Effect of environmental pollutants on the chemiluminescence of hemocytes from the American oyster *Crassostrea virginica*

K. G. Larson, B. S. Roberson, F. M. Hetrick

Department of Microbiology, University of Maryland, College Park, Maryland 20742, USA

ABSTRACT: An assay measuring the chemiluminescent response of hemocytes from the oyster *Crassostea virginica* was used to assess the effects of environmental pollutants on phagocytosis. In vivo and in vitro effects of heavy metals (copper, cadmium, zinc, and aluminum), pesticides (dieldrin and chlordane), and organic compounds (naphthalene and 2,4-dinitrophenol) on the chemiluminescent response of stimulated oyster hemocytes were tested. In most cases, exposure to a high pollutant concentration resulted in a decreased chemiluminescent response. After in vivo treatment, the response of the hemocytes depended on the duration of exposure to the pollutant and pollutant concentration. Instances of both acclimation and cumulative toxicity of pollutants were observed.

INTRODUCTION

Chemiluminescence (CL) is a measurable characteristic of stimulated macrophages and polymorphonuclear leukocytes (PMN). The CL assay was first described by Allen et al. (1972), using human PMN, and has been used to study phagocytosis in many species, including mice (Brummer et al. 1985), channel catfish (Scott & Klesius 1981), striped bass (Stave et al. 1983), and rainbow trout (Elsasser et al. 1986).

Oyster hemolymph contains large numbers of phagocytes which are important in defense against infection (Cheng 1975, Ellis et al. 1975). Similar to other bivalve filter-feeding molluscs, oysters are likely to accumulate a variety of chemicals from the environment including copper (Pesch et al. 1979, Zamuda et al. 1983), cadmium (Zaroogian 1980), and pesticides such as dieldrin (Mason & Rowe 1976, Emanuelson et al. 1978). This concentration thereby exposes tissues and cells including the hemocytes to levels many times those found in the environment.

Exposure to environmental pollutants could depress the phagocytic activity of the hemocytes and thereby increase susceptibility to disease. This study describes the use of a CL assay to determine the effects that chemicals often found in the environment have on the phagocytic activity of oyster hemocytes.

MATERIALS AND METHODS

Oysters. *Crassostrea virginica* collected from a clean area in the Chesapeake Bay (Hog Island) were maintained in a filtered, aerated aquarium filled with 60 gal (225 l) of water containing 16 ppt salt (Instant Ocean, Aquarium Systems, Mentor, OH, USA). These 7 to 10 cm oysters were kept at 18 to 20 °C and were allowed to acclimate for 1 wk prior to use.

Isolation and preparation of hemocytes. A hole was drilled into each oyster at an angle to allow a needle to be inserted into the heart near the adductor muscle. Oysters were allowed to recover from the stress of drilling for 3 d prior to bleeding. Hemolymph was obtained from the pericardial sac using a 19 gauge needle.

The hemolymph from several (3 to 5) oysters was pooled to eliminate possible variation due to the response of individual oysters. Viable hemocytes were counted by trypan blue dye exclusion and numbers were adjusted using hemolymph filtered through a 0.22 μm filter. The cells were dispensed into scintillation vials (6×10^6 per vial) and incubated at 20 °C for 1 h.

Chemiluminescence assay. Luminol was prepared in the manner described by Scott & Klesius (1981) by dissolving 0.618 g boric acid, 0.78 g potassium hy-

droxide and 0.14 g luminol in 10 ml distilled water to prepare a stock solution. A working solution of luminol was prepared by diluting the stock solution 1:1000 in Hanks' balanced salt solution (HBSS). A stock solution of phorbol myristate acetate (PMA) was prepared by dissolving 1 mg of PMA in 0.21 ml of dimethyl sulfoxide (DMSO). Working solutions were made by diluting the PMA in Dulbecco's phosphate buffered saline (PBS). To replicate vials containing 2 ml hemocytes, 2 ml of luminol was added. Finally, 2 ml of the PMA solution (3 ng ml^{-1}, final concentration) was added to initiate the CL response. Negative control vials were prepared by the addition of PBS in the place of the stimulus. Chemiluminescence was read in a Beckman scintillation counter in the out-of-coincidence mode as described by Stave et al. (1983). Data was entered into an IBM 4341 computer and mean values of duplicate vials were plotted. Significant differences between CL responses and treatment groups were determined using an analysis of variance procedure, $p < 0.01$ (Sokal & Rohlf 1973). All experiments were repeated at least once. Responses are presented in units of kilo-counts per minute (kcpm).

