1.

Dissolved oxygen levels within and around the permeable reactive barrier.

(Neap tide: November 16, 2006) The system goes anoxic within the barrier (well 27) and continues up to a relative distance of 9 meters (well 22), the farthest well sampled for dissolved oxygen. For this study, three regions were designated downstream from the barrier (and at an identical distance away from the shore at a control location): the upper region begins at a relative distance of 12 meters (or 3 meters from the water's edge of the barrier), the middle region begins at a relative distance of 15 meters (or 6 meters from the barrier), and the lower region begins at a relative distance of 18 meters (or 9 meters away from the barrier).

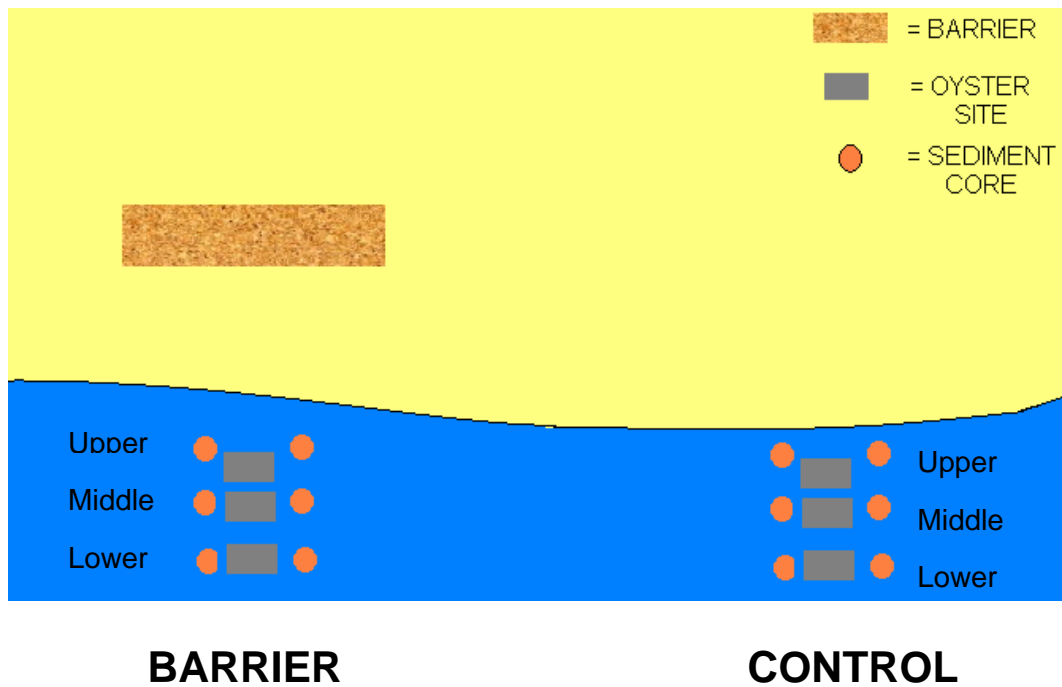


Figure 2 .

Field Sampling Schematic---Oyster cage and sediment core locations.

The barrier setup is shown to the left; an identical setup away from the barrier is shown to the right. Note the designations of upper, middle, and lower regions in order of increasing distance from the shore. The gray rectangles denote locations where oyster cages were deployed. The brown circles show where sediment cores were taken for both benthic infauna and sediment chemistry. Eh readings were taken approximately two feet from the oyster cages at each region.



Figure 3.

**Sediment Cores Taken from the Upper Region
(Control vs. Barrier)**

The photograph depicts visual differences that existed between the barrier and control sediments. These sediment cores were taken from the upper region at both the control (LEFT) and barrier (RIGHT) locations. The sediments downstream from the barrier were found to be much darker in color, primarily due to the presence of black iron-sulfides. These sulfides have been linked to anoxic conditions in aquatic systems.

