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A METHOD FOR EXPOSING LOBSTERS TO MULTIPLE SIMULATED HABITAT BIOGEOCHEMICALS AND TEMPERATURES

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ABSTRACT A flow-through experimental system was designed to simulate habitat conditions encountered by American lobsters (*Homarus americanus*) during an eutrophication-driven mass mortality in the Long Island Sound during 1999. Seawater for the system was chemically conditioned through gas exchange and the addition of inorganic salts, resulting in simultaneous control of multiple environmental variables including: temperature (±0.5°C), dissolved oxygen (±0.3 mg L^{-1}), sulfide (±1 μM) and ammonium (±3 μM). The system consisted of eight gas-tight, 280-L tanks, each capable of accommodating 22 lobsters, supplied with 0.4 L min^{-1} $animal^{-1}$ of conditioned seawater. Outflows were fitted with ozone and ultraviolet sterilization so that lobsters could be exposed to infectious pathogens in varying doses to study effects of habitat on disease resistance, without contaminating the environment. Shelters are supplied in excess and lobsters utilizing them may be monitored to observe behavioral and physiologic responses without opening the tanks. With minimal alterations this system design can be applied to species with diverse structural requirements and to a wide range of ecologic issues including growth, survival and disease resistance under simulated habitat conditions.

KEY WORDS: lobster, habitat, *Homarus americanus*, temperature, hypoxia, sulfide, ammonium

INTRODUCTION

The mass mortality of lobsters in western Long Island Sound (LIS) in 1999 was ascribed to a number of possible causes including the effects of temperature, hypoxia and associated biogeochemicals on the resistance of lobsters to a pathogen (Pearce & Balcom 2005). Seasonal increases in temperature, hypoxia, sulfide, and ammonium (Cuomo et al. 2005) resulting from cultural eutrophication (Parker & O'Reilly 1991) subjects the benthic fauna of western LIS to stressful conditions. The combined effect of these habitat variables on the resistance of lobsters to pathogens needed to be determined. In this article we describe the design and operational characteristics of a multivariable system built to simulate the biogeochemistry of lobster habitat in western LIS in support of experiments to investigate whether habitat may have been a factor in the mass mortality. The actual values and results for the experiments with introduced pathogens are discussed in Robohm et al. (2005); elevated temperatures are discussed in Draxler et al. (2005).

METHODS

The multivariable system was constructed at the James J. Howard Marine Sciences Laboratory on Sandy Hook, New Jersey. The large seawater laboratories provided an ample supply of ambient, heated, and chilled seawater (Olla et al. 1967, rebuilt in 1994) allowing the design to accommodate a large number of market-size lobsters in a flow-through system, which maintains higher water quality than a recirculating system (e.g., Cornick & Stewart 1977).

Water Supply Facility

The Howard Laboratory operates a seawater source drawn from four intakes imbedded in the floor of Sandy Hook Bay at salinities that generally range from 25–27 psu. Because the intakes are buried 2.5 m under course sand, pebble and crushed shells, most suspended matter and fouling organisms are removed. Seawater is pumped from the bay at 1,325 L min^{-1} to an aeration tank, which is followed by a decantation tank to remove precipitated iron, and finally to a deaeration tank before leaving the seawater pump house. On entering the laboratory building, the seawater passes sequentially through three large open-bed biologic filters, filled with gravel and sand. Separate streams of ambient seawater are then heated or chilled, and along with ambient, distributed to seawater laboratories.