Chemicals. Solutions of aluminum chloride, cadmium chloride, copper chloride, and zinc chloride (1 mg ml^{-1}) were prepared in distilled water. Dieldrin and naphthalene (Sigma, St. Louis, MO, USA) were diluted in DMSO to a concentration of 5 mg ml^{-1}. Chlordane (Alltech, West Chester, PA, USA) was prepared in methanol at a concentration of 0.1 mg ml^{-1}. All test dilutions were made in PBS.

In vitro studies. The effect of the chemicals in vitro

was studied by their addition directly to incubation vials containing hemocytes. As some of the pollutants were dissolved in DMSO, controls were included to determine if DMSO had an effect on the CL response of the oyster hemocytes.

In vivo studies. Oysters were drilled, allowed to acclimate and then placed in Pyrex jars containing 4 l of sea salt water (6 oysters per treatment group). The pollutants were introduced directly into the water. At intervals of 24, 48, 72 and 96 h, oysters were bled, the hemolymph was pooled, hemocyte concentrations were adjusted to ca 6 × 10^6 cells ml^{-1} with filtered hemolymph, and the phagocytic activity was tested using the CL assay. No oysters contributing to the pool were bled more frequently than at 48 h intervals.

RESULTS

Heavy metals

Copper depressed the CL response of the oyster hemocytes both in vivo and in vitro. Low levels of copper (0.4 ppm) did not decrease the CL response in vitro; however 4 ppm decreased the response to a level approximately half that of the positive control. Exposure of the hemocytes to 40 ppm copper totally eliminated the CL response (Fig. 1). In vivo studies yielded similar results. Oysters exposed to 40 ppm copper died within 96 h of exposure. Oysters exposed to lower concentrations showed a greater number of dead hemocytes than the untreated control oysters, with the highest concentration of copper resulting in
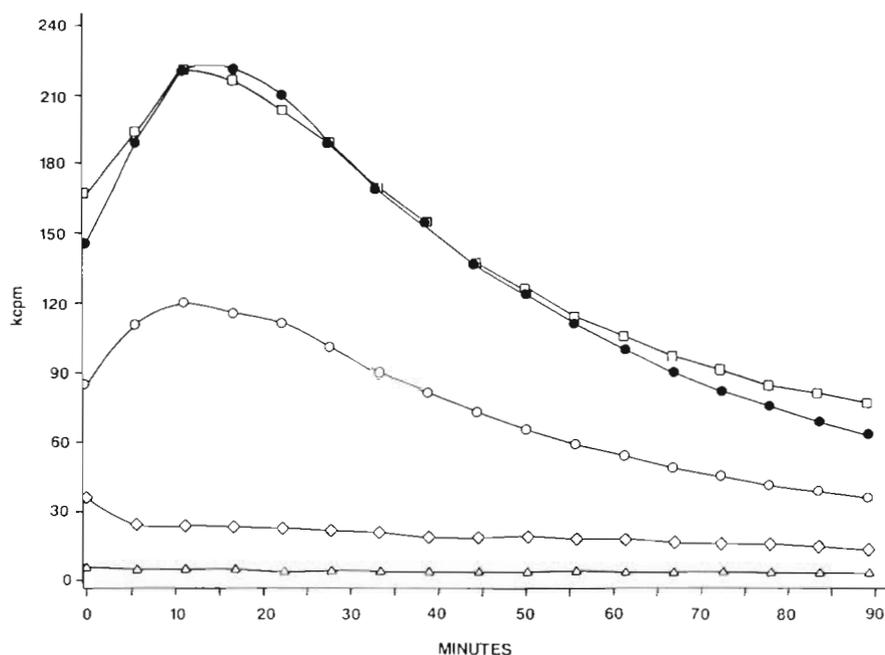