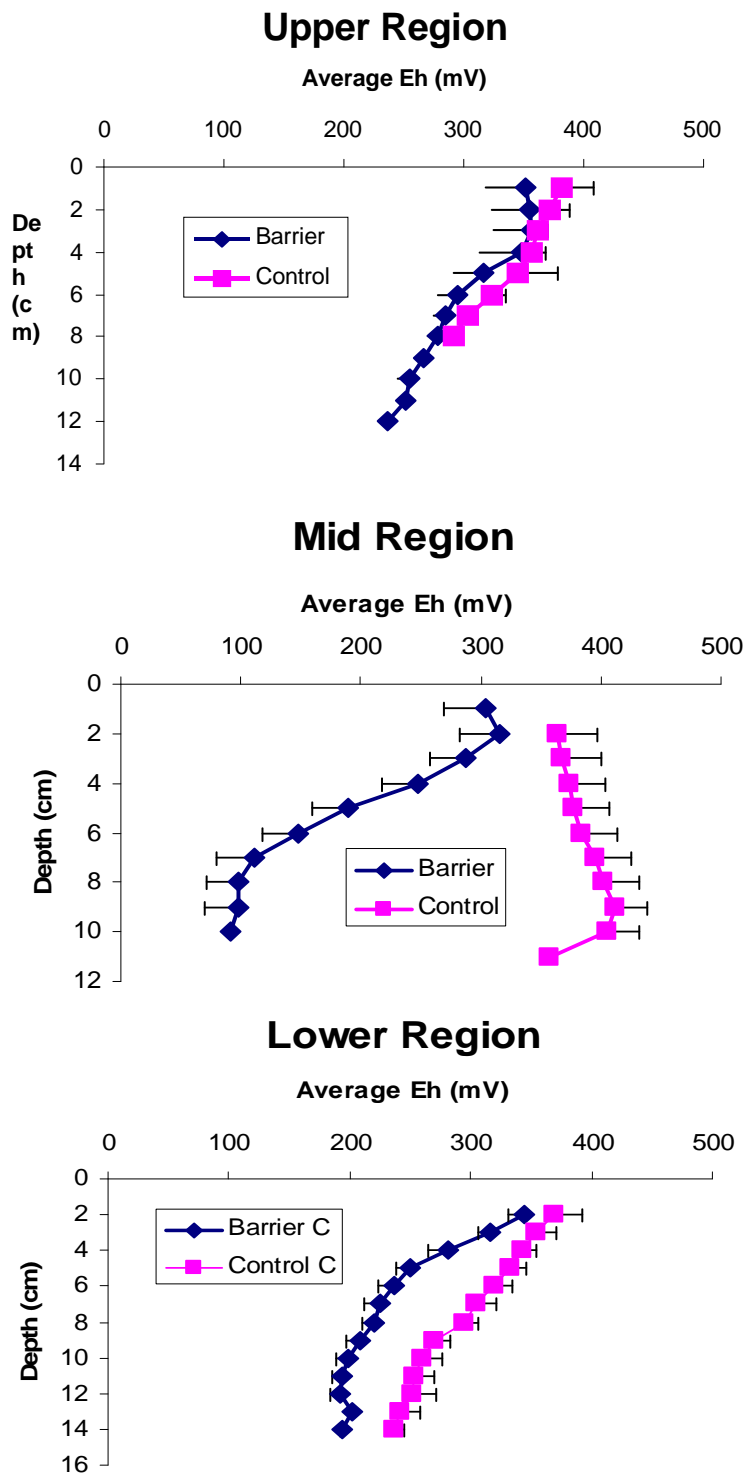


Figure 4.

Average Redox Potential (Eh) values for the 3 distance regions. (Control vs. Barrier)

For all three regions, the barrier sediments (shown in blue) tended to have lower Eh values than the control sediments (shown in pink). The most significant differences in reducing potential between the barrier and the control regions occurred in the middle region.

