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The effects of chronic inorganic and organic phosphate exposure on bactericidal activity of the coelomic fluid of the sea urchin *Lytechinus variegatus* (Lamarck) (Echinodermata: Echinoidea)

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ABSTRACT

The sea urchin *Lytechinus variegatus* can survive chronic exposure to sodium phosphate (inorganic phosphate) concentrations as high as 3.2 mg L^{-1}, and triethyl phosphate (organic phosphate) concentrations of 1000 mg L^{-1}. However, chronic exposure to low (0.8 mg L^{-1} inorganic and 10 mg L^{-1} organic phosphate), medium (1.6 mg L^{-1} inorganic and 100 mg L^{-1} organic phosphate) or high (3.2 mg L^{-1} inorganic and 1000 mg L^{-1} organic phosphate) sublethal concentrations of these phosphates inhibit bactericidal clearance of the marine bacterium *Vibrio* sp. Bacteria were exposed to coelomic fluid collected from individuals maintained in either artificial seawater, or three concentrations of either inorganic phosphate or organic phosphate. Sterile marine broth, natural seawater and cell free coelomic fluid (cfCF) were employed as controls. Bacterial survival indices were measured at 0, 24 and 48 h periods once a week for four weeks. Bacteria were readily eliminated from the whole coelomic fluid (wCF) of individuals maintained in artificial seawater. Individuals maintained in inorganic phosphates were able to clear bacteria following a two week exposure period, while individuals maintained at even low concentrations of organic phosphates failed to clear all bacteria from their coelomic fluid. Exposure to phosphates represses antimicrobial defenses and may ultimately compromise survival of *L. variegatus* in the nearshore environment.

1. Introduction

Seasonal application of phosphate pollutants in agricultural practices, leads to their accumulation in shallow marine waters mainly during spring and summer, though they may be present in lower concentrations throughout the year (Pait et al., 1992). Elevated levels of inorganic phosphates are traditionally linked to increases in algal growth and eutrophication (Justic et al., 1995; Lin et al., 1995) while organic phosphates are a component of insecticides (Pait et al., 1992). The latter are known to directly affect neuromuscular systems through the inhibition of the enzyme acetyl cholinesterase (AChE; Eto, 1974). The inhibition of AChE, an enzyme responsible for muscle relaxation, can cause tetanic stimulation in muscles and eventually mortality. Inorganic and organic phosphates are among the major factors involved in the degradation of the shallow waters of the Gulf of Mexico (Rabalais, 1992; Rabalais et al., 1994; Justic et al., 1995; Lin et al., 1995). Exposure to sublethal concentrations of inorganic and organic phosphates has been shown to adversely influence aspects of nutrition, reproduction and behavior in marine invertebrates, including echinoids (Böttger and Klinger, 1998; Böttger et al., 2001; Böttger and McClintock, 2001).

The echinoid *Lytechinus variegatus* is a common inhabitant of shallow bays and nearshore waters of the Gulf of Mexico (Serafy, 1979). Populations may occur in drainage areas and may be exposed to a wide variety of pollutants, including both inorganic and organic phosphates. Since echinoids are osmoconformers, internal fluids are similar in their ionic composition to the outside environment (Wardlaw and Unkles, 1978). Thus, body tissues within the coelomic fluid may be subjected to pollutants present in the external environment. Antibacterial defenses have been examined in a variety of echinoids (Johnson, 1968; Wardlaw and Unkles, 1978; Yui and Bayne, 1983; Service and Wardlaw, 1984, 1985; Plytycz and Seljelid, 1993). However, little work has been conducted on bacterial infections compromising the health of echinoids. Such studies have focused primarily on the effects of the bacteria *Vibrio anguillarum* and *Aeromonas salmonicida* which cause "bald sea urchin disease" characterized by spine loss and eventual death (Yui and Bayne, 1983; Maes and Jangoux, 1984, 1985; Maes et al., 1986). To date no studies have examined whether immune responses in echinoids may be weakened by chronic exposure to anthropogenic pollutants as occurs in mammalian systems (Colborn et al., 1993).

