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EFFECTS OF ENVIRONMENTAL STRESSORS ON DISEASE SUSCEPTIBILITY IN AMERICAN LOBSTERS: A CONTROLLED LABORATORY STUDY

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ABSTRACT The objective of this work is to determine whether increased (but environmentally realistic) temperature, hypoxia, sulfide and ammonium, alone or in combination, can increase susceptibility of lobsters to microbial infection. Lobsters from eastern Long Island Sound (LIS) were injected with *Aerococcus viridans* var *homari,* a pathogen that causes a disease known as gaffkemia. Injected animals (and controls) were placed in a flow-through seawater-system with mechanisms for control of temperature, dissolved oxygen, sulfide and ammonium levels as well as disinfection of effluent. Exposure variables included 0.1 mL injections of *A. viridans* at doses of 1×10^3 and 1×10^6; dissolved oxygen at 2.5–6.3 mg L^{-1}; sulfide at 0–21 μM; ammonium at 0–80 μM and temperatures at 14.5 and 19.5°C. The criterion for stressor effect was the time at 50% survival in each set of 15 to 22 lobsters per treatment variable. Also, at regular time intervals, lobster hemolymph and hepatopancreas tissues were analyzed for bacterial levels. When lobsters were held under normoxic conditions at 19.5°C, rates of death from gaffkemia were accelerated in the presence of sulfide above 4 μM. When lobsters were subjected to moderate hypoxia (3 mg L^{-1}), death rates were accelerated regardless of the presence of sulfide. Exposure to ammonium up to a level of 80 μM had no effect on death rates. Bacterial counts were similar in lobsters regardless of exposure to stressors. The geometric median count in the hemolymph for all lobsters infected beyond 3 days was 7.7×10^8 ml^{-1} (maximum raw value 1.6×10^9 ml^{-1}) and that for hepatopancreas was 7.7×10^7 g^{-1} (maximum raw value 1.1×10^9 g^{-1}). Our work showed that, at 19.5°C (a peak, summer, bottom-water temperature routinely found in Long Island Sound), relatively moderate levels of hypoxia as well as sulfide in the absence of hypoxia may accelerate deaths in lobsters that are infected with a pathogenic bacterium. Because eutrophication may lead to hypoxia and increased sulfide levels, policies that reduce eutrophication may improve lobster health.

KEY WORDS: lobster, *Homarus americanus,* sulfide, hypoxia, ammonium, *Aerococcus viridans*

INTRODUCTION

In the autumn of 1999 extensive mortalities occurred among American lobsters (*Homarus americanus* H. Milne Edwards, 1837) in western Long Island Sound (LIS) and the US Secretary of Commerce declared the Long Island Sound lobster fishery a marine resource disaster under the Magnuson Stevens Act. Estimated mortalities for this event were between 0.6 and 5.9 million lobsters (P. Howell, Connecticut Department of Environmental Protection, Marine Fisheries Division, pers. comm.).

Histopathology, hematology, microbiology, virology, parasitology and toxicology studies of lobsters taken shortly after the peak of mortalities found, with one exception, no common microbial pathogen and no identifiable toxicologic relationships. The one exception was the presence of a parasitic amoeba, usually associated with systemic inflammation, but primarily affecting the nervous system in many lobsters (French et al. 2000, Russel et al. 2000, Mullen & Frasca 2002). Attempts to culture the organism *in vitro* were unsuccessful (Russel et al. 2000). Evidence for a parasitic amoeba species as the cause of lobster mortalities is not clear. French (pers. comm.) and Shields (2002) found amoebae in lobsters having no clinical symptoms; also, in some tissues of infected lobsters, inflammation was absent even though amoebae were present. Shields (2002) found that the severity of pathology in the eyes of lobsters was not correlated with the intensity of amoebic infection in the optic nerves. A hypothesis proposed at The Long Island Sound Lobster Health Symposium in April 2000 suggested that environmental stressors may have impaired the immune responses of lobsters to the parasitic amoeba and contributed to lobster deaths.