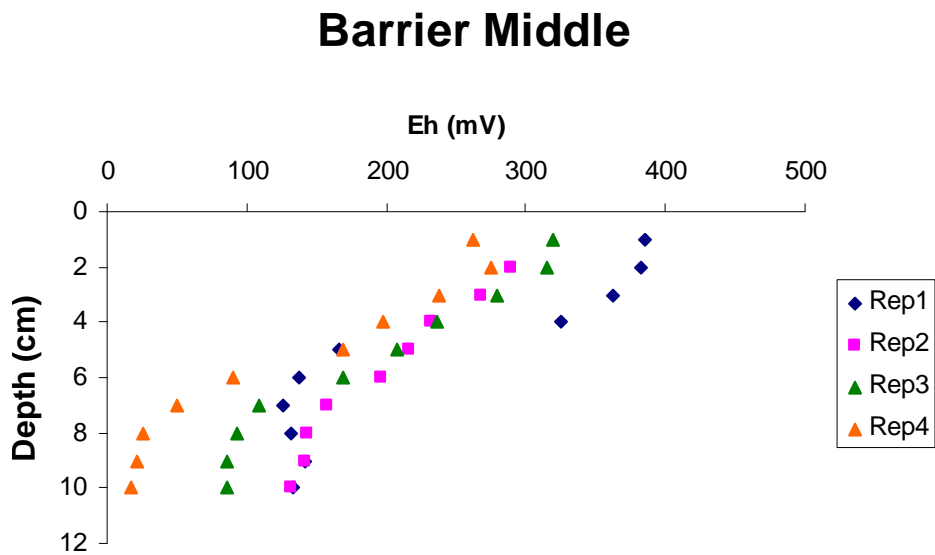
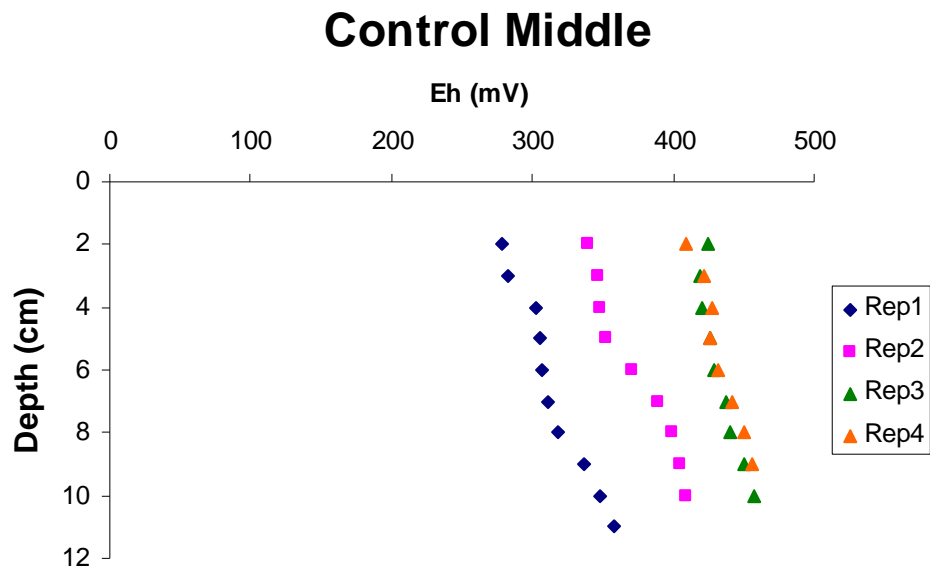


Figure 5.

Representative replicates: Actual Eh values for replicate readings at the middle region (Control vs. Barrier)

Replicates for the middle region show that there was a greater range of Eh values found in the barrier sediments than in the barrier sediments (greater variability between values at each of the different depths for the barrier). The Eh readings remained much more constant in the control regions as depth increased.