The present study investigates the effects of chronic phosphate exposure on the immune response of the common shallow-water echinoid *L. variegatus* exposed to the pathogenic bacterium *Vibrio* sp. The wide distribution and abundance of *L. variegatus* in potentially

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polluted coastal habitats and their important effect on the structure of seagrass communities (Valentine and Heck 1991; Greenway, 1995; McGlathery, 1995; Beddingfield and McClintock, 1999, 2000; Macia, 2000; Watts et al., 2001) makes it important to evaluate the effects of pollutants on the ability of this common shallow-water echinoid to defend itself against virulent microbes. Our results indicate that pollutants can have a negative impact on immune defenses of echinoids exposed to inorganic and organic phosphate pollutants.

2. Materials and methods

2.1. Phosphate pollutants

Sublethal concentrations of presumed environmental concentrations of 0.8, 1.6 and 3.2 mg L^{-1} sodium phosphate (inorganic) and 10, 100 and 1000 mg triethyl phosphate L^{-1} seawater were selected for our experiments.

2.2. Animal collection and maintenance

Lytechinus variegatus of similar size (30–50 mm test diameter) were collected from Saint Joseph Bay in northern Florida in October 1998. Individuals were collected by hand to avoid damaging the animals during collection. Upon return to the laboratory individuals were pretreated for 2 h in an aerated 50 L aquarium with 10 mg L^{-1} gentamicin dissolved in sterile seawater. Following exposure to gentamicin individuals were maintained in a 50 L holding tank with unpolluted artificial seawater for 24 h before being introduced into experimental tanks containing of 20 L of recirculating sea water maintained at ambient field conditions (22 °C and 33‰ salinity). Individuals (n=20) in each tank were maintained under unpolluted conditions and fed *ad libitum* an extruded diet formulated for echinoids (Lawrence et al., 1997) for a period of four weeks. This was done to ensure immune response recovery following potential stress caused by collection or antibiotic exposure (pers. comm. L.C. Smith). Following this four week period, tanks containing echinoids were spiked with either 0.8, 1.6 or 3.2 mg L^{-1} sodium phosphate or 10, 100 and 1000 mg L^{-1} triethyl phosphate. A control group of 20 individuals was held only in artificial seawater. All individuals were fed an *ad libitum* diet (cited above) and maintained in experimental conditions (22 °C water temperature, 33‰ salinity and 12 h light and dark) over a four week period. To maintain concentrations of phosphates in the treatments, phosphate concentrations were measured and adjusted weekly [using a colorimetric assay for inorganic phosphates and spectrophotometric analysis (APHA, 1988) for organic phosphates]. We found that this ensured stable phosphate concentrations. All experimental and control treatments were subjected to partial water changes (10 L) every 48 h and phosphate concentrations were readjusted following the water change.

2.3. Isolation and culture of bacteria

The bacterium used in our *in vitro* bactericidal experiments was isolated from the epithelium covering the test (endoskeleton) of diseased *Lytechinus variegatus* collected from Saint Joseph Bay in July, 1998. The isolate was cultured on marine agar (75% Difco Marine Broth 2216, 25% Difco Bactoagar). Following Koch's postulates, virulence was ascertained in a preliminary experiment by transferring the cultured bacterial agent onto abraded test surfaces of adult *L. variegatus*. Following exposure to the bacterial agent for a 3 day period, infected individuals began to deteriorate, with reduced tube-foot and spine movements and the elevation of the epithelial layer covering the test. Subsequently the epidermis turned white and within a three day period the infected individuals died. The isolated bacterial agent was identified as a *Vibrio* species by MIDI Labs through 16S rRNA gene alignment with GenBank. We further characterized the bacterium in our laboratory using gram stains, and by defining its growth characteristics, utilization of carbon sources (Biolog), and antibiotic inhibition (see Table 1).

