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THE COMBINED EFFECTS OF CHEMICAL AND NATURAL STRESSORS ON PHOSPHAGENS AND NONSPECIFIC IMMUNITY IN TWO SPECIES OF ABALONE

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

DOCTOR OF PHILOSOPHY
in
BIOLOGY

by
Linda Beth Martello

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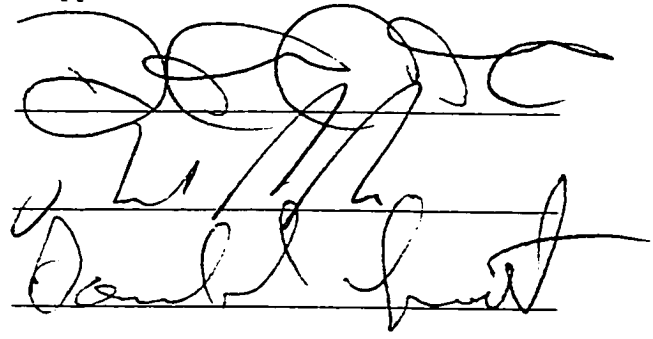
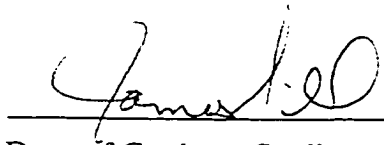
is approved:





Dean of Graduate Studies and Research

THE COMBINED EFFECTS OF CHEMICAL AND NATURAL STRESSORS

ON PHOSPHAGENS AND NONSPECIFIC IMMUNITY

IN TWO SPECIES OF ABALONE

Linda Beth Martello

ABSTRACT

Whereas there is considerable information on the toxicity of xenobiotics for marine invertebrates, as measured in terms of lethality, comparatively little is known of the more subtle physiological effects of chronic exposure to sublethal doses of toxicants. Environmental pollutants can have acute effects on organisms exposed in that the concentration of toxicant may be sufficiently high to cause injury or death in a short period of time. It is more likely, however, that toxicants are present in low concentrations and have sublethal effects on aquatic organisms which predispose them to debilitating disease. By examining phosphagen concentrations after exposure to sublethal levels of a model chlorinated phenol, pentachlorophenol (PCP), it is possible to elucidate the subtle alterations in energy regulation that are vital for the survival of any organism. PCP is a universally toxic compound which is used as a general biocide and because of its fungicidal properties, as a wood preservative. PCP induced reduction in energy production might effect an organism's ability to forage, reproduce, and defend itself from predators as well as endure adverse environmental conditions. This thesis

initially addresses how exposure to pentachlorophenol combined with salinity stress effects concentrations of phosphoarginine (PA), adenosine triphosphate (ATP), intracellular pH (pHi), and inorganic phosphate (Pi) among both red (*Haliotis rufescens*) and black abalone (*H. cracherodii*) using in vivo ^{31}P NMR spectroscopy. Black abalone appear to have a greater overall resistance to PCP exposure and salinity variations as affirmed by ^{14}C PCP uptake analysis demonstrating their slower uptake of the biocide.

The study continues by examining how sublethal exposure to this general biocide might effect an organism's ability to defend itself against microorganisms, thereby representing an experimental approach to describing functional differences between hemocytes that have been exposed to a toxin and hemocytes that are unexposed. Using the same flow-through exposure system as that developed for NMR spectroscopy, abalone were exposed to sublethal concentrations of PCP in combination with salinity variations. A poorly functioning immune system in animals inevitably exposed to a number of invading microorganisms can cause their death. It is therefore important to describe how toxins effect these defense mechanisms. Because the timing of the decline in various species of abalone have shown tremendous variation the defense capabilities in hemocytes of red versus black abalone after exposure to PCP was compared. A tool for comparison used with NMR was adopted for assessment of host defense mechanisms. The metabolic endpoint (MEP) served as a gauge for the level of stress that the abalone were

enduring. It is defined as the *time* for the spectral peak area of inorganic phosphate (Pi) to reach one-half that of phosphoarginine (PA) and was demonstrated to be the maximum amount of time abalone could be exposed and still consistently recover. These exposure periods were employed for assessment of hemocyte defense mechanisms to determine if the effects seen using NMR could be correlated with impaired immune function.