Fig. 1. Chemiluminescent response of oyster hemocytes exposed to copper in vitro for 1 h at 18°C. Stimulus: 3 ng ml^{-1} PMA. (◇) 40 ppm copper; (○) 4 ppm copper; (●) 0.4 ppm copper; (□) no copper; (△) PBS control
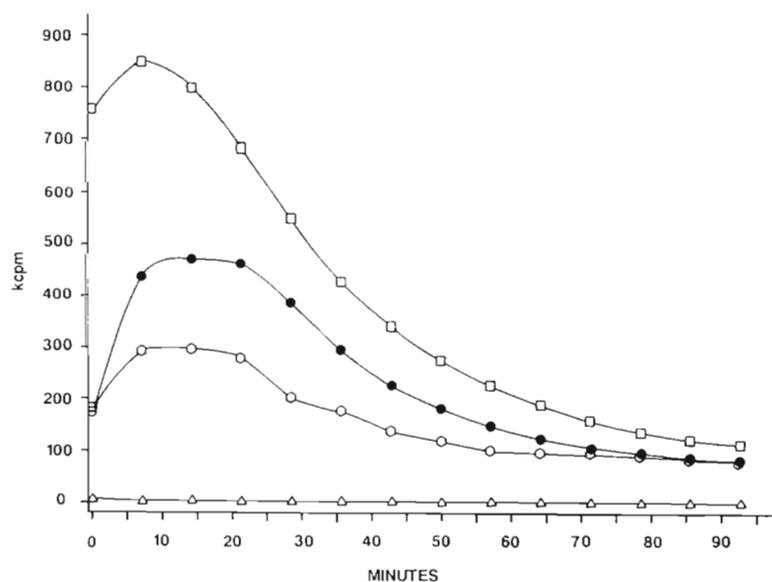
Fig. 2. Chemiluminescent response of hemocytes isolated from cysters exposed to copper for 24 h at 23°C. Stimulus: 3 ng ml^{-1} PMA. (○) 4 ppm copper; (●) 0.4 ppm copper; (□) no copper; (△) PBS control

the highest number of dead cells observed. Oysters exposed to 0.4 and 4 ppm copper for 24 h yielded hemocytes with a decreased CL activity (Fig. 2). The effect of copper on the CL response of the oyster hemocytes was greatest after 48 h exposure. After 72 and 96 h exposures, the hemocytes, though still exhibiting depressed CL, showed an increased percentage of the normal CL response, suggesting that the oysters were acclimating to the copper (Table 1).

Table 1. *Crassostrea virginica*. Chemiluminescent response of oyster hemocytes exposed to copper during a 96 h period

Exposure time[a] (h)	Response as a percent of control
0	100
24	63
48	13
72	32
96	44

[a] Oyster hemocytes were exposed in vivo at 23°C to 0.4 ppm copper for the indicated time

Exposure of hemocytes of aluminum slightly enhanced CL at a concentration of 40 to 80 ppm while no change from the control response was observed upon exposure to 160 ppm. A significant decrease in the response was noted upon exposure to 320 ppm aluminum in comparison to the unexposed controls (Fig. 3). Similarly, cadmium and zinc also enhanced the CL response of the hemocytes over that seen with the unexposed controls at concentrations below 160 ppm, whereas exposure in vitro to 320 ppm of cadmium or zinc decreased the CL response (data not shown).

Pesticides

The DMSO concentrations used in preparation of the pesticides had no effect on the CL response. In vitro, dieldrin did not alter the CL response of the oyster hemocytes at 0.1 ppm; however, incubation with 1 ppm dieldrin decreased the CL response, and incubation with 10 ppm resulted in an even greater decrease (Fig. 4). In vivo exposure to 0.1 and 1 ppm dieldrin for 24 h resulted in a significant increase in the CL response of the exposed hemocytes over the unexposed controls (Fig. 5). However, after 48 h, this enhancement had lessened and after 96 h of exposure the hemocytes exhibited a CL response that was slightly depressed in comparison with that of the control hemocytes. Chlordane, another pesticide, resulted in a dose-dependent decrease in the CL of the exposed hemocytes; however, a concentration of 100 ppm was required to cause significant depression in the CL response of the hemocytes in vitro (data not shown).