2.4. Coelomic and control fluids

Three mL of whole coelomic fluid (wCF) were withdrawn weekly from five randomly selected echinoids from each treatment over the 4 week experiment. A 16-gauge 0.5-in. disposable syringe coated was used to sample coelomic fluid, by rinsing the syringe with an anticoagulant (Plytycz and Seljelid, 1993). Individuals were detached from the aquarium walls by gently rocking them to induce withdrawal of tube-feet and avoid injury.

Each individual was held oral-side down and slightly tilted to drain excess seawater. The needle was inserted through the peristomial membrane surrounding the mouth and angled towards the test to avoid penetrating the lantern or gut. Coelomic fluid (wCF) was withdrawn slowly to avoid damage to the coelomocytes and 1.8 mL aliquots were delivered into sterile tubes coated in anticoagulant. To coat tubes with anticoagulant, tubes were rinsed with 1 mL of EDTA which was removed prior to sample collection. Subsamples (0.1 mL) of coelomic fluid from each individual were plated and incubated at 22 °C immediately post removal to verify sterility.

Controls consisted of exposing *Vibrio* sp. to sterile natural seawater, sterile Difco Marine Broth 2216, or to coelomocyte free coelomic fluid (cfCF) collected from five randomly selected individuals maintained in the control artificial seawater treatment. cfCF was prepared by centrifuging for 15 min at 1789 g and decanting the supernatant to investigate importance of cellular coelomic elements in bacterial clearance. Subsamples (0.1 mL) were plated and incubated at 22 °C immediately following coelomocyte removal to verify sterility. Both natural seawater and marine broth were sterilized at 118 °C for 15 min.

2.5. Bactericidal activity

Cultures of *Vibrio* sp. were grown for 12 h at 22 °C in Difco marine broth 2216. Bacterial suspensions were prepared through serial dilutions to yield an estimated 4000 colony forming bacteria mL^{-1}. Experimental bacterial or control solutions consisted of 1.9 mL coelomic or control fluid and 0.1 mL bacterial suspension. Bacteria were added within 10 min of withdrawal of the coelomic fluid from each experimental animal. Experimental and control bacterial solutions were incubated near ambient aquarium temperature (20 °C) and mortality or growth of bacteria monitored by removing 0.1 mL subsamples at 0, 24 and 48 h. Subsamples of 0.1 mL were plated on marine agar plates (75% Difco Marine Broth 2216+25% Difco Bactoagar) and incubated for 24 h at 22 °C. Bacterial colonies on each plate were then counted and a bacterial survival index calculated using the equation: $\frac{(\text{viable count at time } t_1)\times 100}{(\text{viable count at time } t_0)}$ as given by Wardlaw and

Table 1
Morphology, growth characteristics and antibiotic inhibition of the bacterium *Vibrio* sp

Bacterium	Gram	Cell morphology	Color	Colony	Growth speed	Growth temperature	Medium	Carbohydrate utilization	Antibiotic inhibition
Vibrio sp.	Negative	Coccobacillus, short rods, often pair or chain forming	Cream white	Circular, raised, margin entire, continuous pigmentation	Rapid	15–42 °C, maximum growth at 22 °C	MA, TSA+2%	N-acetyl-glucosamine, maltose, D-trehalose, turanose, inosine, uridine, thymidine	Novobiocin (30 mg) Gentamicin (10 mg) Neomycin (30 mg) Sulfisoxalole (0.25 mg)

(MA) = Marine agar (75% Difco Marine Broth 2216+25% Bactoagar); (TSA+2%) = Tryptic soy agar, enriched with 2% NaCl.

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Unkles (1978). At time zero, subsamples of the control and experimental treatments were plated and compared to 0.1 mL subsamples of the stock solution of bacterial fluid. The equation was modified respectively, comparing time zero count to stock solution count: $\frac{(\text{viable count at time } t_0)\times 100}{(\text{viable count in stock solution})}$.