Seasonal, bottom-water, biogeochemical conditions could contribute to increased stress on the lobster population in western LIS. These conditions are a result of eutrophication in proximity to the New York City metropolitan area. The organic matter believed to result from the nutrient-stimulated planktonic overproduction sinks (Parker & O'Reilly 1991), creating a biological oxygen demand in near bottom water and sediment. Hypoxia alone affects lobster survival (McLeese 1956, Dias & Rosenberg 1995). Seasonal declines in dissolved oxygen result in secondary changes in the biogeochemistry of lobster habitat. Under hypoxic and anoxic conditions, nitrogen in the organic matter is released as physiologically active ammonium (Froelich et al. 1979). In addition, after oxygen is depleted, the heterotrophic benthic microbes use a sequence of terminal electron acceptors (including SO_4^{2-}) to oxidize organic material; when sulfate is reduced to sulfide it becomes biologically active (Wang & Chapman 1999). Lobsters in habitats enriched with organic matter may then be exposed to hypoxia, sulfide and ammonium, simultaneously.

Valente and Cuomo (2005) recently found sulfide concentrations as high as 11 μM and ammonium as high as 12 μM in LIS bottom water. Dissolved oxygen levels in bottom water of LIS recorded by the Connecticut Department of Environmental Protection in August of 1999 revealed levels of 0.0–1.0 mg/L in the extreme western end of LIS, 1.0–2.0 mg/L in an adjacent portion, and 2.0–4.0 mg/L in a wider portion of western LIS (Anonymous 1999). Although the lethal oxygen level in lobsters is believed to be in the range 0.2 mg/L at 5°C to 1.2 mg/L at 25°C (Cooper & Uzmann 1980, summarized from McLeese 1956), it is not known whether exposure to slightly higher, survivable, dissolved oxygen as well as the upper levels of sulfide and ammonium may effect lobster susceptibility to infections.

Meteorological and hydrographic data for LIS in 1999 showed that higher than normal temperatures had occurred in the bottom water in 1999. While American lobsters have a broad temperature

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tolerance (from −1°C to 30.5°C), they have been shown to avoid temperatures in the range of 19°C to 23.5°C (Crossin et al. 1998). Bottom-water temperature at one site in western LIS exceeded 22°C in September 1999; whereas the mean August–September temperature for the decade was about 20°C (Wilson et al. 2001).

Studies of biogeochemical effects on immune-system depression and disease in lobsters are nearly absent in the scientific literature. *In vitro* phagocytosis of a bacterial pathogen, *Aerococcus viridans,* is reduced in lobsters when temperatures are at 22°C and higher (Steenbergen et al. 1978) and there is some indication that stress may be associated with shell disease in crustaceans. Shell disease is contagious in lobsters held in tanks of running seawater (Taylor 1948), it is associated with inadequate diets in juvenile lobsters (Fisher et al. (1976), and it coincides with low serum antibacterial activity (an indication of immune-system depression) in other crustaceans such as blue crabs (Noga et al. 1994).

One of the seven recommendations in the pathology/toxicology section of the LIS Lobster Work Plans (2000) is to conduct controlled laboratory studies to determine whether known environmental stressors can increase susceptibility to microbial pathogens. The following study directly addresses this recommendation. The gram positive, coccoid bacterium, *A. viridans,* seems to be endemic to natural populations of both American and European lobsters (Stewart & Cornick 1967). It causes an infection termed "gaffkemia." We tested 4, interrelated, habitat-quality variables (hypoxia, sulfide, ammonium and temperature) for affects on mortality rates in lobsters infected with this bacterium.

We show here that sublethal levels of sulfide, hypoxia and temperature (habitat quality variables associated with eutrophication in the western portion of LIS) increase the susceptibility of lobsters to *A. viridans* infection.

METHODS

Experimental Animals

Lobsters used in the experiments were caught by commercial fishers from eastern LIS; except that in one of six experiments, because of insufficient numbers available from LIS, one-third of the lobsters in each treatment group was from Rhode Island Sound (RIS). Holm-Sidak, pair-wise, log-rank statistical comparisons of the survival times in the two groups found no significant difference in survival times in five of six comparisons (the survival time in one RIS group was one day shorter than its counterpart from LIS). Therefore, LIS and RIS lobsters were treated as one population for this experiment. All animals were acquired from Garbo Lobster Company (Groton CT). Lobsters ranged in weights from 450 to 560 g and were both males and females in about a 40 to 60 proportion, respectively. Visibly gravid females were not used in any experiments. Claw-banded animals were slowly acclimated (<1° ΔC/day) to within 1° or less of the target temperature before starting an experiment. Lobsters were assigned randomly to exposures, and all were introduced in the same way and in the same part of each tank in the experimental system.