Organic compounds

Exposure to 2 organic compounds, 2,4-dinitrophenol and naphthalene, depressed CL of oyster hemocytes at high concentrations in vitro. Exposure to both compounds at 0.1 and 1 ppm did not significantly alter the CL response of the hemocytes in comparison with the unexposed control. Exposure to 10 ppm slightly decreased the CL response and a greater decrease was seen upon exposure to 100 ppm, as shown in Fig. 6 for naphthalene. In vivo exposure of oysters for 24 h to 1 ppm naphthalene resulted in hemocytes that exhibited an enhanced CL response over the control hemocytes. There was no significant difference
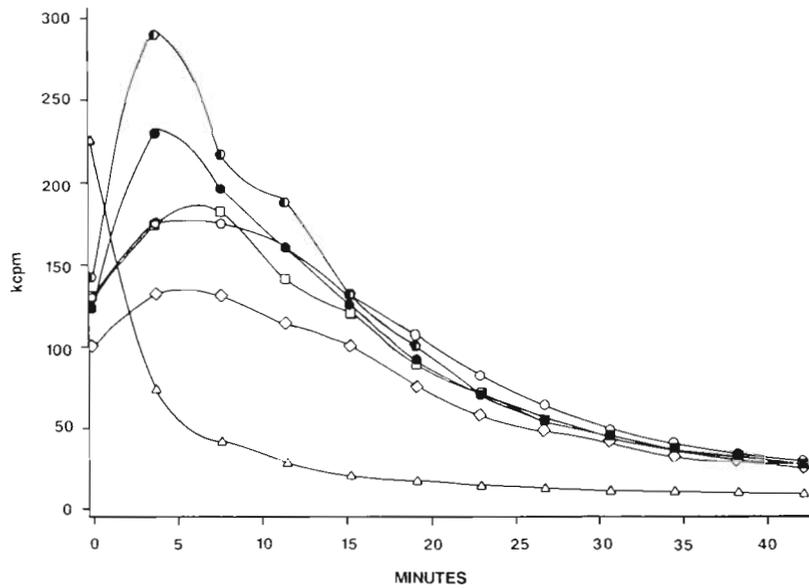
Fig. 3. Effect of exposure to aluminum in vitro for 1 h at 18°C on the chemiluminescent response of oyster hemocytes. Stimulus: 3 ng ml^{-1} PMA. ($\diamond$) 320 ppm aluminum; ($\circ$) 160 ppm aluminum; ($\bullet$) 80 ppm aluminum; ($\triangleleft$) 40 ppm aluminum; ($\square$) no aluminum; ($\triangle$) PBS control
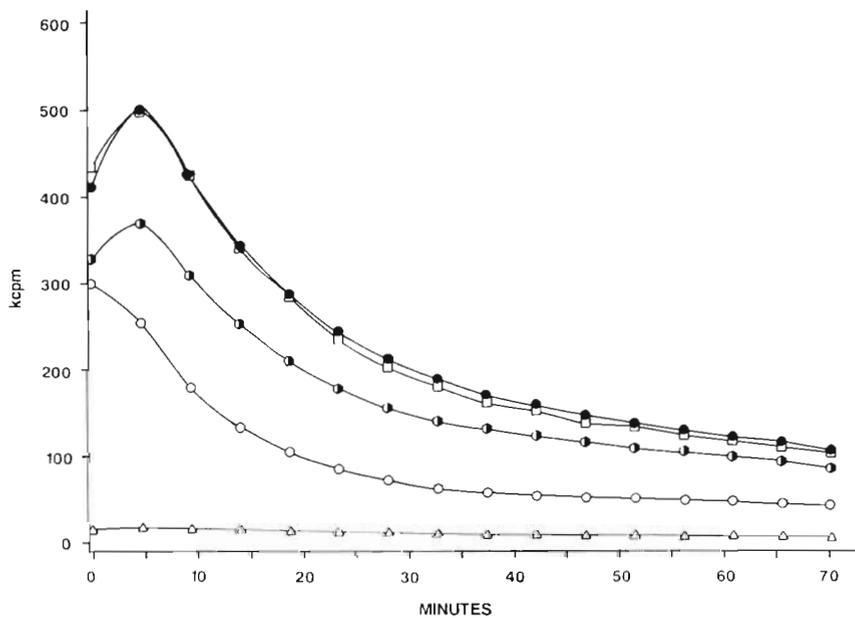

Fig. 4. Effect of dieldrin in vitro on the chemiluminescent response of oyster hemocytes after 1 h at 18°C. Stimulus: 3 ng ml^{-1} PMA. ($\circ$) 10 ppm dieldrin; ($\triangleright$) 1 ppm dieldrin; ($\bullet$) 0.1 ppm dieldrin; ($\square$) no dieldrin; ($\triangle$) PBS control

between hemocytes isolated from oyster exposed to 10 ppm naphthalene and those from unexposed oysters at 24 h. After 72 h, the hemocytes exposed to 1 ppm naphthalene were still enhanced over the response of the control oysters; however, oysters exposed to 10 ppm naphthalene showed a significantly decreased CL response (Fig. 7).