Experimental Habitat Simulation System

The exposure system was divided into two separate sections to provide two closely controlled temperatures at the same time. Seawater from the laboratory supply at three different temperatures was manually mixed with 2-in PVC gate valves to within 1°C of the experimental requirements as it entered 675-L polyethylene reservoirs (Aquatic Eco-Systems, Inc. or AESI, Apopka, FL). Gate valves were chosen over ball valves because their mechanism provided finer and more accurate flow control. The large reservoirs provided temperature control and flow maintenance during brief water supply interruption (<26 min). Two 10-cm coarse air stones supplied with oil-free air aerated and circulated the water to maintain a uniform temperature throughout the reservoir tanks. Further agitation and temperature control was provided by magnetically driven pumps (110 L/min Danner Mag Drive pump, AESI), which moved water from the reservoir to a 1.8-kW in-line chiller with titanium coils (Delta Star, Aqua Logic, AESI), and a Hayward 11-kW in-line, nickel-element heater capable of 45–227 L min^{1} (Hayward Heaters, AESI) on separate loops (A in Fig. 1). The mechanisms of both the chillers and heaters do not engage until the temperature deviates by at least 0.5°C from the programmed temperature. Water was pumped continuously through the temperature control devices regardless of their state of operation.

Tempered water from the reservoirs was pumped (Iwaki magnetic drive pump, AESI) via 1-in vinyl tubing (FDA-approved Vinyl tubing, AESI) through a 1-in gate valve to control flow to twin 160-L clear fiberglass gas exchange columns (29 cm dia. × 2.4 m high, Florida Aqua Farms Inc., Dade City, FL). Seawater from the reservoir was pumped to the gas exchange columns in excess, where the level was maintained by overflows that recycled water back to the reservoir tanks. This ensured constant head pressure to the exposure tanks while providing additional mixing in the reservoirs. As water descended through the columns, dissolved oxygen concentrations were adjusted by counter-current gas flow.

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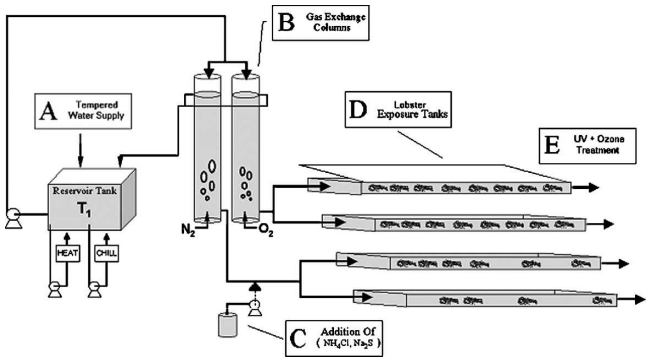

Figure 1. One half of the flow-through system for simulating the biogeochemical habitat of lobsters. Manually tempered seawater enters the reservoir tank (A) where temperature is controlled to within 0.5°C. It is then pumped to the gas exchange columns (B) where dissolved oxygen and carbon dioxide can be adjusted to the desired concentration. If sulfide or ammonium is required, aqueous solutions of inorganic salts are then injected (C) before the seawater enters the exposure tanks (D). Water exiting the tanks (E) is sterilized with ultra violet light and ozone.

In one column of each pair, oil-free air supplied dissolved oxygen to saturation, whereas in the other column, commercial grade nitrogen (Air Products, Manalapan, NJ) stripped oxygen to the desired level. Pressure regulators and micro metering valves achieved the critically sensitive control of nitrogen gas flow from cylinders. Introduction of gasses as fine bubbles to provide adequate surface area on the bubbles for gas exchange was accomplished by diffusers (Pyrex Hirsch-Type Filter Funnel, Fisher Scientific, Agawam, MA) just above the bottom of each column above the point where conditioned water exits the column (B in Fig. 1). Each gas exchange column provided sufficient water to supply two exposure tanks. It was possible to include a third 280-L exposure tank on a single column but maintaining control of flow rates, and hence, sulfide and ammonium levels became more difficult, requiring more frequent monitoring and manual adjustments.

Water from gas exchange columns was directed through 3/4-in vinyl tubing (AESI) to PVC sampling ports where pH, dissolved oxygen (DO), and temperature were measured before concentrated solutions of sulfide and ammonium were introduced. Sampling ports consisted of a 3/4-in PVC Tee fitting, a 3/4-in ball valve, and a 3/4-in to 2-in reduction fitting to directly accommodate a DO probe and drawing tube for discrete samples. Fine adjustments in flow rates were made with gate valves and monitored with upright, in-line flowmeters (Ultrapure, 1.8–18 L min^{-1}, Cole-Parmer, Vernon Hills, IL) calibrated with catch bucket measurements at each system outlet.