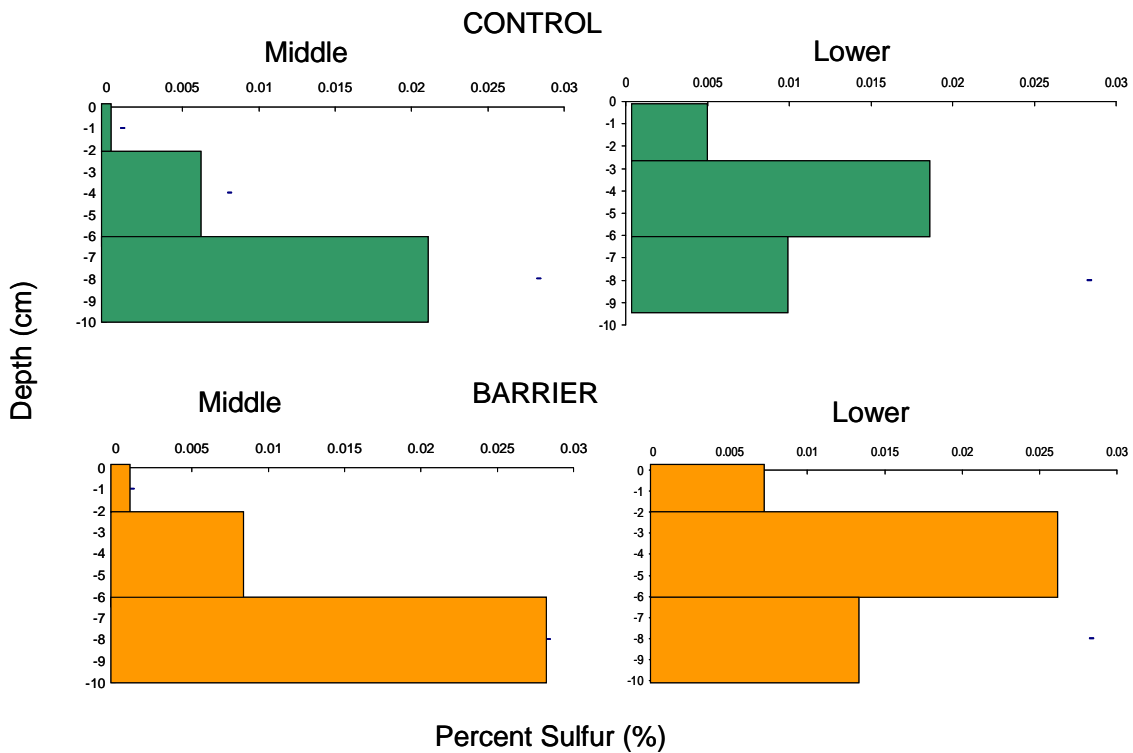


Figure 6.

**Total sulfur content of the middle and lower sediment regions
(Control vs. Barrier)**

Total percent sulfur was lower in the control sediments (top---green) than in the barrier sediments (bottom---orange) for both the middle and lower regions. Note: Sulfur readings were not detectable for the sediments found in the upper region for both the control and barrier locations. The greatest differences between the barrier and control for the upper and middle regions were found at the 2-6cm depth. The least amount of sulfur was found at the 0-2 cm depth for both locations.