Experimental System

A flow-through experimental system at the J. J. Howard Marine Laboratory, Highlands, New Jersey was used throughout the study. A complete description of the system is provided by Wieczorek and Draxler (2005); however, a brief description follows. The system consisted of eight gas-tight, 250-L tanks, each capable of accommodating 26 lobsters and supplied with 0.37 L min^{-1} $animal^{-1}$ of conditioned seawater. Outflows were fitted with ozone and ultraviolet sterilization to prevent dissemination of a lobster pathogen used in the study. Individual refuges were supplied in the tanks to minimize social stress. Water for the system, with a salinity from 25 to 27 psu (a range similar to the bottom salinity of western LIS) was pumped from Sandy Hook Bay, New Jersey through coarse sand, pebble and shells; followed by aeration, decantation and passage through three, open-bed biologic filters. Seawater was then chemically conditioned through counter-current gas exchange (oxygen and nitrogen) to adjust dissolved oxygen levels. Sodium sulfide and ammonium chloride were added through a metering pump. Temperature was controlled by pumping water from a reservoir through an in-line chiller and a heater. The system allowed simultaneous control of temperature (±0.5°C), dissolved oxygen (±0.3 mg L^{-1}), sulfide (±1 μM) and ammonium (±3 μM).

Variables were controlled by monitoring the following three times per day. Sulfide levels were determined colorimetrically (Strickland & Parsons 1972), ammonium levels by ion chromatography (Dionex DX500, CS16 cation column, eluent 18 mM H_2SO_4 at 1 mL min^{-1}, ion suppression 300 mA, conductivity detection, and Li internal standard), dissolved oxygen levels by metering (YSI 550 DO meter, AESI) and titration (Brinkmann 665 Dosimat titrator), pH levels by handheld probe (pHep5, 0–14.00, Hanna), and temperature was determined by computer integrated thermistors.

Exposure Variables

Exposure variables in the study included 0.1 mL injections of *A. viridans* (in sterile seawater) into the ventral abdominal aorta at doses of 1×10^3 and 1×10^6 (or control injections of sterile seawater); dissolved oxygen at 2.5, 3.0, 6.0 and 6.3 mg L^{-1}; sulfide at 0, 3, 4, 6, 7, 9, 15, 20 and 21 μM; ammonium at 0, 10, 24 and 80 μM; and temperatures at 14.5°C and 19.5°C. A single table showing the complete design of the study proved to be needlessly complex; tables in the results section provide an overview of the exact treatment combinations for each of five experiments. Fifteen to 22 lobsters (with the exception of 10 animals for some non-treated controls) were used in each treatment combination for a total of 504 lobsters. Only exposure tanks for 4-μM sulfide were replicated. However, internal consistency of the other exposure variables was shown by the increasing death rates associated with increasing doses of the variable. Lobsters were observed twice daily for behavior and evidence of mortality. Animals identified to have ceased maxilliped activity were removed to tanks with normoxic conditions to confirm death. At the termination of each experiment, tanks were cleaned, disinfected with 1% household bleach (600 ppm sodium hypochlorite solution), rinsed thoroughly, and flushed with flowing seawater for at least 2 days.

Bacteriology

A. viridans var. *homari* was isolated from a moribund lobster at a commercial lobster pound. The bacterial species identity was verified by its typical tetrad morphology and phenotypic characteristics when compared with a reference strain (ATCC 10400) from the American Type Culture Collection (Manassas, VA) using tests in the Biolog Identification System (Hayward, CA). It also was verified to be pathogenic in lobsters. This organism was used

throughout the study after periodic re-isolation from moribund lobsters. The bacterium was grown overnight at 20°C in Trypticase Soy Broth (Becton Dickinson, Sparks, MD) supplemented with 2% NaCl, washed twice by centrifugation in sterile (0.45 μ membrane-filtered) seawater, and adjusted to contain 1×10^3 or 1×10^6 cells per 0.1 mL in sterile seawater (based on microscopic counts using a 1:1 ratio of bacterial suspension and precalibrated latex beads).