DISCUSSION

Of the pollutants tested in this study, copper appears to be the most toxic to the cells both in vivo and in vitro. This is indicated by the fact that the

levels of copper that depress chemiluminescence are much lower than the levels needed of the other pollutants. Copper concentrations in polluted waters have been measured as high as 0.1 ppm. As the oysters may accumulate the copper at 1000 times the environmental level (Pesch et al. 1979) the actual exposure may be as high as 100 ppm, a level that will depress phagocytic ability of the hemocytes. The effect of the copper may be due to killing hemocytes as well as damage to the cells while still alive. However, cell killing cannot account for the decrease in the response as the viable cell concentrations of in vivo treatment groups were adjusted just before assay so that they were equal.
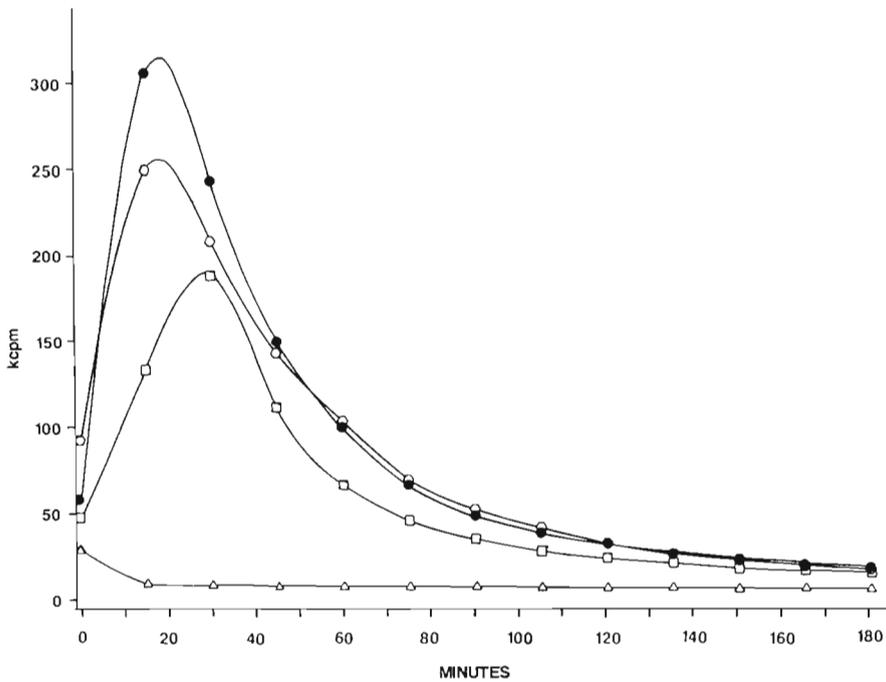
Fig. 5. Chemiluminescent response of hemocytes isolated from oysters exposed to dieldrin for 24 h at 23 °C. Stimulus: 3 ng ml⁻¹ PMA. (○) 1 ppm dieldrin; (●) 0.1 ppm dieldrin; (□) no dieldrin; (△) PBS controls
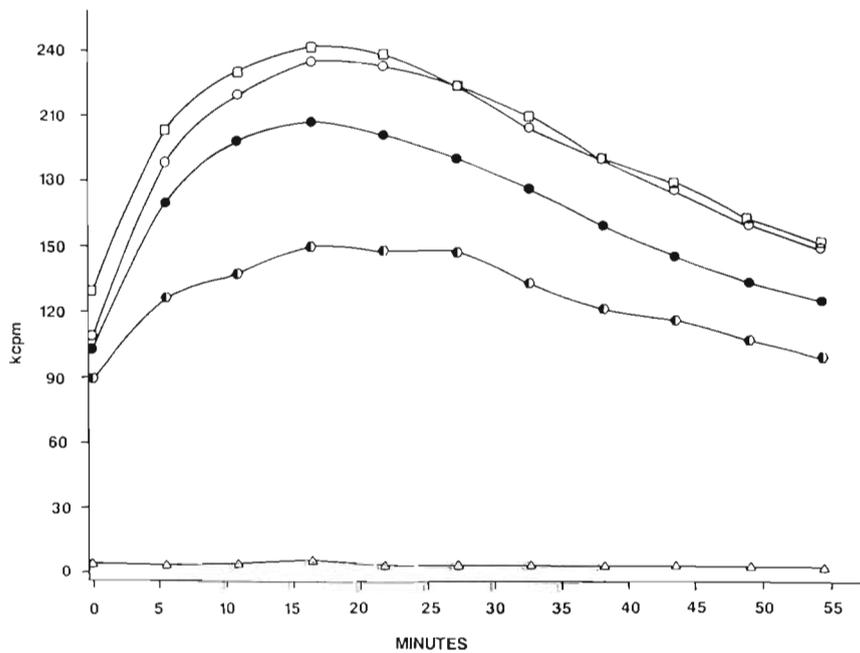
Fig. 6. Effect of naphthalene in vitro on the chemiluminescent response of oyster hemocytes after 1 h at 18 °C. Stimulus: 3 mg ml⁻¹ PMA. (◑) 100 ppm naphthalene; (●) 10 ppm naphthalene; (○) 1 ppm naphthalene; (□) no naphthalene; (△) PBS control

