Effects of Temperature, Salinity & Pesticides on Oyster Hemocyte Activity

Gene B. Rodrick

Oyster production represents an important global economic resource (USDA 2006). Periodic increases in oyster deaths have become a major problem globally, severely limiting production, but the causes of the sudden increase in oyster deaths is unknown (Glude, 1974, Koganezawa, 1974, Goulletquer et al. 1998, Cheney, et al., 2000).

The oyster's estuary is subjected to large variations in both temperature and salinity (Turner 2006, Pomeroy et al. 2000). Temperature is elevated (80-92°F) during the summer months, and salinity fluctuates (2-32 parts per thousand [°/oo]) with tidal cycles, rainfall, and drainage carrying pollutants from adjacent terrestrial sites such as farmland and lawns (Turner, 2006, Pomeroy et al. 2000).

Among pollutants, pesticide contamination of shellfish has become more common in estuarine areas over the past several decades, in part because of chemical run-off from farmland (EPA 2006). Pesticides are introduced into rivers when rainfall occurs and then can enter marine areas, particularly estuarine and coastal zones (EPA 2006). These pollutants may have major ecological consequences and could endanger shellfish growth, reproduction, or survival (Banerjee et al. 1996).

In the estuaries in the Apalachicola Bay, where 95 percent of Florida's oysters are harvested, three common pesticides that have been reported to contaminate water are imidacloprid, permethrin and fipronil (EPA 2006).

Oysters are "sessile" or attached organisms, so they are continually exposed to the physiochemical modifications of their seawater environment. Their circulatory system is open, exposing the blood (hemolymph) and circulating cells (hemocytes) and tissues to variations in temperature, salinity, and pollutants such as pesticides in the seawater (Shumway, 1977). Like other bivalve mollusks, they are both osmo- and thermo-conformers; therefore, the hemolymph readily acquires the salinity and temperature of its seawater environment (Shumway, 1977).

Pathogenic organisms including bacteria, viruses, and parasites contaminate the oysters' estuaries (EPA 2006), and the number of infectious agents such as Escherichia coli and Vibrio vulnificus) have been correlated previously with low salinities and high temperatures (Cochran and Paul, 1998). Since the oyster does not possess a thymus, spleen, or bone marrow, the primary defense mechanism against foreign organisms such as bacteria involves circulating hemocytes which are the invertebrate blood cells required for the immune response (Cheng, 1975).

Hemocytes constitute the main line of defense against "non-self" material such as bacteria and pollutants. They also are involved in phagocytosis, pinocytosis, encapsulation, and wound healing (Cheng, 1996, Fournier et al. 2000).

Granulocytes are one of the two types of hemocytes and previously have been shown to be involved in phagocytosis and pinocytosis of foreign material. They are thought to contain hydrolytic enzymes and possibly to produce reactive oxygen compounds that play a key role in killing and degrading deadly invading pathogenic organisms (Pipe 1992, Cheng 1996).

Hemocytes have been used as immune capacity indicators in many bivalve species (Gelder and Moore 1986), but even though some previous studies have been conducted, the factors that control oyster hemocyte phagocytosis and mortality remain unclear and have not been definitively characterized (Hegaret et al. 2003; Gagnaire et al. 2006; Pruzzo et al. 2005).

Previous studies have provided evidence that unidentified non-cellular components of hemolymph (serum) are also essential for the immune function of hemocytes (Leclerc 1996),

PHOTO BY ADRIENNE CHILD, Oak Hall School

Gene Rodrick is a 17-year-old junior at Oak Hall High School in Gainesville who won the 2008 Stockholm Junior Water Prize Competition in the state of Florida.

Gene Rodrick accepts the Florida Stockholm Junior Water Prize award from Jamie Hope of Gainesville Regional Utilities, left, representing the Florida Water Environment Association.

Rodrick's winning project paper was entered into this year's U.S. Stockholm Junior Water Prize Competition, hosted June 19-21 by the Florida Water Environment Association June in Orlando. See next month's Journal for details on the national competition, which was held after our July issue was printed.

but little is known about whether changes in temperature, salinity, or exposure to pesticides could alter these protective serum factors.

Previous studies have failed to identify how the changes in water temperature and salinity may affect the ability of the hemocytes to protect the oyster from invading organisms, or to determine whether the presence of pollutants may also affect the oysters' immune response negatively. It is proposed that a cause of oyster mortality outbreaks may be related to increased susceptibility to bacterial infection because of increased temperature or changes in salinity during the summer months, coupled with exposure to pollutants such as pesticides.

The present study was designed specifically to determine if changes in temperature, salinity, and exposure to pesticides, in addition to alterations in the non-cellular components of hemolymph serum, may have negative effects on the viability of hemocytes and normal immune function as assessed by hemocyte phagocytosis of bacteria. In this investigation, the specific species of oyster of interest was the economically important eastern oyster, *Crassostrea virginica*, for which information is limited regarding how environmental changes influence hemocyte activity (Hegaret et al. 2003).