Samples of injected and control lobsters, after various treatments, were placed in insulated chests containing ice packs and transported by van from the J. J. Howard Laboratory in New Jersey to the Milford Laboratory in Connecticut (2.5 h driving time) or shipped by overnight express for subsequent bacteriological examination. Zero-time and 1-day samples of lobsters were taken for bacteriology followed by samples every other day from each treatment tank or until complete mortality in the tank; usually only one lobster per tank was removed at each time interval; but replicate tanks frequently provided two or more lobsters per treatment at the time interval.

Bacterial counts in lobster hemolymph were made using the following procedure. An area on the ventral abdomen was disinfected using two 70%-ethanol swabs and then dried with a sterile swab. A half milliliter aliquot of hemolymph was drawn from the ventral abdominal aorta under the disinfected area into a syringe preloaded with 4.5 mL of cold, sterile seawater containing l-cysteine free-base as an anticoagulant (Paterson & Stewart 1974). Testing of this anticoagulant showed no detrimental effect on *A. viridans.* The initial 10^{-1} dilution of hemolymph as well as an additional 10^{-3} dilution were placed in sterile cups for bacterial counts using an Autoplate 4000 spiral plater (Spiral Biotech, Norwood, MA). Plastic Petri-plates containing tripticase soy agar (TSA) supplemented with 2% NaCl were spread in triplicate and counted for colony-forming units (after overnight incubation at 20°C) using the spiral grid method specified by the manufacturer.

A. viridans colonies on TSA medium were easily recognizable and periodic wet mounts from picked colonies showed the typical tetrad grouping of cells when observed by phase-contrast microscopy. Also, the pathogenicity of organisms from picked colonies was verified by reinjection of random colonies into healthy lobsters.

Bacterial counts in the hepatopancreas also were made with the spiral plater; however, initial processing was different. The carapace was opened to expose the hepatopancreas and a small portion of tissue (about 0.25 g was removed with sterile instruments and placed in a preweighed, sterile microtube). After reweighing the microtube plus tissue, 0.75 mL of sterile seawater was added, a fitted pestle (Kimble/Kontes) inserted, and the tissue was ground for about 10 s with a cordless motor attached to the pestle. The tissue suspension was then diluted in cold, sterile seawater (10^{-1} and 10^{-3}) and the suspensions placed in the spiral plater cups as in the hemolymph procedure. Calculations were made using the exact tissue weight to calculate the final dilution for each sample and determine bacteria per gram of tissue.

Statistics

Statistical analyses of survival curves for each treatment were done by Kaplan-Meier (product limit) survival curve estimation using the SigmaStat 3.0 (SPSS, Inc., Chicago, IL) software program. This method calculates median and mean survival times based on deaths and on those periodically removed from the study (censored) for bacterial analysis. Holm-Sidak, log-rank multiple comparison tests were done using the SigmaStat 3.0 program to identify significant differences between treatments (see Glantz 2002) for detailed descriptions and evaluations of these tests). The Student *t*-test was applied to evaluate mean differences in bacterial growth between hemolymph and hepatopancreas.

RESULTS

In the first experiment, we examined the effects of two temperatures, two bacterial doses, two oxygen levels and one combination of sulfide and ammonium (Table 1). Survival curves for this and subsequent experiments followed typical patterns; however, for comparative purposes the median (50%) points are shown in the table. At either 14.5°C or 19.5°C, 50% of the lobsters survived for 8 days to >10 days with variations depending on the temperature or bacterial load (the experiment was terminated at 10 days). When the oxygen level was dropped to 2.5 mg L^{-1} and sulfide plus ammonium were added, mortality rates were dramatically accelerated (highly significant differences were seen between the top four and the bottom four treatments). This first experiment served as a range-finding experiment and helped us select conditions of temperature and bacterial dose level for subsequent experiments. With adequate oxygen and no sulfide and ammonium, lobsters injected with 10^6 bacteria at 19.5°C had a significantly increased mortality rate over similar lobster at 14.5°C; the same was true for lobsters treated with sulfide and ammonium. Also, animals exposed to sulfide and ammonium and injected with 10^6 bacteria at 19.5°C had a significant increased mortality rate over that shown by lobsters injected with the lower (10^3) bacterial dose. Because the 10^6 bacterial dose provided an increased response and better discrimination than the 10^3 dose, it was selected for subsequent experiments. The 19.5°C exposure-temperature was selected for subsequent experiments not only because it was shown to accelerate death rates, but also because it was near the mean August–September temperature for the decade (20°C) at a site in western LIS (Wilson et al. 2001).