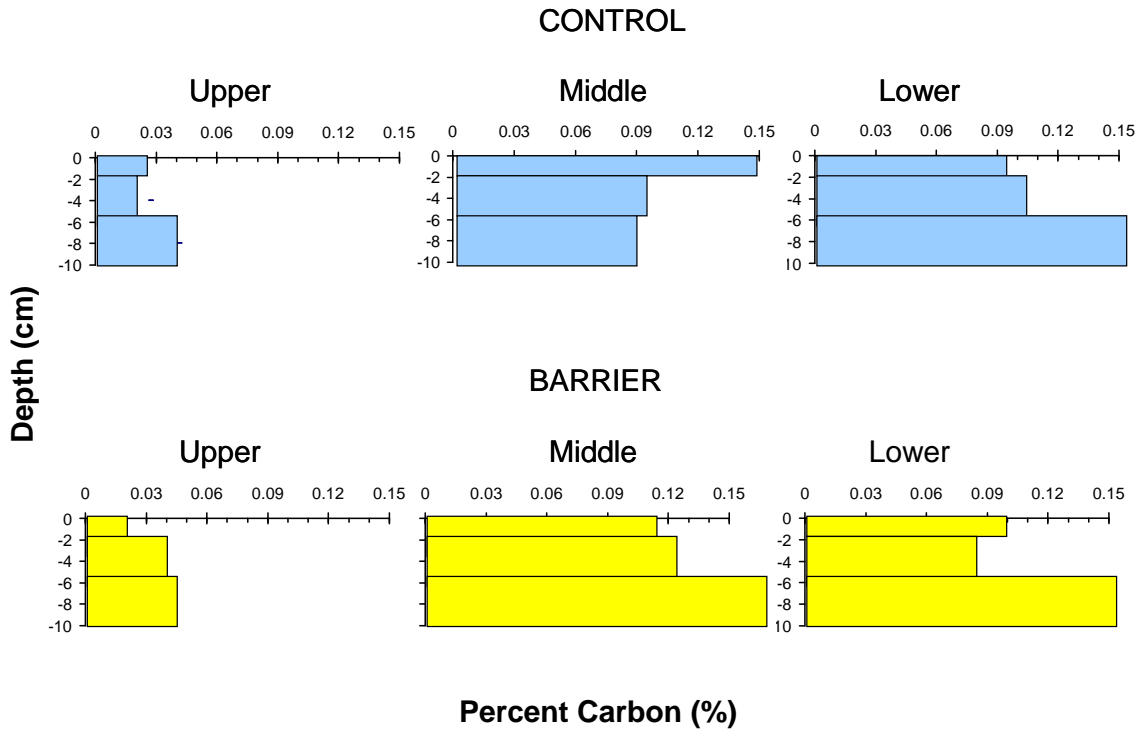


Figure 7.