The hypothesis of the present study was that *in vivo* oyster hemocyte mortality and phagocytic activity against bacteria could be affected negatively by an increase in temperature, salinity, and pesticides, and that normal hemocyte activity is dependent on the non-cellular serum component of hemolymph that may be influenced by common pesticides that pollute estuaries.

The specific objectives of the study were:
(1) To determine by flow cytometry if *in vivo* exposure to different temperatures (4, 25, or 37°C), salinities (1, 15, or 32 °/oo), and a mixture of three commonly used pesticides (imidacloprid, permethrin and fipronil) would affect the mortality of oyster hemocytes or the hemocyte phagocytic activity against *E. coli* and *V. vulnificus.*
(2) To determine if the serum component of the oysters' hemolymph is required to prevent hemocyte mortality and for normal phagocytosis of *E. coli* and *V. vulnificus.*
(3) To determine if the in vitro exposure of pesticides will affect oyster hemocyte mortality and normal phagocytosis of *E. coli* and *V. vulnificus.*

Overview & Rationale for Experimental Design

The Eastern oyster, *Crassostrea virginica*, was collected from approved shellfish harvesting waters from Apalachicola Bay. Flow cytometry was used to evaluate the effects of different temperatures, salinities, and hemolymph serum on hemocyte mortality and phagocytosis of common pathogenic bacteria (*E. coli* and *V. vulnificus*) at timed intervals in acclimatized oysters. The specific salinity treatments (1, 15, 32 °/oo) were selected because they are representative of the average salinity (15 °/oo), and the hypo and hyper-salinities (1 and 32 °/oo, respectively) represent the range of salinities that may result from fluctuations in tides and rainfall in the normal aquatic environment of the *C. virginica* species (Berrigan et al. 1991).

The three seasonal water temperatures that the *C. virginica* species would be exposed to are on average 4, 25, or 37°C (EPA 2006). The three pesticides were chosen (imidacloprid, permethrin, and fipronil) because they are commonly used in the area of Apalachicola Bay, and the concentrations (0.7 mg/L) represents the concentration that may occur in a Florida estuary (EPA 2006). The time periods (zero, three, five, and seven days) were selected because the time required for the oyster to acclimate to different temperatures and salinities previously had been estimated to be up to three to five days (Shumway 1977).

Experimental Design for Objective 1

Temperature Treatments

Oysters were acclimated at 4, 25, or 37°C, respectively, in one of nine tanks (225 L) of recirculating artificial seawater. The temperatures of the tanks were controlled by placing the tanks in one of three large temperature-controlled incubators.

Salinity Treatments

Three tanks at each of the temperatures were adjusted to a different salinity (1, 15, or 32 °/oo) by using Instant Ocean Aquarium Sea Salt® (Aquarium Systems Inc.).

Pesticide Treatments

Oysters were exposed to seawater with a mixture of pesticides (imidacloprid, permethrin and fipronil) at a concentration of 0.7 mg/L.

Time Period for Treatments

Oysters (n=5) were removed from each of the nine tanks for hemocyte measurements on Days 0, 3, 5, and 7.

Hemolymph/Hemocyte Collection

A hole was notched in each oyster using a drill. Hemolymph was then withdrawn from the pericardial cavity and posterior adductor muscle sinus of five oysters using a 10-ml. syringe equipped with needle (0.9x25mm). Hemolymph samples were pooled, centrifuged for 10 minutes at 100 x g, resuspended in the serum fraction, and used for flow cell cytometry analysis and microscopy observation. Hemolymph samples were pooled to reduce inter-individual variation and to provide sufficient hemocytes for experiments.

Flow Cytometry Analysis of Hemocyte Mortality

Hemolymph samples were monitored within two hours of collection for hemocyte mortality by a modification of the method of Hegaret et al. (2003) using an EPICS XL 4, Beckman Coulter Flow Cytometer. Mortality was quantified using 200 μl of the oyster hemocyte suspension. Hemocytes were incubated in the dark for 30 minutes at room temperature with 10 μl of propidium iodide (1.0 mg/L) (Interchim). Propidium iodide binds to double-stranded DNA and fluoresces at wavelengths above 630 nm; it enters and stains non-viable cells but can not cross the membrane of viable cells, thereby making the dead hemocytes more fluorescent in the flow cytometer's light detector (Hegaret et al. 2003).

For each sample, 10,000 hemocytes were counted. Results were depicted as cell cytograms indicating cell size (FSC value) and cell complexity (SSC value) and the fluorescence channel(s) corresponding to the marker used. Hemocyte mortality was measured using red fluorescence and was expressed as the percentage of dead hemocytes relative to the total number of hemocytes.

Bacteria Preparation

Both *E. coli* and *V. vulnificus* were cultured separately in heart infusion (HI) broth at 37°C for 24 hours. The culture broths were then centrifuged for 10 minutes at 500 x g. The bacterial pellets were washed two times with sterile phosphate buffer, pH 7.2. The bacterial phosphate buffer mixture was adjusted by adding phosphate buffer until an optical density of 0.69 at 540 μm wavelength light was achieved. This is equivalent to 1×10^6 bacterial cells per ml.