Next, metering pumps (LMI Solenoid diaphragm type, Cole-Parmer, Vernon Hills, Illinois) injected concentrated aqueous solutions of sodium sulfide ($Na_2S{\cdot}9H_2O$) and ammonium chloride into the system (C in Fig. 1). Target concentrations of chemicals were calculated taking into consideration the metering pump injection rate (speed and stroke) and assuming a nominal system flow rate of 9.5 L min^{-1} for each tank to achieve a desired dilution and final concentration to which the lobsters were exposed. Concentrated sulfide and ammonium solutions were mixed in, and then injected from, 9-L Pyrex bottles (Thomas Scientific, Swedesboro, NJ). This volume supplied the system for 6–16 h depending on the pump settings and concentration of solution.

To facilitate temperature and flow control, the fiberglass exposure tanks (280 L, 198 × 84 × 17 cm; Gemini Fabrication Co., Denver, CO) were plumbed with equal lengths of 3/4-in vinyl tubing (D in Fig. 1, Fig. 2). Water entered the tanks through two 3/4-in inlets (dispersed by vertical baffles of PVC sheeting), traveled the length of the exposure tank subjecting lobsters to the selected conditions, and exited through two 3/4-in outlets. Before going to waste, used seawater was treated with ultraviolet and ozone sterilization to remove pathogens and reduce excess sulfide. Lids were constructed of 12-mm plexiglas sheets, reinforced with a frame of pressed fiberglass angle stock (Structural Fiberglass Inc., Bedford, PA). Gaskets of neoprene foam were glued to the 1-in lip around the top edge of the fiberglass tanks to form a watertight seal between the tank and lid. Bolts were attached to the lip of the tank so the lid could be tightened securely down with wingnuts. The outlet end of the tank was elevated 1.5 cm above the inlet end to facilitate purging the tanks of gas bubbles during sealing. Because these exposure tanks were intended specifically for lobsters, they were equipped with an excess number of shelters in which the lobsters could take refuge. Equal sized partitions (19 × 14.5 cm) of 1/8-in PVC sheeting were arranged vertically about 15 cm apart along both sides for the full length of each tank (occupying 51% of the area). Each shelter had a volume of 5,600 cm^3 with a 3-cm gap along the back and 1.25 cm gaps along the top and bottom that allowed seawater to pass through the shelters, exposing the lobsters without creating circulation dead spots (Fig. 2). Two removable PVC sheets (25 × 198 cm) were placed on top of the clear lids to complete the shelters. These removable covers excluded light from the shelters while permitting close observations of the lobsters. Experiments were conducted under subdued lighting (1.05×10^{-5} μE m^{-2} h^{-1}), with a daily cycle of 12 hours on (0700 h) and 12 hours off (1900 h).

Analytical Methods

After an initial trial period of frequent observations, we found we could manually control system variables with three samplings per day. We also monitored the consistency of seawater flow rates, as well as rates of reagent usage. The following analytical methods were used in monitoring the environmental conditions to which the lobsters were exposed.

Dissolved oxygen concentration in the system was monitored by both Winkler and electrometric methods. The DO meter (YSI model 550, Yellow Springs, OH) permitted frequent, convenient and rapid determination of all critical DO concentrations in the system. During the three daily samplings, readings were made at sampling ports after each gas exchange column and at the outlet of each exposure tank. The instrument was calibrated in water-