Using these equations an index value >100 represents bacterial growth, while an index value <100 indicates bacterial clearance from the coelomic fluid.

2.6. Statistical analyses

A repeated measures ANOVA followed by a Tukey-test was used to compare bacterial survival indices in experimental and control treatments over the 4 week test period. Prior to statistical analyses, assessments of the assumptions of normality (Kolmogorov–Smirnov Test) and homoscedacity (Spearman–Rank Correlation) were conducted. An arcsine transformation was conducted to normalize the data prior to statistical analysis.

3. Results

Inorganic phosphates are discharged into the environment in the form of fertilizers and urban discharges. Sodium phosphate (NaH_2PO_4), selected as the inorganic pollutant in the present study, is a common component of fertilizers (Lovejoy, 1992). Concentrations of inorganic phosphates in streams entering the northern Gulf of Mexico may reach levels of 3.2 mg L^{-1} (Lovejoy, 1992), while ambient concentrations as high as 0.8 mg L^{-1} are known to occur in pristine environments (Rafaelli pers. comm.). Concentrations of inorganic phosphates in the northern Gulf of Mexico are also known to attain levels of 0.4 to 0.8 mg L^{-1} in the spring and summer and 1.6 mg L^{-1} in the fall (Lovejoy, 1992).

Organic phosphates are composites of a variety of insecticides (Eto, 1974; Lowe et al., 1991; Pait et al., 1992). Triethyl phosphate ($(C_2H_5O)_3P(O)$), an ingredient of a wide range of organophosphorous insecticides, is known to have effects on both nerves and muscles (Eto, 1974). A half life of up to four weeks and break down products that include inorganic phosphorous and carbon dioxide (Cartwright pers. comm.) make triethyl phosphate an ideal representative of organophosphorous insecticides for experimental analysis. The use of organophosphorous pesticides has been more common since the ban of chlorinated pesticides but concentrations in the Gulf of Mexico have not yet been investigated extensively (Lytle pers. comm.). However, triethyl phosphate has been the subject of toxicological research with concentrations as high as 1000 mg L^{-1} evaluated in bioassays (Gumbmann et al., 1968).