Flow Cytometry Analysis of Hemocyte Phagocytosis of Bacteria

Phagocytosis was measured using two different methods that included:
(1) Oyster hemocyte ingestion of fluorosphere carboxylate-modified beads (1 μm) (Interchim). A 200 μL aliquot of cell suspension was incubated for 60 minutes at 25°C with 10 μL of a 1/10th dilution of fluorescent beads coated with heat-killed *E. coli* or *V.vulnificus*; or
(2) Stained *E. coli* or *V. vulnifucus* with propidium iodide (1.0 mg/L, (Interchim). Phagocytosis was expressed as the percentage of hemocytes that had ingested the fluorescent bacterial coated beads or ingestion of propidium iodide fluorescent *E. coli* or *V. vulnificus.*

Experimental Design for Objective 2

Hemolymph from oysters acclimated at 25°C in 15 °/oo salinity for three days was collected and centrifuged for 10 minutes at 500 x g. The resulting cell-free serum was removed and artificial seawater (ASW) (15 °/oo) was added back to the untreated hemocytes and evaluated by flow cytometry for cell mortality and phagocytosis activity against *E. coli* and *V. vulnificus* and compared to that observed when hemocytes were suspended in normal hemolymph or 15 °/oo artificial seawater after two and four hours incubation at 25°C.

Experimental Design for Objective 3

Hemolymph from oysters acclimated at 25°C in 15 °/oo salinity for three days was collected and centrifuged for 10 minutes at 500 x g. The resulting cell-free serum was collected and 0.7 mg/L seawater of pesticides were added and mixed. The resulting pesticide-treated serum was added back to the untreated hemocytes and evaluated by flow cytometry for cell mortality and phagocytosis activity against *E. coli* and *V. vulnificus* and compared to that observed when hemocytes were suspended in normal hemolymph or 15 °/oo artificial seawater after two and four hours incubation at 25°C.

Microscopy Observation

Light microscopy was used to determine how uniform the oyster hemocyte preparations were and to assess if there were any osmotic or temperature effects on the morphology of the hemocytes (Ford et al. 1994). Hemocytes were placed on a microscope glass slide with cover slip and observed using a Nikon light microscope equipped with a camera.

Statistical Analysis

The means and standard deviations were determined for hemocyte mortality at 4, 25, or 37°C at 1, 15, or 32 °/oo salinity with and without pesticide exposure and hemocyte phagocytic activity against *E. coli* and *V. vulnificus* in oysters acclimated at 4, 25, or 37°C in 1, 15, or 32 °/oo salinity on Days 0, 3, 5, and 7 in the presence and absence of the pesticide mixture.

Analysis of variance (ANOVA) was used to test the null hypothesis that the hemocyte mortality and phagocytosis of *E. coli* and *V. vulnificus* at 4, 25, or 37°C at 1, 15 and 32 °/oo salinity on Days 0, 3, 5, and 7 with and without pesticide exposure would be the same, and whether interactions among temperature, salinity, and pesticide exposure existed. ANOVA was also used to test for significant differences between means of phagocytosis of *E. coli* and *V. vulnificus* by hemocytes resuspended in normal hemolymph, positively treated hemolymph and 15 °/oo artificial seawater after two and four hours incubation at 25°C.

The level of significance selected was (α) = .01, which means that there was only a 0.01 chance that the observed differences in means were due to chance and a 99.99 percent probability that the differences were due to the temperature, salinity, and hemolymph treatments.

Results

Objective 1

Oyster *in vivo* hemocyte mortality was significantly ($p < 0.01$) increased for oysters acclimated for Days 0, 3, 5, and 7 in 1 °/oo and 32 °/oo salinity without pesticide exposure when compared to hemocyte cell mortality in oysters acclimated in 15 °/oo at 4, 25, or 37°C without pesticide exposure. Hemocyte mortality was significantly ($p < 0.01$) increased for the oysters exposed to the mixture of pesticides when compared to oysters that were not exposed to the mixture of pesticides for three, five, and seven days, regardless of salinity or temperature treatment.