Phagocytic and chemotactic function after *in vivo* exposure of abalone to PCP and salinity stress was examined. Most noteworthy was the dramatic effect that high salinity, particularly in combination with PCP, had on both red and black abalone; red abalone demonstrating the most profound effects. As was previously demonstrated using ^{31}P NMR, it took black abalone nearly twice as long to manifest toxic effects of PCP on phagocytic and chemotactic ability as that for red abalone. Furthermore, phagocytic function proved to be significantly more robust overall among black abalone.

Finally, mechanisms employed to kill microorganisms among red and black abalone were examined. These included the quantification of reactive oxygen intermediates as well as lysozyme. While exposure of red abalone to salinity variations plus PCP caused a stimulatory effect on the production of microbicidal reactive oxygen intermediates (ROIs) the same exposure parameters caused an inhibitory effect on ROI production among black abalone. Furthermore, no lysozyme-like activity was discernable for any of the exposure parameters even upon stimulation with a variety of antigens.

As aquatic organisms are rarely exposed to one stress at a time the research performed in this dissertation has contributed to the understanding of the impacts of multiple environmental stresses. Those responsible for managing threatened aquatic environments will hopefully find the information herein useful to the understanding of the processes and mechanisms that govern such effects as stress.

Table of Contents

Chapter One

Introduction: Stress Effects and Their Impact on Energetics and Nonspecific Immunity

There is a considerable amount of information linking stress with the inability of aquatic organisms to resist disease as a result of modulation of the immune system (Snieszko, 1973; Sindermann, 1979; Cheng, 1990; Anderson, 1993). Stress can be induced by a number of factors including temperature, salinity or anthropogenic factors such as release of xenobiotics into the environment. The phagocytic cells of an organism are the first line of defense against invading microorganisms and potential pathogens and therefore any modulation of either the specific or non-specific aspects of immunity could potentially effect resistance to disease and parasitism.

Abalone Life History

The largest of the abalone species is the Red abalone (*Haliotis refescens*), which inhabits the low intertidal and subtidal zone. Along the West coast, Abalone are found from Sunset Bay, Oregon to Bahia de Tortugas, Baja Mexico (Abbott and Harderlie, 1980). Adult abalone occur as separate sexes, and reproduce through the release of gametes. Twelve to twenty-four hours after the eggs are fertilized trochophone larvae hatch and develop into shelled veliger larvae. The veliger larvae remain swimming for an additional 4-14 days until settlement and metamorphosis occur (Abbott and Harderlie, 1980). Because larval abalone are considered to be highly sensitive to environmental conditions and toxicants, they have been used extensively in the state of California to evaluate toxicity of effluent discharges.

The Decline of the Abalone Population and Withering Syndrome

Eight north American abalone are found along the western coast of California. Significant declines in populations have occurred over the last 20 years. Figure 1 illustrates an approximate 70% decline in abalone fisheries production in California alone. Along the coast of the California channel islands the abundant black abalone *Haliotis cracherodii* began to die in large numbers in 1985 (Haaker et al., 1992). This die-off has spread to most of the islands and still continues. Assessment of the changes in abalone populations at numerous locations suggests that this is the result of an infectious disease spreading from the southern side of Santa Cruz Island (Lafferty, 1993). Extensive infection of digestive diverticular cells and the resultant deficiency in digestive enzymes correlates to the degree of foot muscle atrophy and the severity of signs associated with a condition known as withering syndrome (WS). WS is a progressive wasting disease characterized by the shrunken appearance of the foot muscle, retracted visceral tissues, and the inability to tightly adhere to the substrate (Haaker, 1992). Other symptoms include color changes of the foot and epipodium and reduced gonadal size (Haaker *et al.*, 1992). Reductions in populations of other abalone species have all contributed to a general decline in harvest of abalone as illustrated in Figure 1. Such hypotheses as over-harvesting, increased market demand, increased sport fishing demands, expanding sea otter populations, loss of habitat, marine pollution, or factors related to physiological stresses induced by warm temperatures, starvation and competition have been invoked (Lafferty, 1993).