**Percent carbon composition of the upper, middle, and lower regions
(Control vs. Barrier)**

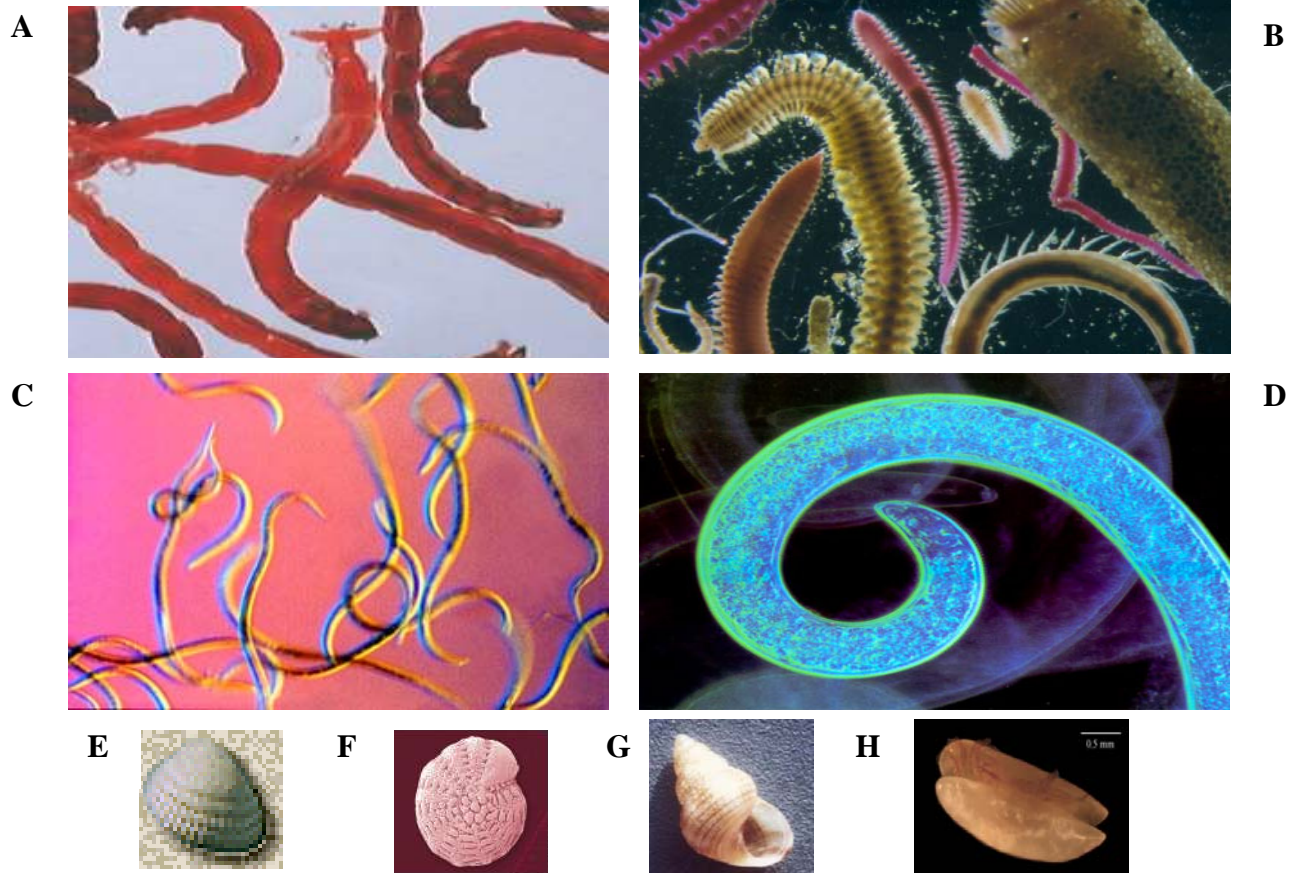


Figure 8.

Diversity of benthic infauna found in sediment cores.

- A) oligochaetes B) polychaetes C) nematodes D) nematodes
- E) small clams F) foraminifera G) snails H) ostracods