The data illustrated in Figure 1 indicate that exposure to either low (1 °/oo) or high (32 °/oo) salinity significantly ($p<0.01$) increased the mortality of oyster hemocytes
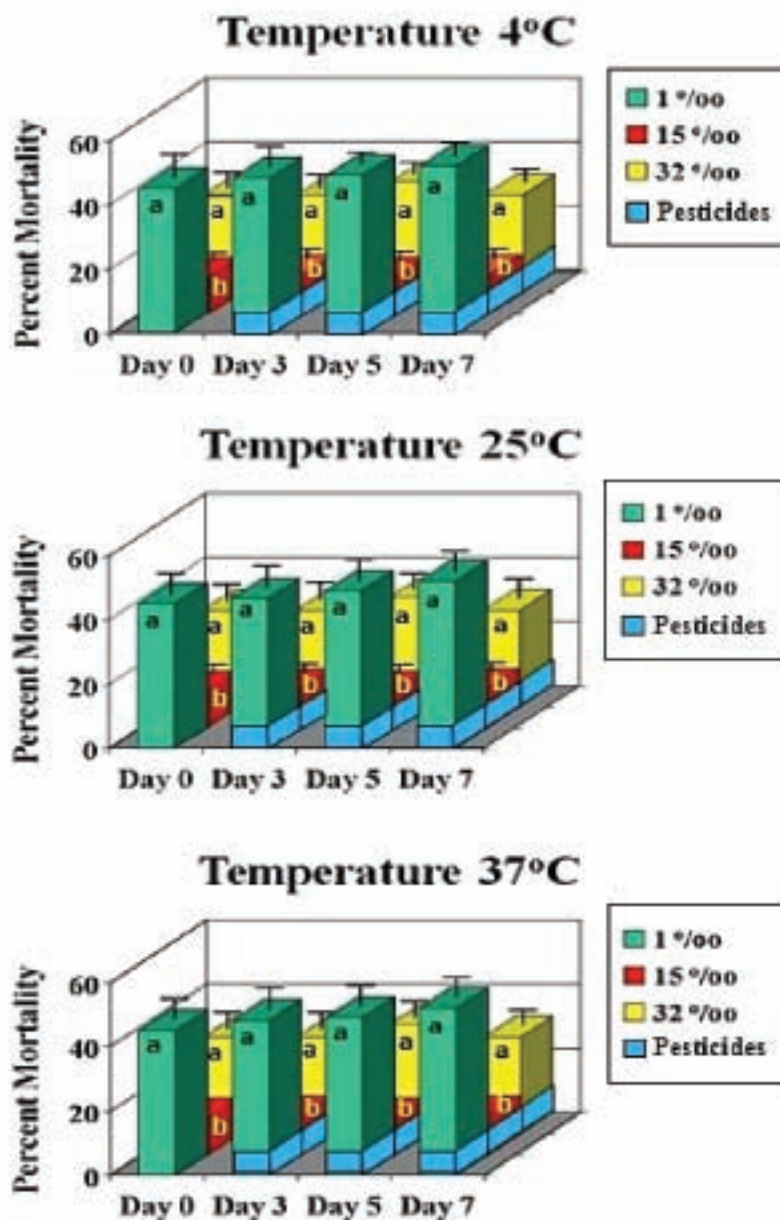
Figure 1. In vivo percent oyster hemocyte mortality at 4, 25, or 37°C at 1, 15, and 32 °/oo salinity at Days 0, 3, 5, and 7 with and without pesticide exposure. Values are means of three replicates ± SD. Different letters indicate significant differences (p< 0.01) between salinity groups.