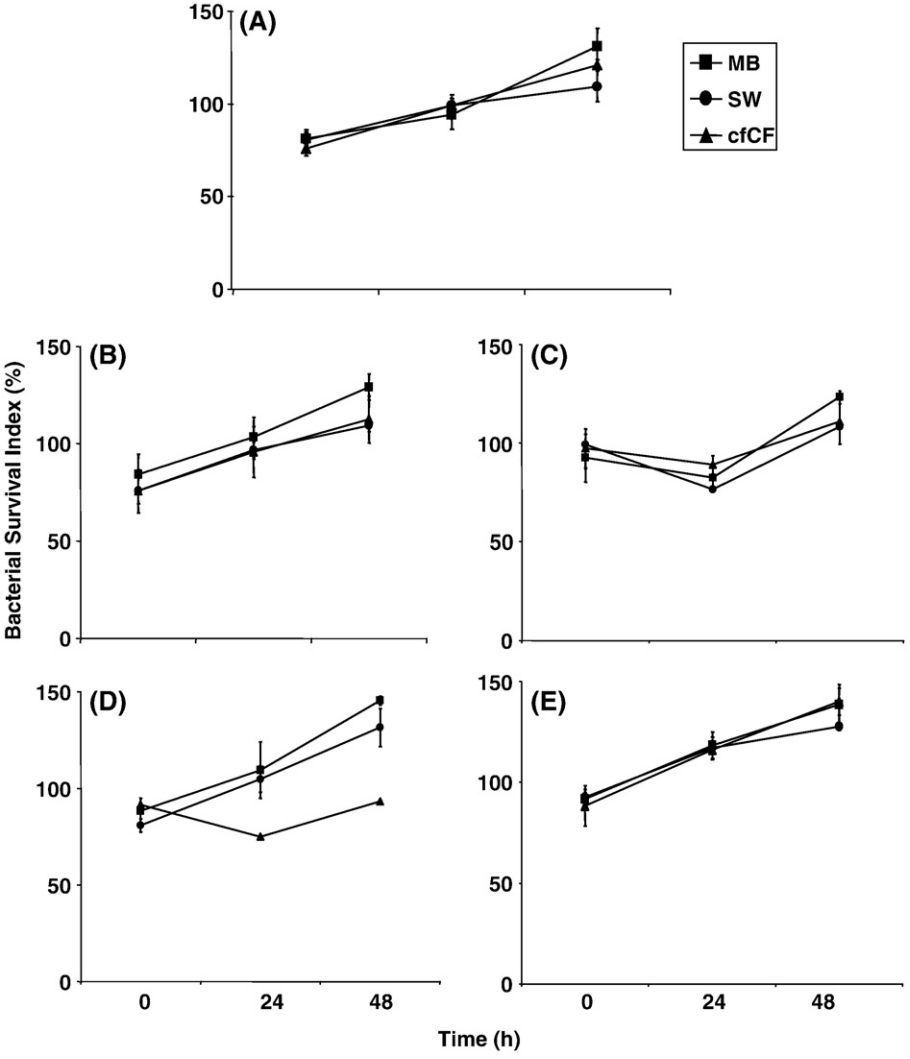

Fig. 1. Controls: Bacterial survival indices measured at time zero (A) and over a four week exposure period (B–E) in the control media [(MB) = sterile Difco Marine Broth 2216, (SW) = sterile natural seawater, and (cfCF) = coelomocyte-free coelomic fluid from individuals maintained in artificial seawater]. An index value >100 indicates bacterial growth, while values <100 represent bacterial clearance from the coelomic fluid. (mean±SE; n=5 individuals treatment $^{-1}$).

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To our knowledge there are no data available on organic phosphate concentrations in the Gulf of Mexico, though drainage of river systhems, especially the Mississippi river should ensure their presence. We therefore conducted preliminary studies to ascertain sublethal concentrations by exposing adult *Lytechinus variegatus* to increasing concentrations of triethyl phosphate over a 4 week period. Preliminary studies were conducted using logarithmically increasing concentrations of triethyl phosphate in seawater ranging from 0 to 10 g L^{-1} ($n=10$ individuals per treatment). Low concentrations of triethyl phosphate (10 mg L^{-1}) led to individuals displaying a high degree of spine and tube-foot movement and decreased locomotory and feeding behaviors. Spine and tube-foot movements were greatly reduced at 1000 mg L^{-1}, however, no mortality was observed. When maintained in concentrations of 10 g L^{-1} of triethyl phosphate, individuals displayed slowed movements and did not survive exposure longer than a period of three days.

Bacterial survival indices for the control and experimental phosphate treatments are shown in Figs. 1 and 2. Survival indices for bacteria maintained for 48 h in all three control treatments were positive and above 100%. Bacterial growth, however, varied when exposed to the different control treatments over time. During the first 3 weeks of the experiment *Vibrio* sp. grew significantly ($p<0.01$) faster when cultured in marine broth (Fig. 1) compared to bacteria cultured in sterile natural seawater or cfCF. However, no significant differences in bacterial growth indices ($p=0.79$) were detected in sterile natural seawater and cfCF during the first two weeks of the experiment. During week 3 a significant decrease in bacterial survival indices was detected in cfCF. In the fourth week of the experiment there were no significant differences ($p=0.081$) in rates of bacterial growth in all control treatments.

After a one week period, *Vibrio* sp. maintained for 48 h in wCF collected from *L. variegatus* held in artificial seawater showed complete clearance from the wCF compared to all other experimental treatments (Fig. 2). Bacterial survival was also significantly ($p<0.01$) lower in wCF from individuals held in sea water alone than in treatment containing either inorganic and organic phosphates. Patterns of bacterial survival differed when measured in wCF collected from *L. variegatus* maintained in inorganic versus organic phosphates. After a one week period bacterial survival rates in all inorganic phosphate concentrations was <15% at 24 h. These levels that were not significantly different from bacterial survival indices measured in wCF collected from *L. variegatus* maintained in artificial seawater.