The bacterial growth curves for the hemolymph and hepatopancreas mirrored the temperature and dose effects. Figure 1

TABLE 1.

Initial experiment (18 lobsters per treatment) to establish effects of stressors on lobsters.

Exposure Conditions				
Temperature (C°)	Oxygen (mg/L)	Bacterial Dose	Sulfide, NH_4^+ (μM)	Median (50%) Survival Time[a]
14.5	6.3	1×10^6	0, 0	$>10^{1,5,7,10,19}$
19.5	6.3	1×10^3	0, 0	$>10^{4,12,15,16}$
14.5	6.3	1×10^3	0, 0	$>8^{2,6,8,11}$
19.5	6.3	1×10^6	0, 0	$9^{3,9,13,14,19}$
14.5	2.5	1×10^3	20, 70	$5^{7,8,13,15,18*}$
14.5	2.5	1×10^6	20, 70	$4^{10,11,14,16,17}$
19.5	2.5	1×10^3	20, 70	$4^{5,6,9,12,20*}$
19.5	2.5	1×10^6	20, 70	$3^{1,2,3,4,17,18,20}$

[a] Survival time in days is based on Kaplan-Meier (product moment), survival analysis. Values with identical number superscripts are significantly different at $P < 0.01$ level (* at $P < 0.05$ level) based on Holm-Sidak, pair-wise, log-rank, statistical analysis.

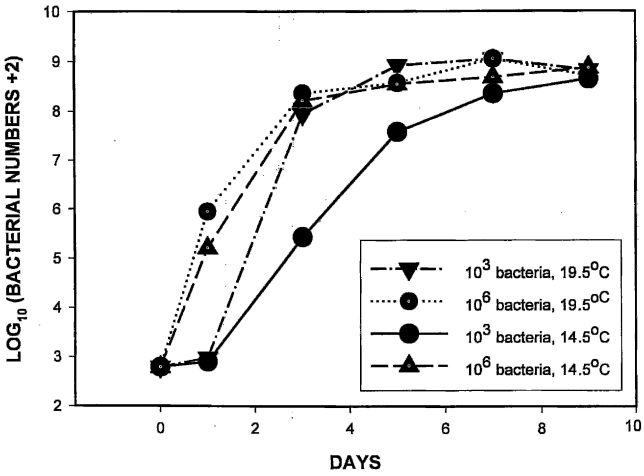

Figure 1. Growth of bacteria in hepatopancreas tissue of lobsters infected with two different doses of *A. viridans* and held at two temperatures.

shows the growth of bacteria in the hepatopancreas of lobsters injected with two bacterial doses and held at either 14.5°C or 19.5°C. A temperature lag and a dose lag is evident in the figure, with the greatest lag in those lobster injected with 10^3 bacteria and held at 14.5°C. However, even the counts in these lobsters reached very high levels within 9 days. Therefore, it may be stated that both temperature and dose affected survival rates in lobsters and that the introduction of hypoxia, sulfide and ammonium further accelerated lobster death rates; however, the design of the initial experiment did not allow separate analyses of these stressors.

In the second experiment, three levels of sulfide and ammonium were tested at 19.5°C (Table 2). With adequate oxygen and no sulfide or ammonium, 50% survival was 12 days in infected lobsters (longer in noninfected lobsters; the experiment was terminated after 13 days). When 6 and 24 μM sulfide and ammonium, respectively, were added in the presence of adequate oxygen, time to 50% mortality was significantly shortened to 8 days. However, when oxygen was reduced to 3 mg L^{-1} with the same concentrations of sulfide and ammonium, time to 50% mortality was reduced to 3 days. Paradoxically, when sulfide and ammonium were absent at 3 mg L^{-1} oxygen, 50% survival time also was 3 days. This moderate hypoxia effect was supported by the result seen after exposure to 3 μM sulfide and 12 μM ammonium; again, 50% survival time was only 3 days.