Temperature 4°C

Temperature 25°C

Temperature 37°C
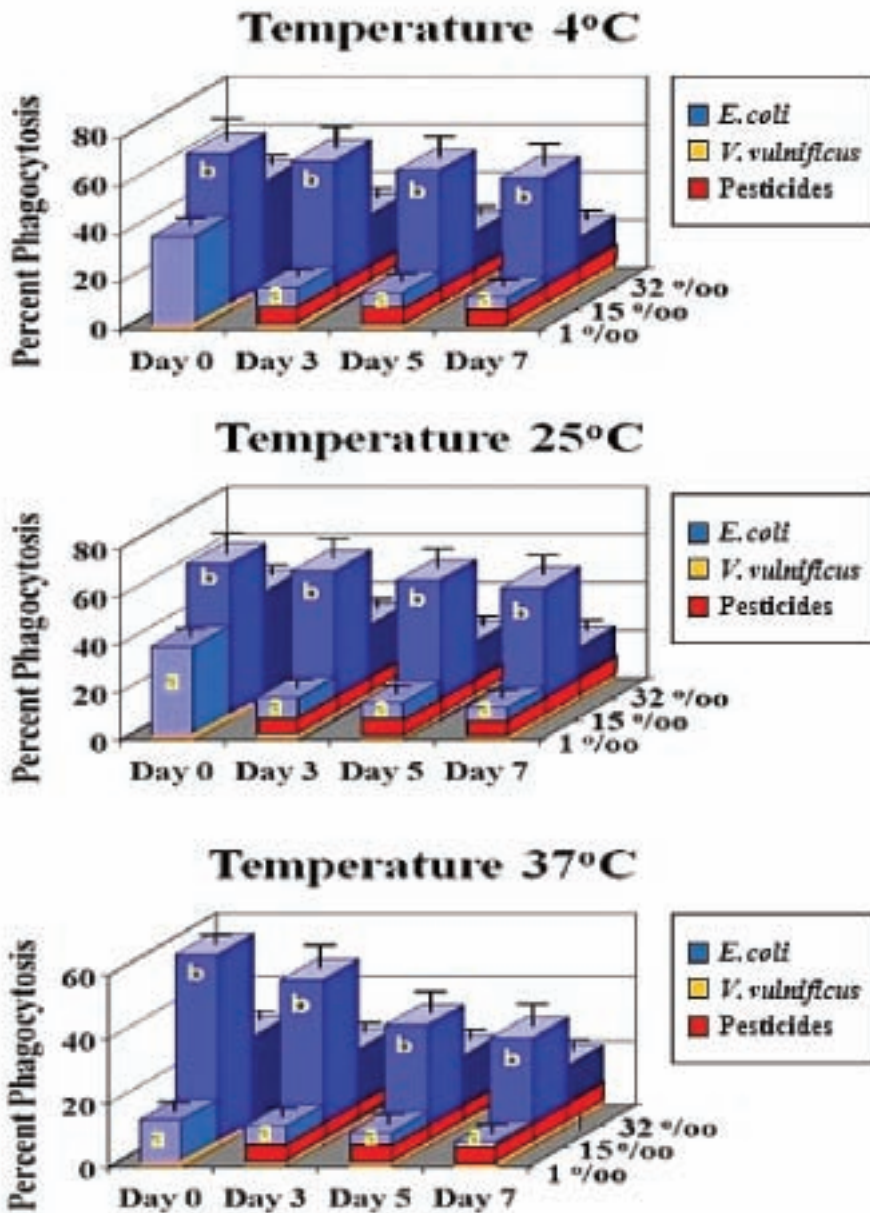

Figure 2. In vivo percent oyster hemocyte phagocytosis of E. coli, V. vulnificus measured by flow cytometry at 4, 25, or 37°C at 1, 15, and 32 °/oo salinity at Days 0, 3, 5 and 7 with and without pesticide exposure. Values are means of three replicates ± SD. Different letters indicate significant differences (p< 0.01) between salinity groups for E. coli. For V. vulnificus no differences were detected between the same salinity groups.
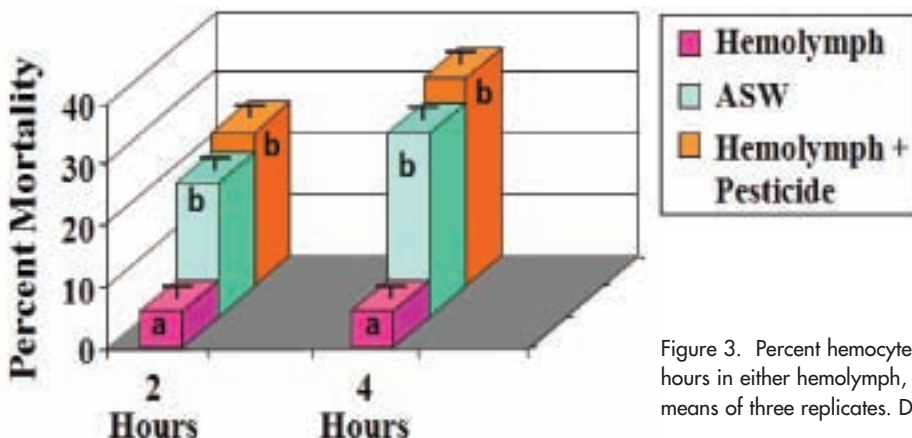

Figure 3. Percent hemocyte mortality in hemocytes incubated for either two or four hours in either hemolymph, ASW, or pesticide-treated hemolymph. Values are means of three replicates. Different letters indicate significant (p<0.01) differences.

relative to 15 °/oo. The negative effect of either hypo (1 °/oo) or hyper (32 °/oo) salinity on hemocyte viability was independent of the water temperatures evaluated in this study.

Regardless of temperature, the mortality of hemocytes exposed to 15 °/oo was on average 10 percent, compared to either 40 percent or 30 percent for the 1 and 32 °/oo salinities, respectively (Figure 1). No significant differences (p>0.01) were detected between acclimatization days. No significant (p>0.01) interactions among temperature, salinity, and incubation times were detected.

Oyster *in vivo* hemocyte phagocytois of *E. coli* and *V. vulnificus* monitored by flow cytometry was significantly lower (p< 0.01) in oysters acclimated for Days 0, 3, 5, and 7 at 4, 25, or 37°C in 1 and 32 °/oo salinity, compared to hemocyte phagocytosis in oysters acclimated in 15 °/oo salinity (Figure 2). The effect of salinity on hemocyte phagocytosis was independent of the water temperature. Regardless of temperature, the percent hemocyte phagocytosis for the 15 °/oo was approximately two-fold higher than in the 1 and 32 °/oo salinity groups (Figure 2).

In addition, the percentage of phagocytosis of *E. coli* by oyster hemocytes was markedly higher than the phagocytosis of *V. vulnificus*, regardless of the temperature or salinity. The percent hemocyte phagocytosis of *V. vulnificus* was only a fraction of 1 percent regardless of treatments, in contrast to a 59 percent maximum hemocyte phagocytosis of *E. coli*. No significant (p>0.01) differences were detected between acclimatization days, and no significant (p>0.01) treatment interactions were detected.

In vivo pesticide exposure to the oysters significantly (p>0.01) increased hemocyte mortality and decreased hemocyte phagocytosis of both *E. coli* and *V. vulnificus* at Days 3, 5, and 7, regardless of salinity or temperature treatment.