A recent study has suggested a pathogen-disease association in WS. The pathogen implicated is an intracellular prokaryote that infects epithelial cells lining the gut and enzyme-secreting cells of the digestive diverticula (Gardner *et al.*, 1995). This pathogen is a rod-shaped, ribosome-rich, gram-negative, prokaryote with a trilaminar cell

wall consistent with Rickettsiales (Gardner *et al.*, 1995). Foot muscles used for locomotion and attachment on rocky substrate are weakened by atrophy in WS and all abalone examined from the California Channel Islands where WS prevails were infected with the rickettsiales organisms. Metaplasia of the digestive diverticula was the most common and the most serious aspect of GI tract involvement (Gardner *et al.*, 1995). Together with widespread atrophy of uninfected diverticula that further reduced enzyme production, the progressive involvement of the alimentary system was correlated to the degree of foot muscle atrophy and the increasing severity of the gross signs of WS (Gardner *et al.*, 1995). Special stains used by Gardner *et al.* demonstrated degenerating myofibers, loss of orientation of myofibrils and myofilaments, degeneration of connective tissue, and infiltration by hemocytes. Gardner *et al.* hypothesized that abalone rickettsiales is directly communicable via the gastrointestinal tract (GI) because of its high rate of intracytoplasmic division and release from GI tract lining cells into the gut lumen. Also, black abalone from a sewer outfall in Los Angeles County that had WS like symptoms transplanted to Santa Catalina Island transmitted the bacteria to otherwise unaffected Santa Catalina Island black abalone (Young, 1964). Moreover, WS is no longer found only among black abalone as red, pink and green abalone have also been targeted.(C.Friedman, person. commun.).

The immune systems of molluscs, including abalone, depends on circulating hemocytes present in blood sinuses which are capable of migrating throughout tissues to protect against potential pathogens and undertake immunoserveillance. The primary defense strategies involve phagocytosis (Renwrantz and Stahmer, 1983), as well as the release of reactive oxygen metabolites and degradative enzymes (Pipe, 1990b, 1992; Coles and Pipe, 1994). Both locomotion and microbial killing are energy requiring

processes so any reduction in available phosphagens could effect the integrity of the immune system.

Mammals posses two branches of the immune system: innate or nonspecific immunity as well as acquired or specific immunity. The innate branch of the immune system comprises defensive barriers such as anatomical barriers, physiological barriers, endocytic and phagocytic barriers as well as inflammatory barriers. Furthermore, mammals possess an acquired immune system which can be described as the functional part of the immune system capable of recognizing and selectively eliminating foreign microorganisms and molecules (Kuby, 1992). Unlike innate immunity, acquired immunity displays specificity, diversity, memory, and self/nonself recognition. Invertebrates, including molluscs, typically possess only the inate branch of the immune system. So, unlike mammals and teleost fishes, invertebrates are generally incapable of generating antibodies against foreign invaders which is an aspect of acquired immunity. They therefore depend on the innate branch of the immune system which is a primitive arm of the sophisticated mammalian immune response.

Assays capable of quantifying phagocytosis, an arm of nonspecific immunity, can provide an overall measure of the status of the internal defense system of invertebrates as well as be used to study fundamental aspects of invertebrate immunology. Possible links between pollution and the incidence of disease and various abnormalities in fish and shellfish has led to immunocompetence assays being used to study aquatic environments. Several examples of potentially important environmental contaminants which alter specific components of the immune system of shellfish species have now been reported. (Anderson, 1981; Cheng and Sullivan, 1984; Pickwell and Steinert, 1984; Cheng, 1988a, 1988b, 1990; Larson *et al.*, 1989; Suresh and Mohandas, 1990; Anderson *et al.*, 1992; Anderson, 1993; Coles *et al.*, 1994; Pipe *et al.*, 1995; Coles *et al.*, 1995). These studies

have not examined combined effects of environmental stress and toxicant exposure and have typically focused on only one aspect host defense. The research performed here has included a battery of tests for evaluation of host defense mechanisms. The study of invertebrate immunotoxicology is early in its development but will presumably extend our understanding of the physiological basis of disease in natural populations.

The hematology of abalone is markedly different than that of other molluscan species studied (Shields et al., 1997, unpublished data). Foremost, abalone lack circulating granulocytes. Functional granulocytes are only found associated affixed to tissues. Conversely, granulocytes comprise the majority of cell types found in pulmonate gastropods (Cheng, 1975; Yssel and Wolmarans, 1989), oysters (Cheng, 1975; Ruddell, 1971, Auffret, 1988, 1989), venerids (Cheng, 1975; Auffret, 1988) and mytilids (Moore and Lowe, 1977; Rasmussen et al., 1985). Pectinids, like the abalone, do not possess free granulocytes (Auffret, 1988; Auffret, 1989), but the patellids, which are closely related to abalone do possess circulating granulocytes (Davies and Partridge, 1972).