In other words, it appeared that, at adequate oxygen levels, sulfide and ammonium strongly accelerated mortality rates in lobsters. However, moderate hypoxia alone was sufficient to accelerate lobster deaths regardless of the presence of sulfide and ammonium. Therefore, it was important to separate the effects of sulfide and ammonium at normoxic conditions (given that a combination of sulfide and ammonium had no added effect under hypoxic conditions). Table 3 illustrates the first of three experiments to do this. The table shows that no apparent statistical difference was found between 0 sulfide and 3 μM sulfide in infected lobsters; however, there was a highly significant difference between 3 μM sulfide and 9 μM sulfide. No difference was seen between 9-μM and 15-μM sulfide, but highly significant acceleration of the mortality rate was seen at 21-μM sulfide compared with all other treatments. The result of an experiment to define the break point between 3-μM sulfide and 9-μM sulfide is shown in Table 4. The table shows that 6-μM sulfide was significantly more effective ($P < 0.01$) than 4 μM sulfide in accelerating lobster mortality. A final experiment depicted in Table 5 shows that ammonium alone had no significant effect on lobster mortality even at a level of 80 μM. In this experiment, there was a system failure after 8 days, which required termination of the exposures; however, up to 8 days, few deaths had occurred—unlike the early deaths seen in the sulfide exposures.

Other parts of the work and additional high-temperature experiments showed that stressors alone did not cause the accelerated death; infection also was necessary. For example, at 19.5°C in the presence of hypoxia, sulfide and ammonium, infection with 10^6 bacteria significantly accelerated deaths over those caused by 10^3 bacteria (Table 1). Also, at 19.5°C and 3 mg L^{-1} oxygen, infection with 10^6 bacteria resulted in extremely short survival time (Table 2); whereas, in an additional experiment, at an even higher temperature of 24°C, and a lower oxygen level of 2.5 mg L^{-1}, but without bacteria, survival was beyond 11 days (see Draxler et al. 2005).

The growth of *A. viridans* in the hemolymph and hepatopancreas followed a nearly identical pattern in all experiments. The pattern depicted in Figure 2 is typical of that seen at 19.5°C for all treatments. No differences in bacterial numbers were seen ($P > .05$ in a *t*-test) between lobsters exposed to only bacteria and those

TABLE 2.

Experiment to test effects of additional stressor levels on lobsters at 19.5°C.

	Exposure Conditions			
Number of Lobsters	Oxygen (mg/L)	Bacterial Dose	Sulfide, NH_4^+ (μM)	Median (50%) Survival Time[a]
20	6	0	0, 0	$>13^{1,2,3,5,6}$
21	6	1×10^6	0, 0	$12^{4,6,7,8}$
21	6	1×10^6	6, 24	$8^{5,9}$
21	3	1×10^6	6, 24	$3^{3,8}$
21	3	1×10^6	0, 0	$3^{2,7}$
21	3	1×10^6	3, 12	$3^{1,4,9}$

[a] Survival time in days is based on Kaplan-Meier (product-moment), survival analysis. Values with identical number superscripts are significantly different at $P < 0.01$ level based on Holm-Sidak, pair-wise, log-rank, statistical analysis.

TABLE 3.

Experiment to test the effects of sulfide and infection (in the absence of ammonium) on lobster survival at 19.5°C.

	Exposure Conditions			
Number of Lobsters	Oxygen (mg/L)	Bacterial Dose	Sulfide (μM)	Median (50%) Survival Time[a]
10	6	0	0	$>10^{2,5,7}$
15	6	1×10^6	3	$9^{1,3,8}$
16	6	1×10^6	9	$5^{4,7,8}$
16	6	1×10^6	15	$4^{3,5,6}$
15	6	1×10^6	21	$2^{1,2,4,6}$

[a] Survival time in days is based on Kaplan-Meier (product-moment), survival analysis. Values with identical number superscripts are significantly different at $P < 0.01$ level based on Holm-Sidak, pair-wise, log-rank, statistical analysis.

TABLE 4.

Experiment to test the effects of lower levels of sulfide and infection (in the absence of ammonium) on lobster survival at 19.5°C.

Number of Lobsters	Exposure Conditions: Oxygen (mg/L)	Exposure Conditions: Bacterial Dose	Sulfide (μM)	Median (50%) Survival Time[a]
10	6	0	0	$>12^{2}$
16	6	1×10^6	0	$>12^{1*}$
32	6	1×10^6	4	$>11^{3}$
15	6	1×10^6	6	$9^{1*,2,3}$

[a] Survival time in days is based on Kaplan-Meier (product-moment), survival analysis. Values with identical number superscripts are significantly different at $P < 0.05$ level (* at $P < 0.01$ level) based on Holm-Sidak, pair-wise, log-rank, statistical analysis.