Objectives 2 & 3

After incubation in either pesticide-treated cell-free serum or 15 °/oo artificial seawater (ASW), hemocyte mortality was significantly (p< 0.01) increased when compared to hemocytes incubated in normal hemolymph serum (Figure 3). The hemocyte percent mortality was approximately five-fold higher, regardless of incubation time (two or four hours) in hemocytes incubated

in either ASW (15 °/oo) or pesticide-treated serum, compared to hemocytes incubated in normal hemolymph serum (Figure 3).

Also, the phagocytic activity of the hemocytes toward *E. coli* and *V. vulnificus* was significantly (p< 0.01) decreased when compared to hemocytes incubated in normal hemolymph serum for two and four hours (Figure 4).

Light microscopy of hemocyte

Hemocytes taken from the acclimated oysters were observed to be intact morphologically and normal by examination of photographs taken by light microscopy (Figure 5).

Discussion

The results of this study indicate that in contrast to our hypothesis, the viability and phagocytic activity of hemocytes did not change when the oysters were exposed to different water temperatures. The surprising and dramatic effects of hypo- or hyper-salinity on the oysters' defense mechanism provide new evidence for potential negative effects of salinity changes on oyster production (Turner 2006).

The hypothesis that pesticides would have a negative effect on the oysters' immune system was supported by the in vitro data which indicated that exposure of the oyster hemocytes to pesticides significantly increased hemocyte mortality and depressed hemocyte phagocytosis of both *E. coli* and *V. vulnificus*. In contrast to the in vitro exposure, however, *in vivo* exposure of oysters to pesticides unexpectedly decreased the hemocyte mortality, yet still depressed hemocyte phagocytosis of *E. coli* and *V. vulnificus*.

The reasons for the differences between in vitro and *in vivo* hemocyte mortality when exposed to pesticides may possibly involve a biological transformation of the pesticides once these chemicals entered the oyster's digestive tract. It is known that many chemicals can be altered through digestive tract enzymes and the P-450 microsomal enzymes (Woodburn et al. 2003).

The design of the study also provided the opportunity to observe if there were differences in the ability of the oysters' hemocyte to phagocytize *E. coli* compared to *V. vulnificus*. The data clearly indicate that the oysters' hemocyte is much less capable of phagocytizing *V. vulnificus* compared to *E. coli*. One potential explanation is that there may be differences between how the hemocyte membrane receptor recognizes either *E. coli* or *V. vulnificus* before these bacteria are engulfed and phagocytized.

These findings indicate that salinity and the presence of pesticides may play a more important role in the oyster hemocyte viabil-
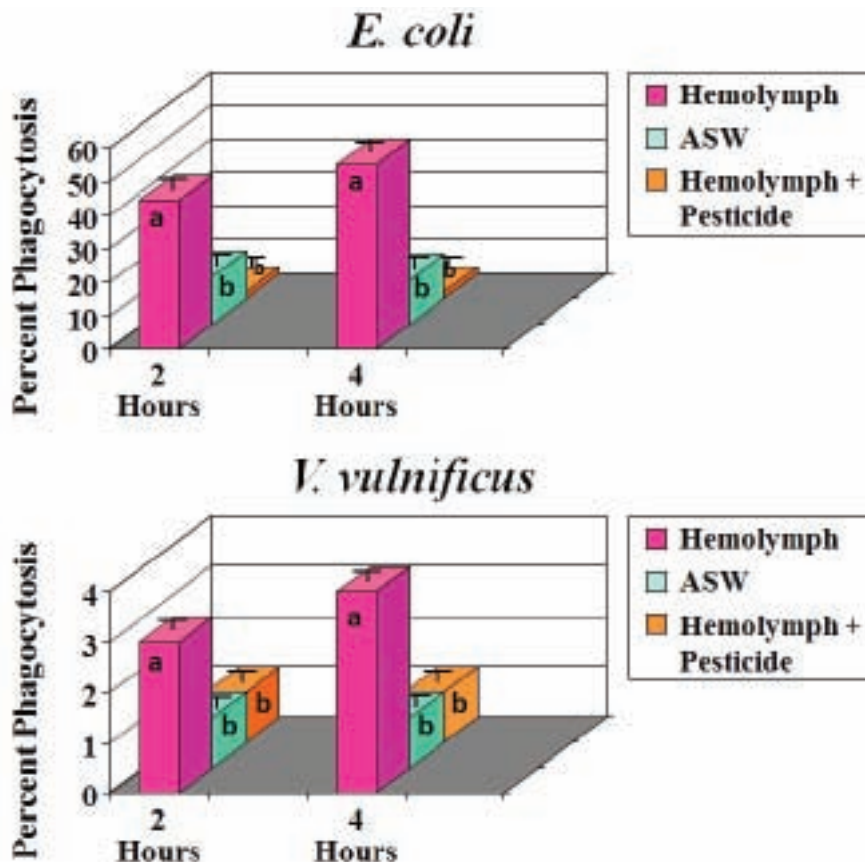

Figure 4. Percent in vitro phagocytosis of either E. coli or V. vulnificus when hemocytes were incubated in hemolymph, ASW, or pesticide-treated hemolymph. Values are the means of three replicates. Different numbers indicate significant (p<0.01) differences among treatments (note different scale-higher percent phagocytosis for E. coli).

ity and activity than previously thought. The current study alerts the scientific community to the negative effects that alterations in salinity have on the oysters' defense mechanism that may be linked to the observed oyster mortality outbreaks.