Shields et al. (1997, unpublished data) identified three subpopulations of free hemocytes. Small hemocytes that are most likely stem cells that would develop into hyalinocytes. The Type I hyalinocyte possesses moderate to large pseudopodia and occupy approximately 20% of the cells in the hemolymph. The Type II hyalinocyte are rounded, with small thin pseudopodia when not in contact with a surface. They possess a large cytopplasm to nucleus ratio, and a few small, electron-rich granules. Upon contact with a surface, Type II hyalinocytes spread quickly and extend numerous pseudopods. This cell type comprises over 80% of the cells of abalone hemolymph.

Natural environmental variation is known to play an important role in the defense response for many animals, but may be particularly important for marine molluscs, which are poikilothermic osmoconformers. As such their internal environment mimics the

salinity and temperature of the external environment, which may change with seasonal, diurnal, and tidal cycles. Temperature and salinity variations have been shown to effect endocytosis, inflammation, wound repair, encapsulation, and enzyme secretion (Feng and Feng, 1974; Cheng and Foley 1975; Moore, 1976). Fisher *et al* (1986) found that increased salinity not only caused hemocytes to spread more slowly (an indication of the response-time of the hemocytes) but also caused a reduction in the rate of hemocyte locomotion towards an antigen. This in vitro experiment focused on the time from stimulation to locomotion of hemocytes thereby indicating how salinity variations were able to adversely effect the hemocytes ability to react to a pathogen in a timely manner. These results imply that the time period was being used by the hemocytes for osmotic adjustment and that the hemocytes might have to be iso- or hyperosmotic to their environment in order to become ameboid. Hemocyte spreading may be linked to intracellular volume regulation and the salinity effects on hemocyte locomotion may illustrate a fundamental characteristic of ameboid locomotion.

This research will contribute to the identification of cell processes involved in host-environment interactions and will examine the combined effects of environmental stress and toxicant exposure on hemocyte function

Pentachlorophenol

The compound employed in these experiments, pentachlorophenol, was widely used in wood preservation while also serving as a general biocide. This phenolic compound is toxic to animals, plants, and microorganisms and has been widely used as a pesticide. PCP acts as a potent uncoupler of mitochondrial oxidative phosphorylation whereby it effectively shuttles protons across the inner mitochondrial membrane thereby dismantling the gradient required for the production of ATP proving highly toxic to many

marine organisms as well. Whereby PCP uncouples mitochondrial oxidative phosphorylation at low concentrations (Weinbach, 1956, 1957) it causes complete inhibition of the electron transport chain at higher concentrations (Desaiah, 1978), the locus of action being the cell membrane and mitochondrial ATPases. A wide variety of secondary effects of PCP have been described in mammals, including liver histopathology, and interference with the drug-metabolizing hepatic microsomal cytochrome P-450 mixed-function oxygenase system (Debets et al., 1980). Marine organisms have also demonstrated a senstivity to this biocide. Injected PCP is accumulated in the hepatopancreas of marine animals where it is esterified to various metabolites (Bose and Fujiwara, 1978). There is also a change in lipid composition of the hepatopancreas of PCP-dosed marine animals (Bose and Fujiwara, 1978). Furthermore, several glycolytic and tricarboxylic acid cycle enzymes are either inhibited or stimulated by *in vivo* or *in vitro* exposure to Na-PCP (Fox and Rao, 1978) and oxygen consumption was found to be significantly inhibited by PCP exposure in crustaceans (Cantelmo and Rao, 1978).

Sublethal concentrations of PCP have been shown to elicit a rapid elevation in blue crab hemolymph glucose concentration, accompanied by a gradual depletion of hepatopancreatic and gonadal glycogen reserves (Coglianese and Neff, 1982; Fingerman et al., 1981). Several effects of PCP on intermediary metabolism in a variety of invertebrates have been described (Bose and Fujiwara, 1978; Rao et al., 1979). These include inhibition of oxygen consumption by high PCP concentrations and inhibition at lower concentrations of key pentose phosphate cycle, glycolytic, and tricarboxylic acid cycle enzymes and membrane ATPases certainly causing a rapid decrease in glycogen concentration in tissue.