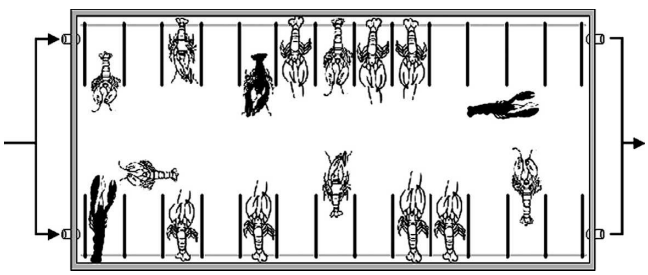

Figure 2. Plane view of exposure tank showing position of treated seawater inlets and outlets, shelter partitions with 3 of 16 lobsters dead (black) in this example.

saturated air according to the manufacturer's instructions. Two additional checks were made to ensure accuracy. At the beginning and conclusion of each trial, a zero reading was confirmed for the YSI electrode in DI water sparged with nitrogen (DO undetectable), and once per day DO concentrations were measured by the Azide Modification of the Winkler procedure (Standard Methods 1995) using commercial reagents, standards and titrant (Hach Chemical, Loveland, CO) and a motorized burette (Dosimat 665, Metrohm Ltd., Herisau, Switzerland).

Sulfide concentrations were determined colorimetrically (Strickland & Parsons 1972) on 55-mL samples drawn from the outlet of each tank. When the target sulfide concentration was met and stabilized (about 30 min), measurements were made 3 times per day. Samples were treated with *p*-phenylenedimine followed by a ferric chloride solution, which developed a purple color, and the absorbency was measured on a spectrophotometer (Hewlett Packard 8452a, Palo Alto, CA) at 600 nm in a 1-cm cell, 0.3-μM limit of detection. Sulfide concentrations in the sulfide-ammonium concentrate (diluted 1:1000 with DDI water) were analyzed as in the seawater procedure.

Ammonium analyses were preformed on 60-mL samples (collected in parallel to sulfide samples) by ion chromatograph (Model DX500, Dionex, Sunnyvale, CA) using a CS16 cation column, 18 mM H_2SO_4 eluent at 1 mL min^{-1}, and 300-μA ion suppression. Seawater samples were diluted 1:10 with DDI water to prevent sodium from saturating the analytical column and lithium was added as an internal standard. The limit of quantitation (LOQ) in seawater is high (8 μM), but the method is a good choice in this application for a number of reasons including: simplicity of daily startup, stable and relatively nonaggressive chemicals compared with phenol-hypochlorite methods (e.g., Liddicoat et al. 1975), internal standardization, and absence of sulfide interference. Control of ammonium exposure concentrations was ensured by other factors. Initial trials using 80 μM ammonium showed that it behaves conservatively in the system so accurate dilution of a concentrate in the flowing seawater will produce the target concentrations. Ammonium chloride salt for each batch of sulfide-ammonium concentrate was weighed and the ammonium concentration determined. Analysis is straightforward at the higher concentrations (to ~30 mM) and the LOQ in DI water is lower (<3 μM). Rates of concentrate usage and seawater flow are then monitored and controlled to achieve the desired exposure concentration.

Values of pH were measured (O'Reilly & Thomas 1983) using a hand-held meter (pHep5, Hanna Instruments, AESI). A two-point calibration was performed daily according to the manufacturer's instructions by submerging the probe in pH 7.0 buffer until the reading stabilizes, then in pH 4.0 buffer. The instrument was then equilibrated for a minimum of 30 min in ambient seawater before it was ready for measurements.

Temperature was recorded continuously in the room, reservoirs, gas exchange columns and exposure tank outflows by a system wired to a desktop computer (DAQ System, Ocean Optics, Dunedin, FL) via type T thermistors (Ocean Optics, Dunedin, FL). Room temperature was recorded in 500 mL of water at tank height. All thermistors were calibrated relative to an NIST traceable thermometer.