Benthic Infauna Abundance

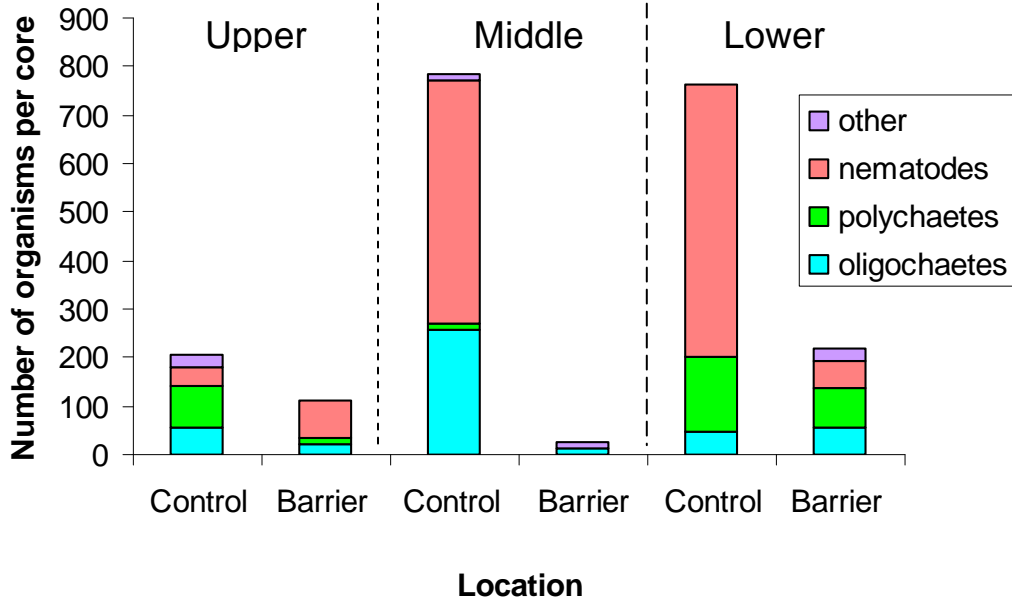


Figure 9.

Benthic Infauna Abundance by Region (Control vs. Barrier)

The barrier locations had much lower abundance of benthic infauna.
The greatest differences were seen in the middle region.