Moreover, after a one week of pollutant exposure bacterial survival decreased significantly after 24 h exposure to wCF from animals maintained in all concentrations of inorganic phosphates. However, bacterial survival increased to levels significantly ($p=0.031$) higher in wCF collected from individuals maintained in the highest inorganic phosphate concentration (18 ± 1.4%) after 48 h, but did not change in wCF collected from individuals maintained in low and medium inorganic phosphates concentrations. Survival indices of *Vibrio* sp., when exposed to wCF from individuals maintained in organic phosphate concentrations were significantly ($p<0.01$) higher than in inorganic phosphate treatments and a clear concentration dose–response was evident. A decline in viable bacteria was observed at 24 h, while bacterial survival had increased again at the 48 h measurement. During the second through fourth week of pollutant

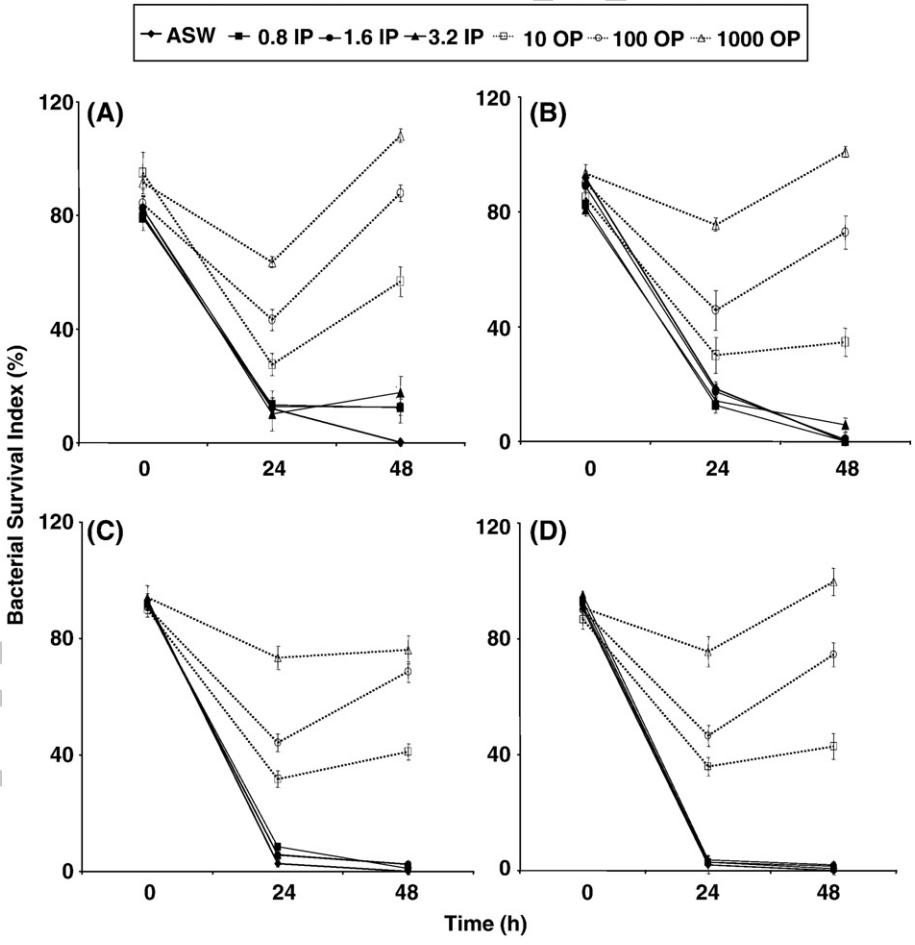

Fig. 2. Treatments: Bacterial survival indices measured over a four week exposure period (A–D) in the whole coelomic fluid (wCF) from *L. variegatus* exposed to artificial seawater (ASW), and three concentrations of either inorganic (IP) or organic phosphate (OP). An index value >100 indicates bacterial growth, while values <100 represent bacterial clearance from the coelomic fluid. (mean ± SE; $n=5$ individuals treatment $^{-1}$).