OBSERVATIONS

From September 2002 through February 2004 we conducted sixteen experiments ranging in duration from 3 to 21 days, depending on the lethality of exposure conditions. Lobsters used in our experiments were caught by commercial fishers from eastern Long Island Sound and were acquired from Garbo Lobster Company (Groton, CT). They ranged in weights from 450 to 560 g and were both males and females, but no gravid females were used. The animals were acclimated (<1°C day^{-1}) to within 1°C or less of the target temperature before starting an experiment. Lobsters were assigned randomly to exposures, and all were introduced the same way and by placing them in the same part of the tank. Lobsters were outfitted with a numbered poultry tag (Nasco Farm and Ranch, Fort Atkinson, WI) on the right cheliped between the carpopodite and meropodite segments for identification.

Control of all experimental variables (except sulfide, see later) was established rapidly. The required flow of 40 L min^{-1} for each side of the system required substantial heating and cooling capacity to control the system. With the 1.8 kW chiller we found that we were able to overcome a difference of slightly more than 1°C between the inlet water temperature and the reservoir target temperature. For example, if the target temperature was 19.5°C, the manually mixed inlet water temperature could be 20.5°C. To achieve the same control at lower temperatures (<16.5°C) as we did at higher temperatures (>19°C), we found that we had to increase the size of the heater to the 11 kW unit.

Target dissolved oxygen values were easily met and maintained in the system. We found that very slight manual adjustments in the settings of the micrometering valve were sufficient to increase or decrease the concentration of DO supplied to the exposure tanks by ±0.05 mg L^{-1}. DO control was ± 0.3 mg L^{-1} across all treatments (1.5–6.4 mg L^{-1}). With the DO probe inserted in the sampling port, we found the response time for an adjustment in the column was <1 min.

Analysis of water samples from the inlet and outlet of the exposure tank revealed a sulfide gradient lengthwise through the tank with a loss of 2 μM in our first set of conditions (19.5°C, 3 mg L^{-1} DO, 20 μM sulfide, 80 μM ammonium). Averages of the inlet and outlet values were linearly related, allowing us to calculate a tank average along this gradient (mean sulfide = 1.0884 (outlet sulfide) + 0.5913), providing a value of sulfide in the center of the tank by sampling from the outlet (Fig. 3). Using this formula we recalculated the concentration of the aqueous solution being metered into the system to achieve the desired sulfide exposure to ±1 μM.

When the experimental design called for higher temperatures and more severe hypoxia, sulfide values in the system changed

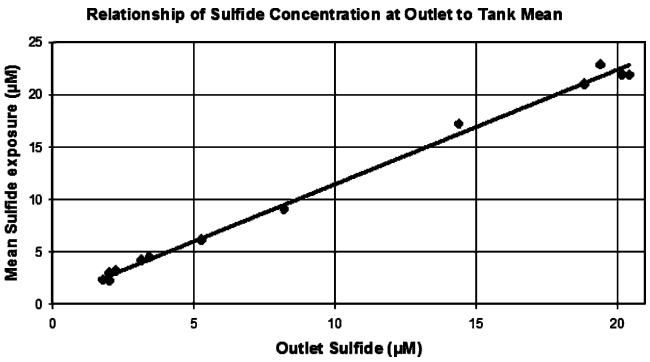

Figure 3. Mean sulfide concentration in the exposure tank as a function of outlet concentration. Target values for each tank were achieved using outlet concentrations and regression relationship [mean tank sulfide] = 1.0884 [Outlet sulfide] + 0.5913 to adjust the amount of sodium sulfide metered into the inlet seawater.

further. At temperatures >22°C and in combination with low dissolved oxygen (<2 mg L^{-1}), long, light brown filaments of microaerophilic chemotrophs grew in the tubing downstream of the sulfide injection, presumably generating energy by oxidizing the sulfide. This resulted in a loss of sulfide in excess of 2.5 μM from inlet to outlet as the water passed through a tank. When experiments were run long enough, the filaments reached the exposure tanks. This appears to be a partially self-compensating phenomenon, as lobsters survived shorter times as sulfide and hypoxia increased, and did no apparent harm to the lobsters.