The results of this study could also lead to a better understanding of the possible interactions between abiotic environmental factors (temperature, salinity, pesticides) and biotic factors associated with the "immune" system of oysters, and the resulting effect on susceptibility to infections. The finding that the oysters' hemocyte is much less capable of phagocytizing *V. vulnificus* compared to *E. coli* provides an explanation for the previously observed *in vivo* natural decay of *E. coli* compared to *V. vulnificus* (Wright et al. 1996).

It was not possible to investigate all of the potential environmental factors that also may have affected the oyster's hemocyte activity. It is recognized that the hemocyte response to the specific temperature, salinity, and pesticide treatments may actually be somewhat different within the estuary, where the oyster is exposed to other variables that include other pollutants, different amounts

of dissolved oxygen, and changes in pH. Since estuaries in different regions of the country are exposed to different seasonal changes in temperature and salinity, the application of these data may be more relevant to the southeastern U.S.

The fact that it was possible to evaluate only a limited number of environmental factors in these studies sets the stage for future investigations which are needed to characterize how other pollutants such as chemicals used in fertilizers and contaminants from industrial wastes also may impact the observed seasonal increase in oyster mortality. In addition, future studies are needed to identify and characterize the serum factors required for the oysters' immune response and to investigate the role of these factors in hemocyte viability. Future investigations are needed to determine the physiological basis of the observed differences in hemocyte phagocytosis of *E. coli* and *V. vulnificus*.

Since the study identified the fact that the oyster's immunity to common bacteria may be impaired when salinity is either too high or too low and pesticides are present, monitoring systems should be put in place to track

changes in salinity and pesticide contamination of the estuaries. This is important because the bacterial content of the oysters harvested under these conditions is likely to be elevated, which may lead to the previously unexplained increase in oyster deaths. When the oysters' ability to kill invading bacteria is impaired because of changes in environmental conditions, the increased bacterial content also may pose a health risk to humans when the oysters are consumed raw (Potassman et al. 2002).

The findings from this study should be reported to the U.S. Environmental Protection Agency (EPA), whose National Estuary Program (NEP) could take a variety of regulatory and non-regulatory approaches to reduce pesticide contamination of oyster estuaries (EPA 2006).

The mission of the NEP is to identify, restore, and protect nationally significant estuaries in the U.S. To accomplish this, the program involves local communities, including oyster farmers and others, with an economic and environmentally related process. When the NEP is notified of the published results of this study, the new information can be communicated through this organized program to design ways to monitor environmental changes and to take steps to reduce contamination by pollutants such as pesticides. In this way, the people and communities that have the most to gain from an economic, environmental, and health-related perspective can develop specific action plans.

For example, by controlling runoffs from farms and lawns, contamination of estuaries with toxic chemicals such as the pesticides investigated in this study can be reduced. Also, efforts can be made to eliminate the use of specific pesticides by changing to alternative systems of controlling insect infestation on cropland and lawns.

The results of this study will be important to initiate and implement new NEP programs to protect the oysters' estuaries, since this program relies on new scientific evidence regarding the biological effects of chemicals on the animals inhabiting the contaminated estuaries and the potential effect of the contaminates on the seafood and on human health if the seafood is consumed (Banatvala et al. 1997).

Conclusions

1. *In vivo* variations in salinity could affect the ability of oyster hemocytes to resist foreign bacterial invasion.
2. Significant differences in the eastern oyster hemocyte phagocytic activity towards *E. coli* compared to *V. vulnificus* may be partially related to changes in salinity.
3. Non-cellular serum components are required for the hemocyte to phagocytize *E. coli* and *V. vulnificus*.
4. In vitro exposure of pesticides to the oyster hemocytes significantly increased oyster hemocyte mortality and depressed hemocyte phagocytosis of both *E. coli* and *V. vulnificus*.
5. In contrast to the in vitro exposure, *in vivo* exposure of oysters to pesticides decreased the hemocyte mortality yet still depressed hemocyte phagocytosis of *E. coli* and *V. vulnificus*.