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exposure bacterial survival indices declined significantly ($p=0.02$) in wCF from individuals maintained in all concentrations of sodium phosphate (Fig. 2). Bacterial survival indices in wCF collected from individuals maintained in all inorganic phosphate concentrations were not significantly ($p=0.07$) different from survival indices of bacteria cultured in wCF from individuals maintained in artificial seawater. After a 48 h exposure period *Vibrio* sp. were absent in wCF of *L. variegatus* exposed to artificial seawater or sodium phosphates. Bacteria exposed for 48 h to wCF collected from *L. variegatus* exposed to medium and high concentrations of organic phosphate, did not display significantly ($p=0.71$) different bacterial survival indices from each other during week three (Fig. 2). During week two and four bacterial survival following 24 and 48 h exposure was significantly higher ($p>0.001$) in wCF from individuals maintained in the highest organic phosphate concentration when compared to wCF from individuals maintained in the medium organic phosphate concentration. Nonetheless, *Vibrio* sp. showed a significantly ($p<0.01$) lower bacterial survival index when cultured in wCF from individuals maintained in the lowest concentration of organic phosphate.

4. Discussion

The *in vitro* clearance of the virulent marine bacterium *Vibrio* sp. from the wCF of *L. variegatus* maintained in artificial seawater is similar to results reported for the clearance of *V. anguillarum* from the coelom of the sea urchins *Strongylocentrotus purpuratus* (Yui and Bayne, 1983) and *S. droebachiensis* (Plytycz and Seljelid, 1993). *Lytechinus variegatus* maintained in phosphate-free conditions efficiently cleared all *Vibrio* sp. from the coelomic fluid within 48 h, with the highest efficiency of clearance (90–99%) evident after only 24 h. Bacterial clearance is potentially related to the phagocytic capacity of coelomocytes, specifically those of an amoeboid nature (Johnson, 1969; Wardlaw and Unkles, 1978; Yui and Bayne, 1983; Plytycz and Seljelid, 1993; reviewed by Gross et al., 1999). Phagocytic coelomocytes are not only involved in engulfing foreign particles but contain high concentrations of enzymes to subsequently degrade and dispose of previously phagocytized material (Canicatti, 1990). Phagocytic coelomocytes comprise only one component of the immune response of echinoids (Boolootian and Giese, 1958; Karp and Coffaro, 1980; Bertheussen, 1981; Smith, 1981; Dybas and Frankboner, 1986; Gross et al., 1999). Additional coelomocytes are involved in allograft rejection (Hildemann and Dix, 1972; Karp and Hildemann, 1976), infiltration of injury (Höbaus, 1979) and cytotoxicity (Bertheussen, 1979). Additional mechanisms of echinoid immunity rely on humoral factors, including cytolytic, bactericidal and agglutinating factors (Gross et al., 1999). Survival indices of bacteria exposed to humoral factors in colomocyte-free coelomic fluid were similar overall to bacterial survival indices measured in sterile natural seawater and marine broth. Our experiments demonstrate the importance of the cellular factors only and do not provide insights into the role of humoral factors. Nonetheless, humoral factors may play an important role in bacterial clearance (Gross et al., 1999) and could be adversely affected just as cellular factors by phosphate exposure.