Evidence of acclimation is seen in oysters that are exposed to copper in vivo. The oyster hemocytes were reduced to their lowest response after 48 h exposure and then began to make a recovery during the time period studied (96 h).

Levels of dieldrin have been reported up to 0.1 ppm in polluted waters. As the oysters have been found to accumulate dieldrin at a level of up to 5000 times the environmental level (Emanuelson 1978), the levels of dieldrin the oyster hemocytes would actually be exposed to would be in the range 0.1 to 500 ppm. The levels of dieldrin used in this study fall within this range and were found to depress the phagocyte activity at levels as low as 10 ppm, well below the potential upper limit of exposure.

In vivo exposure to low levels of naphthalene and dieldrin initially stimulate and increase the CL response of hemocytes isolated from exposed oysters. This may be an initial reaction on the part of the hemocytes in response to a stressful agent. It has been
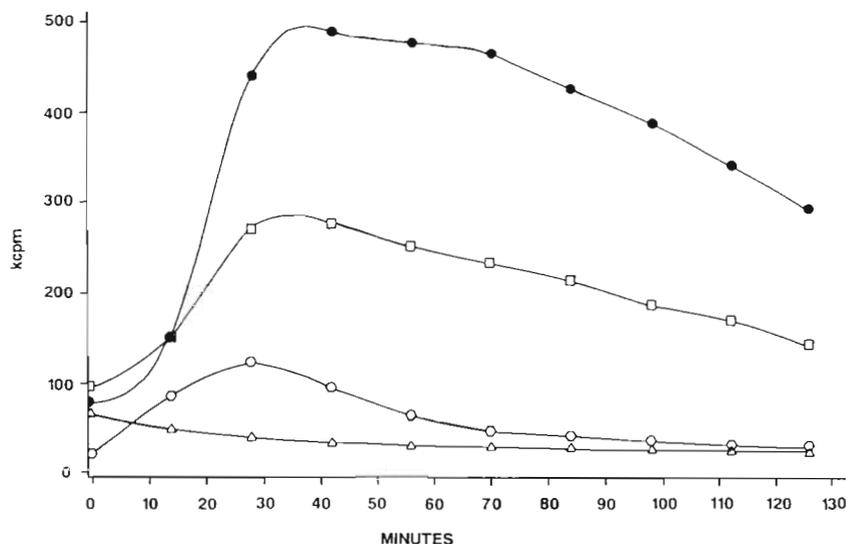
Fig. 7. Chemiluminescent response of hemocytes isolated from costers exposed to naphthalene for 72 h at 23 °C. Stimulus: 3 ng ml^{-1} PMA. (O) 10 ppm naphthalene; (●) 1 ppm naphthalene; (□) no naphthalene; (△) PBS control

observed that exposure to sublethal levels of pollutants may also enhance the phagocytic response of rainbow trout phagocytes (Elsasser et al. 1986). Although an initial increase is observed, the final effect over time is a decrease in the CL response of the exposed hemocytes. This may be due, in part, to the accumulation of the pollutant in the oyster tissues.

As seen in this study, exposure to pollutants can decrease the phagocytic ability of oyster hemocytes as measured by CL response. The sensitivity and rapidity of the CL assay, as well as the ease and practicality of the in vitro test are attractive features. Studies are currently in progress to determine if any correlation exists between depression of the CL response and increased susceptibility to infection. If there is a correlation, then the CL assay may prove to be a useful predictor of those pollutants that are harmful to finfish and shellfish.

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