Nuclear Magnetic Resonance Spectroscopy

Nuclear Magnetic Resonance Spectroscopy (NMR) is a technique that combines high sensitivity with the use of unstressed animals to describe sublethal effects of toxicant exposure. This non-invasive technique is able to detect nuclei that possess a magnetic moment ie. ^{1}H, ^{15}N, ^{31}P. The net magnetization can be perturbed whereupon spectra can be collected upon the return of the magnetic field to its equilibrium. The use of ^{31}P NMR spectroscopy allows the measurement of endogenous energy compounds such as phosphoarginine, phosphocreatine, nucleoside phosphates, inorganic phosphate as well as intracellular pH (Gadian, 1982). Quantifying these compounds allows for the assessment of chemical and natural stressors on mitochondrial electron transport and oxidative phosphorylation.

It seems plausible that the diverse group of microorganisms that inhabit the mantle fluid of the abalone might act opportunistically. Environmental stresses combined with stresses such as overharvesting and pollution may support growth of this diverse group of organisms. Although the influence of pollution in the decline of abalone has not been observed directly these chemicals may indirectly influence infectious organisms by increasing their virulence or reducing host resistance thereby making the host more susceptible to pathogens. The objective of the studies carried out in this thesis were to establish whether a relationship could be confirmed between a reduction in energy charge by sublethal doses of a model uncoupler of oxidative phosphorylation and the functioning of the immune system in abalone. These studies require confirmation and

expansion, and the extent to which they can be extrapolated to field situations of pollutant exposure needs to be determined.

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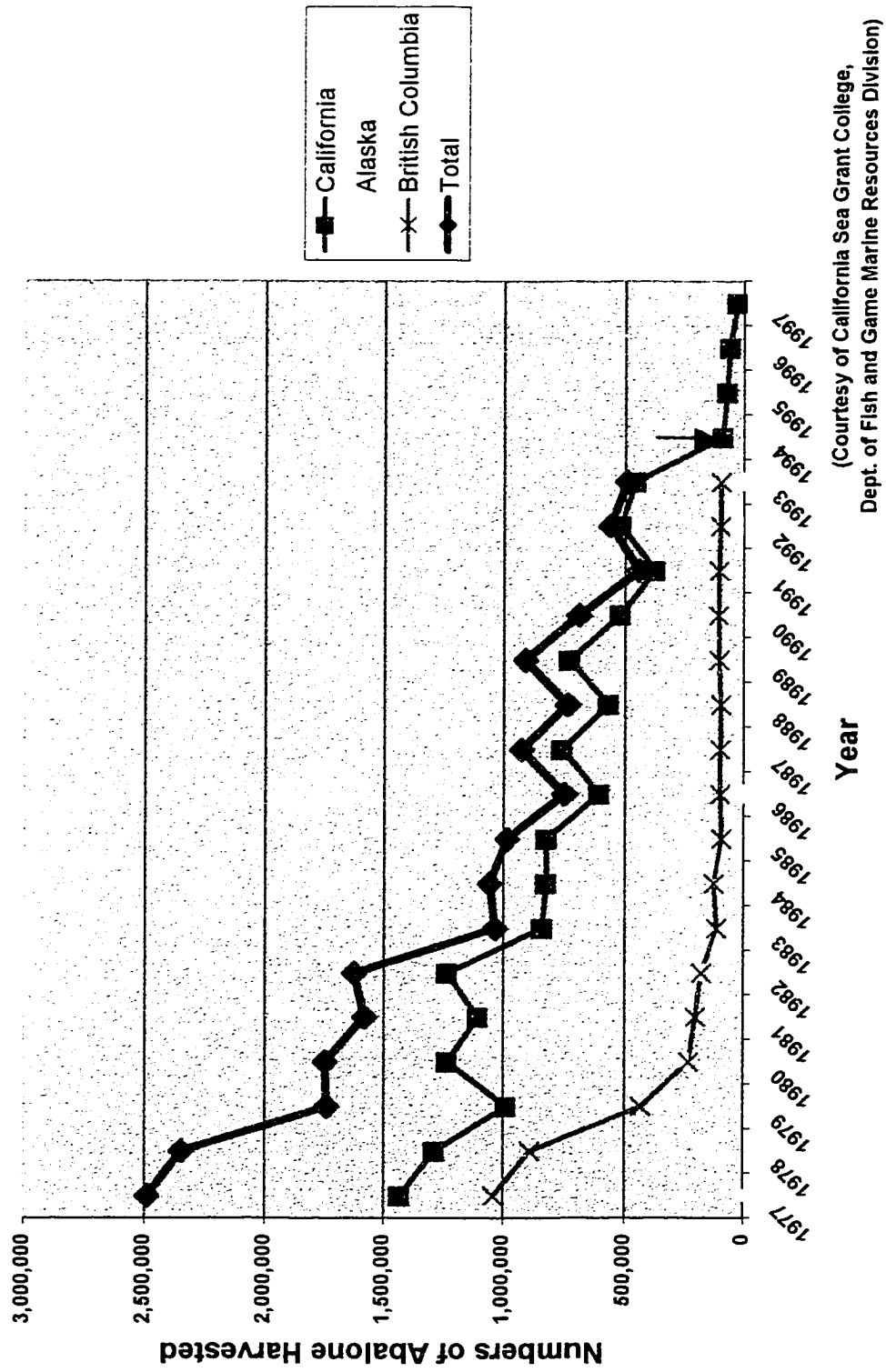
Fig 1. Abalone fisheries production in three regions from 1977-1997 (California, Alaska, British Columbia). Species include red, black, pink, green, white, pinto, and threaded Abalone