Our results indicate that chronic exposure to sublethal concentrations of inorganic (sodium) and organic (triethyl) phosphate decreases bacterial clearance rates in wCF extracted from the sea urchin *Lytechinus variegatus*. The level of reduction of bacterial clearance appears to depend on the type of pollutant, its concentration, and the time of exposure. These declines in bacterial clearance could be explained by reduced phagocytic activity of the coelomocytes. Since echinoids have concentrations of solutes in the coelomic fluid that are similar to those found in the outside aqueous environment, exposure to increased levels of phosphates are likely to lead to increased concentrations within the coelomic fluid (Robertson, 1980). The coelomic fluid of echinoids (*Echinus esculentus* and *Paracentrotus lividus*) under natural ambient conditions ranges from 0.18–0.22 mg L^{-1} inorganic phosphate (Robertson, 1980). Thus, exposure to significantly increased concentrations of inorganic and organic phosphates and concomitant increases in coelomic phosphate concentrations could stimulate rapid intracoelomic bacterial growth. Should bacterial growth be increased beyond the capacity of the coelomocytes then observed reductions in bactericidal activity could in fact be attributable to rapid bacterial growth rather than reduced coelomocyte activity. Moreover, the introduction of organic triethyl phosphate ($(C_2H_5O)_3P(O)$) to the marine environment also results in increased carbon loading within the coelom. These increases in carbon could further enhance intracoelomic bacterial growth and reduce associated bactericidal clearance.

When initially exposed to low, medium and high concentrations of inorganic and organic phosphate, wCF from individuals held in all phosphate treatments showed a reduction of bactericidal activity as evidenced by increased bacterial survival when compared to the bactericidal activity of coelomic fluid from individuals held in artificial seawater. However, after a one week exposure period, wCF from *L. variegatus* maintained in all inorganic phosphates displayed an acclimatory immune response, meaning full bactericidal clearance activity by the coelomocytes following an initial lag, with complete bacterial clearance after a 48 hr exposure period. This indicates that stress induced by exposure to inorganic phosphates can temporarily inhibit bactericidal activity, as reported for organisms experiencing stress caused by both abiotic and biotic factors (Colborn et al., 1993). When exposed to organic phosphates, bactericidal clearance was dose dependent and acclimation did not occur over the four week experimental period. Partial acclimation with decreased survivorship of *Vibrio* sp. was observed in wCF collected from individuals maintained in the lowest organic phosphate concentration where levels of bacterial survival decreased from 51 to 34% over the four week exposure period. Exposure to the medium and high organic phosphate concentrations, however, did not cause a decrease in bacterial survival. This indicates that *L. variegatus* maintained in sublethal but chronic medium to high concentrations of organic phosphate will be compromised in their ability to defend themselves against microbial infection (Johnson, 1968; Wardlaw and Unkles, 1978; Yui and Bayne, 1983; Service and Wardlaw, 1984, 1985; Plytycz and Seljelid, 1993).

In summary, *Lytechinus variegatus* maintained under unpolluted conditions were capable of effectively eliminating the bacterial pathogen, *Vibrio* sp., known to be lethal to this species. In contrast, *L. variegatus* chronically exposed to sublethal concentrations of inorganic phosphates required an acclimation period of one week before eliminating the bacterial pathogen, while individuals exposed to organic phosphates never cleared this pathogenic bacterium from wCF during the four week experimental period. Aspects of nutrition, reproduction and behavior are similarly compromised in *L. variegatus* due to stress induced by exposure to inorganic and organic phosphates (Böttger and Klinger, 1998; Böttger et al., 2001; Böttger and McClintock, 2001). Thus, phosphate-induced changes in bactericidal activity add yet another dimension to the overall compromised health of echinoids under conditions of phosphate pollution. Our results indicate that *L. variegatus* occurring in estuarine and riverine drainage areas within the northern Gulf of Mexico that contain phosphate pollutants may become immunologically compromised against pathogens in their natural environment. As *L. variegatus* plays an important ecological role in determining the community structure of nearshore seagrass communities (Valentine and Heck, 1991; Beddingfield and McClintock, 2000; Watts et al., 2001), changes in population demography resulting from increased susceptibility to microbial infection may have community-wide ramifications.

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