The system was designed to control pH by either altering the composition of the metered solution or by adding CO_2 to N_2 streams in the gas exchange columns. However, early trials demonstrated a very small difference, both among treatments and from inlets to outlets, with a range of pH for all 16 experiments of 7.2 to 7.7. This 0.5 variation in pH was most likely due to seasonal variation in the water chemistry of the seawater pumped in from the bay.

DISCUSSION

The system described here has a high level of inherent stability. Control of most variables was achieved within a few days of initial assembly and required about a half hour from the startup for each experiment. The direct control achieved with this approach successfully simulates hypoxic habitats. It has the advantage that it does not depend on allowing respiration to decrease DO (e.g., McLeese 1956) with the attendant accumulation of biogeochemical artifacts. For practical reasons, experiments on lobsters (Draxler et al. 2005, Robohm et al. 2005) were conducted at unnaturally high densities. This was partially compensated for by the provision of an excess of well-ventilated individual shelters, high water flow rates to prevent the accumulation of signaling chemicals (e.g., Bushmann & Atema 1997), metabolic chemicals, and by carefully tracking individual lobster locations within the tanks to discern biased results for either down- or upstream individuals. Once in the tank, virtually all the lobsters quickly found refuge in a shelter, suggesting that the partitions were successful in reducing unintended stress and increasing the lobster's comfort within the tank environment. Experiments at more natural densities are planned using exactly the same biogeochemical controls but in a much larger experimental tank.

Though it has not yet been used, a means of controlling pH can be critically important, because it is the master variable in determining the degree of ionization of both ammonium and sulfide (Whitfield 1978, Millero et al. 1988). The degree of ionization affects toxicity (Burrows 1964). For ammonium, as pH increases (hydronium ion concentration decreases), the amount of unionized ammonium increases. Unionized species can more readily traverse membranes, so higher pH values result in increased ammonium flux and, all other conditions remaining equal, higher ammonium toxicity (Vismann 1996).

In the experiments on lobsters, we accepted the uncontrolled pH of the system for another reason. It was not clear what other pH value might have been chosen. Carbonate equilibria dominate seawater buffering and microbial respiration in the sediment produces carbon dioxide, which can accumulate, lowering the pH to <5 (Stumm & Morgan 1996). Like sulfide and ammonium, carbon dioxide also diffuses from the sediment into the overlying water, generating a decreasing CO_2 gradient away from the sediment-water interface. The pH observed at the interface is a function of multiple variables (CO_2 production, diffusion rate which depends on temperature, CO_2 concentration gradient, sediment porosity, etc.) so that a useful estimate of the pH to which an epibenthic organism is exposed to is difficult to make. For the large-scale experiments planned, and for which the system was designed, we chose to accept the very narrow range (0.5 pH units for all 16 experiments conducted) that resulted naturally due to seasonal changes in the bays water chemistry, and leave the variation of habitat biogeochemical toxicity to lobsters as a function of pH to experiments dedicated to that variable.

We have successfully used this system to study survival of lobsters exposed to environmental biogeochemicals (Draxler et al. 2005) and the resistance of lobsters to pathogens (Robohm et al. 2005) under simulated habitat conditions of Long Island Sound. The approach would be applicable to a variety of other ecologic problems with easy modifications to accommodate the structural requirements of other species (system size, flow, water chemistry). For example, as more areas of the marine environments experience seasonal hypoxia (e.g., Gulf of Mexico, Rabalais et al. 1996, Grantham et al. 2004) it will be important to determine responses of fisheries species to these events, including blue crabs living in Chesapeake Bay and shrimp in the Gulf of Mexico. We have also used this approach to determine avoidance thresholds of sediment biogeochemicals in flatfish (Draxler & Siclare 2000). It could be applied to explore effects of habitat chemistry on fish growth (*cf.* Bejda et al. 1992), clearly separating hypoxia from effects of other biogeochemicals, to physiology by utilizing a modified heart monitor (Jury & Watson 2000), or to diseases beyond *Aerococcus viridans* var. *homari* (causing gaffkemia or red tail disease, Stewart 1980), such as chitonoclasia.

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