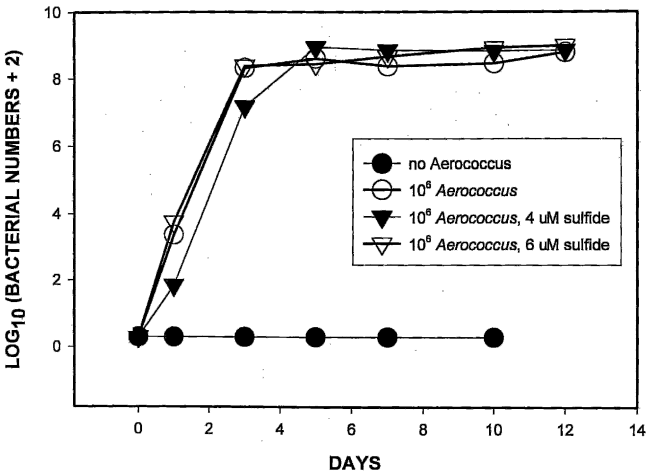

Figure 2. Typical *A. viridans* growth pattern in the hemolymph for all experiments. Growth in the hepatopancreas was similar except counts were generally one-$\log_{10}$ lower.

exposed to stressors as well. The bacteria reached a peak in 3–4 days regardless of whether the lobsters were exposed to stressors. However, median counts in the hemolymph were higher than those in the hepatopancreas. When the counts at 3 days and beyond were averaged in four experiments, the geometric median for hemolymph was 7.7×10^8 mL^{-1} (maximum raw value 1.6×10^9 mL^{-1}) and that for hepatopancreas was 7.7×10^7 g^{-1} (maximum raw value 1.1×10^9 g^{-1}).

DISCUSSION

This study provides evidence that exposure at 19.5°C either to moderate hypoxia or to normal oxygen conditions at sulfide levels that are known to occur in LIS may accelerate mortality rates in lobsters that are infected with the bacterial pathogen, *A. viridans.* Whereas the experimental conditions emulated, as near as possible, some of the biogeochemical conditions known to occur in LIS and flowing seawater was used in the experiments, it is recognized that a contained system, in which lobsters were more crowded than in the natural environment, cannot match exactly natural conditions. On the other hand, the system allowed us to control the dosing of variables at a level that could never be achieved in a natural setting. Further, the crowding of lobsters in this system was less than that which occurs in commercial lobster traps.

As noted previously, sulfide levels as high as 11 μM were found recently in western LIS (Valente & Cuomo 2005). Our data showed that a level between 4 and 6 μM sulfide accelerated mortality in lobsters that were infected with *A. viridans;* further, 21-μM sulfide had an extreme effect on lobster survival. Sulfide levels in other marine areas are known to exceed those recently reported in LIS. Draxler and Byrne (unpublished) found sulfide levels as high as 63 μM in an oxygen-depletion event in the New York Bight in 1976; measurements in a wider area for that event showed levels of about 17 μM (Thomas et al. 1979).

The mechanisms contributing to oxygen depletion in marine sediments are associated with microbial metabolism of organic material. After oxygen is depleted, the heterotrophic benthic microbes (Wang & Chapman 1999) use a sequence of terminal electron acceptors (Mn^{4+}, NO_3^-, NO_2^-, Fe^{3+}, SO_4^{2-}) to continue oxidization of organic material. Sulfate is abundant in seawater (100 times the dissolved oxygen concentration); its reduced product (sulfide) is biologically active. Sediment becomes anoxic below some point where microbial respiration exceeds the oxygen resupply from overlying water. As bottom water oxygen concentrations decline and respiration rates increase with increasing temperature, less dissolved oxygen diffuses into the sediment to meet the increased consumption, and the sulfide-generating stratum moves upward toward the sediment-water interface (Kristiansen et al. 2002). If microbial metabolic rates become sufficiently elevated and the bottom water sufficiently hypoxic, sulfide may diffuse into the overlying water even though it may still be oxic. Therefore, sulfide could have an effect even at somewhat elevated oxygen levels; we found that under these conditions, sulfide affects lobster survival rates (Table 2). While Table 2 includes sulfide in the presence of ammonium, we found (Tables 3, 4 and 5) that the accelerated lobster mortality was caused by sulfide alone; ammonium had no effect up to a level of 80 μM.