Acknowledgements

References

- Banatvala, N, Hdady WG, Ray, B J., McFarland LM, Thompson S, Tauxe RV (1997). Vibrio vulnificus infection reporting on death certificates: the invisible impact of an often fatal infection. Epidemiology Infection. 118: 221-225.
- Banerjee BC, Koner BC and Ray A (1996). Immunotoxicity of pesticides: perspectives and trends, Indian Journal Experimental Biology 34: 732-733.
- Cheney, DP, MacDonald, BF and Elston, RA (2000). Summer mortality of Pacific oysters, Crassostrea gigas (Thunberg): Initial findings on multiple environmental stressors in Puget Sound, Washington, 1998. Journal of Shellfish Research. 19: 353-359.
- Cheng, TC (1975). Functional morphology and biochemistry of molluscan phagocytes. Annuals New York Academy of Sciences. 266: 343-379.
- Cheng, TC (1996). Hemocytes: Forms and Functions. In: The Eastern Oyster, Crassostrea virginica. (eds. VS Kennedy, RIE Newell and AF Eble), pp.299-333. Maryland Sea Grant, College Park, Maryland.
- Cochran, PK and Paul, JH (1998). Seasonal abundance of lysogenic bacteria in a subtropical estuary. Applied and Environmental Microbiology 64: 2308-2312.
- Environmental Protection Agency (2006). Challenges facing our estuaries. Key management issues. http://www.epa.gov/owow/estuaries/about3.htm (date accessed June 21, 2007)
- Fournier, M, Cyt, D, Blakley, B, Boermans, H and Brousseau V (2000). Phagocytosis as a biomarker of immunotoxicity in wildlife species exposed to environmental xenobiotics. American Zoologist 40: 412-420.
- Ford, SE, Ford, KA and Kanaley, SA (1994). Comparative cytometric and microscopic analyses of oyster hemocytes. Journal of Invertebrate Pathology 64: 114-122.
- Gagnaire, B, Frouin, H, Moreau, K, Thomas-Guyon, H and Renault, T (2006). Effects of temperature and salinity on haemocyte activities of the Pacific oyster, Crassostrea gigas (Thunberg). Fish and Shellfish Immunology 20: 536-547.
- Gelder, SR and Moore, CA (1986). Cytochemical demonstration of several enzymes associated with processing of foreign material within hemocytes of Mercenaria mercenaria. Transactions of the American Microscopical Society 105: 51-58.
- Glude, JB (1974). A summary report of Pacific Coast oyster mortality investigations 1965-1972. In: Proc of the Third U.S.-Japan Meeting on Aquaculture at Tokyo. pp 28. Tokyo Press, Tokyo, Japan
- Goulletquer P, Soletchnik P, Le Moine O,

Razet D, Geairon P, Faury N, and Tailade,S (1998). Summer mortality of the Pacific cupped oyster Crassostrea gigas in the Bay of Marennes-Oleron (France). ICES Mariculture Committee CM, Copenhagen.

· Hegaret H, Wikfors G H, Soudant, P (2003). Flow cytometric analysis of haemocytes from eastern oysters, Crassostrea virginica, subjected to a sudden temperature elevation. Journal Experimental Marine Biology and Ecology 293: 249-265.

· Koganezawa, A (1974). Present status of studies on the mass mortality of cultured oysters in Japan and its prevention. In: Proceedings of the Third U.S.-Japan Meeting on Aquaculture at Tokyo, pp 29-34. Tokyo Press, Tokyo Japan.

· Leclerc M (1996). Humoral factors in marine invertebrates. In: Invertebrate Immunology. (eds. B Rinkevich, and WEB Muller), pp. 1-10. Springer Verlag, Berlin, Germany

· Pipe, RK (1992). Generation of reactive oxygen metabolites by the hemocytes of the mussel Mytilus edulis. Developmental and Comparative Immunology. 16: 111-122.

· Pomeroy, LR, Sheldon JE, Sheldon Jr, WM, Blanton JO, Amft J, Peters F (2000). Seasonal changes in microbial processes in estuarine and continental shelf waters of the south-eastern U.S.A. Estuarine, Coastal and Shelf Science 51: 415-428.

· Potassman, I, Pax, A, and Odeh, M (2002). Infectious outbreaks associated with bivalve shellfish consumption: a worldwide perspective. Clinical Infectious Disease 35: 921-928.

· Pruzzo, C, Gallo G, Canesi L (2005). Persistence of vibrio in marine bivalves: the role of interactions with haemolymph components. Environmental Microbiology 7: 761-772.

· United States Department of Agriculture (2006). Census of Aquaculture (2005), Vol. 3, Part 2, National Agricultural Statistics Service, USDA, Washington, D.C. 1-114.

· Shumway, SE (1977). Effect of salinity fluctuation on the osmotic pressure and Na+, Ca2+, and Mg2+ ions in the hemolymph of mollusks. Marine Biology 41: 153-177.

· Turner R.E. 2006. Will lowering estuarine salinity increase Gulf of Mexico oyster landings? Esturaries and Coasts 29: 345-352.

· Woodburn KB, Hansen SC, Roth GA, and Strauss K (2003). The bioconcentration and metabolism of chlorpyrifos by the eastern oyster, Crassostrea virginica. Environmental Toxicology and Chemistry 22: 2766-284.

· Wright AC, Hill, RT, Johnson, JA, Roghman, MC, Colwell RR, Morris, JG, Jr (1996). Distribution of *V. vulnificus* in the Chesapeake Bay. Applied and Environmental Microbiology 62: 717-724. ⬦