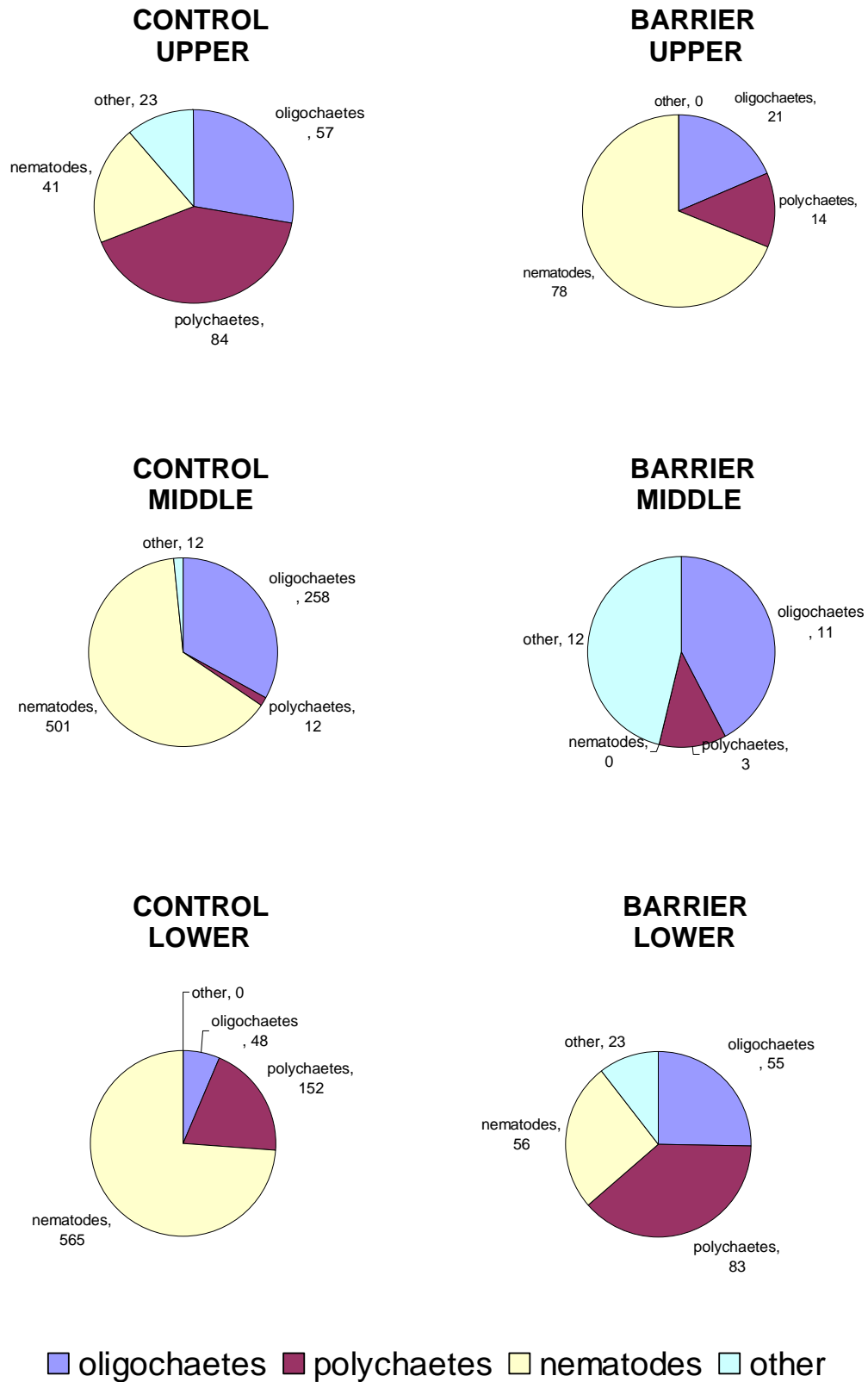


Figure 10.

Diversity of benthic infauna by sample

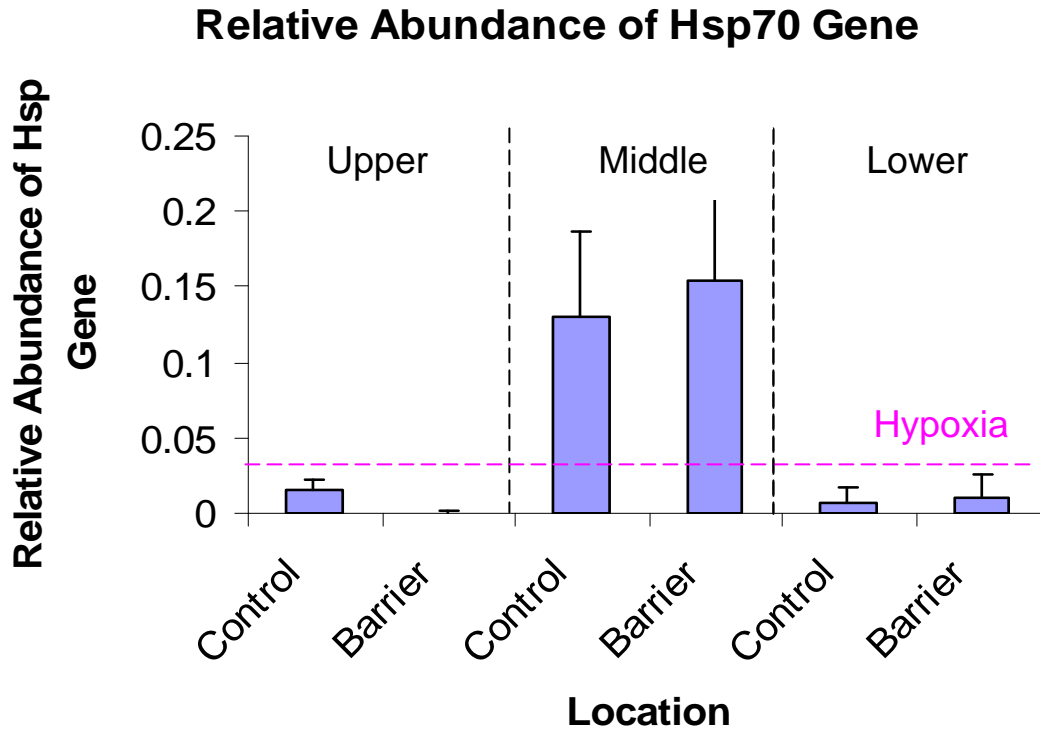


Figure 11.

Relative abundance of Hsp gene expression by region (Control vs. Barrier)

The middle region showed the highest expression of the Hsp gene. The barrier showed slightly higher expression than the control at this region. Differences between the barrier and the control locations for all three regions (upper, middle, and lower) were not found to be statistically significant. The pink dotted line that extends across the graph denotes the relative expression of the Hsp gene in the oysters that were exposed to 12 hours of hypoxia under the conditions outlined.