Also, we found that moderate hypoxia alone has an effect on lobster survival, which over-rides any effects of sulfide (Table 2). Whereas it is known that low levels of dissolved oxygen are lethal to disease-free lobsters (i.e., levels of 0.2 mg L^{-1} at 5°C to 1.2 mg L^{-1} at 25°C) (Cooper & Uzmann 1980, summary of the work of McLeese 1956); our findings showed that a bacterial disease in lobsters is accelerated at the moderately hypoxic level of 3 mg L^{-1} dissolved oxygen (Table 2).

TABLE 5.

Experiment to test the effects of ammonium and infection (in the absence of sulfide) on lobster survival at 19.5°C.

Number of Lobsters	Exposure Conditions: Oxygen (mg/L)	Exposure Conditions: Bacterial Dose	Ammonium (μM)	Median (50%) Survival Time[a]
10	6	0	0	>8
19	6	1×10^6	0	>8
19	6	1×10^6	10	>8
22	6	1×10^6	20	>8
20	6	1×10^6	80	>8

[a] Survival time in days is based on Kaplan-Meier, log-rank (product-moment) survival analysis. The experiment was terminated after 8 days due to system failure. Holm-Sidak, pair-wise, log-rank, statistical analysis on all pair combinations was not significant ($P > 0.05$).

The physiological mechanisms involved in these accelerated mortalities are not known. It is known that *A. viridans* infections cause a drastic decline in hemocyte numbers; resulting in a risk of fatal hemorrhage, and that hemocytes are a major factor in lobster immunity (Paterson & Stewart 1974). In addition, *A. viridans* infection causes a significant decline in glycogen and ATP levels in the hepatopancreas, heart, and tail muscle; also, glucose, lactic acid, and nonprotein nitrogen disappear from the hemolymph (reviewed by Stewart 1980). The microorganism successfully competes with the lobster for its own energy reserves and causes a nontoxic, noninvasive bacteremia (Johnson et al. 1981). We found that growth of *A. viridans* in infected lobsters can result in hemolymph counts as high as 1.6×10^9 ml^{-1} and hepatopancreas counts as high as 1.1×10^9 g^{-1}. It would have been ideal to use the parasitic amoeba in this study as well; however, this was prevented by the inability to culture the organism.

Published information on effects of environmental pollutants on lobster immunity is sparse; however, information available in studies on immunity in fish and other species leads us to suspect similar effects on lobster immunity. Sindermann (1979) and Weeks et al. (1986) reviewed the literature associating pollution with disease in a number of marine organisms. Zeeman & Brindley (1981) and O'Connor & Huggett (1988) reviewed data showing that aquatic pollutants can depress the immune systems of many fish species. There seems to be a definite correlation between fish disease and point sources of pollution; however nonpoint-source pollution and disease are more difficult to correlate (reviewed by Vethaak & Rheinallt 1992). Fijan (1972) found that poor immune status in carp makes them prone to viral infection, which leads to secondary and tertiary disease caused by opportunistic infections. Examples of the effects of sublethal doses of single pollutants on immune-system suppression include the effect of cadmium exposure on antibody response in the cunner, *Tautogolabrus adspersus* (Robohm 1986) and on cellular response in rainbow trout (Thuvander & Carlstein 1991) as well as the effects of copper in increasing susceptibility of salmonids to IHN virus (Hetrick et al. 1979), *Yersinia ruckeri* (Knittel 1981) and *Vibrio anguillarum* (Baker et al. 1983).

Our work showed that, at 19.5°C, relatively moderate levels of hypoxia as well as sulfide in the absence of hypoxia can accelerate death rates in lobsters that are infected with a pathogenic bacterium, *A. viridans* var *homari.* Although this did not prove that similar effects occur with other microorganisms such as the parasitic amoeba found in the 1999 mortality event, it showed the potential for such effects—particularly since immune system stressors are shown to increase diseases in other marine species. At this time the amoeba cannot be used in controlled laboratory studies because attempts to grow the organism *in vitro* were not successful (Russel et al. 2000). However, exposure to *A. viridans* proved to be a useful tool to show the effects of environmental stressors on lobster disease resistance.

Our study showed potential impacts of eutrophication and elevated temperature on lobster immunity. Because eutrophication may lead to hypoxia and increased sulfide levels, policies that reduce eutrophication in Long Island Sound may improve lobster health.

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