Chapter Two

Influence of Salinity on the Actions of Pentachlorophenol in *Haliotis* as Measured by *In Vivo* ^{31}P NMR Spectroscopy

Introduction

Pentachlorophenol (PCP), an uncoupler of mitochondrial oxidative phosphorylation, acts by destroying the electrochemical potential across the inner membrane of mitochondria (Mitchell, 1961, 1966; Mitchell and Moyle, 1967). A potent fungicide, herbicide, bactericide, and algaecide, it is introduced into California coastal waters from pressure treated wood pilings, anti-fouling paints and urban runoff sources. PCP has been detected in coastal waters of California and in the tissues of a variety of marine animals (Pierce and Victor, 1978).

Along the California coast, the red abalone (*Haliotis rufescens*) resides in both the low intertidal and subtidal zones, while the black abalone (*H. cracherodii*) resides in the mid to high intertidal zones. Typically, they are exposed to water salinities ranging between 32 $^{0}/_{00}$ and 35 $^{0}/_{00}$, although they may be exposed to salinities as low as 25 $^{0}/_{00}$ during seasons of high precipitation or, conversely, higher salinities during evaporative water loss in tide pools (J. Pearse, pers. commun., 1994). During a normal tidal cycle, abalone are exposed to a variety of environmental stresses including air exposure, temperature variations, as well as salinity variations . Changes in salinity have been hypothesized to contribute to withering syndrome (WS), which has caused black abalone populations to decline by as much as 99% around the Channel Islands (Ebert, 1990). Although *Rickettsiales* has been implicated as the responsible pathogen (Gardner *et al.*, 1995), pollutant exposure combined with environmental stresses may enhance its activity, thus contributing to the overall decline in the abalone population in California.

Due to their sedentary nature and large foot muscle, intact abalone are ideal for surface-probe localized nuclear magnetic resonance (NMR) spectroscopy. Application of *in vivo* ^{31}P NMR for illustrating the sublethal biochemical actions of toxicants in intact aquatic organisms, and the influence of natural stress factors, has previously been

demonstrated (Tjeerdema *et al*, 1991a, b, c; 1993). The ^{31}P nucleus is highly useful for *in vivo* NMR because of its high natural abundance and presence in important endogenous compounds. Measurable changes include those in the levels of phosphoarginine [PA] or phosphocreatine [PC], nucleoside phosphates (NPs), inorganic phosphate (Pi), and intracellular pH (pHi).

Invertebrate muscle contains a reservoir of high potential phosphoryl groups in the form of PA. The phosphoryl transfer potential of PA is higher than that of ATP which allows PA to maintain a high concentration of ATP during periods of exertion. When an organism is stressed, ATP is rapidly consumed and ADP can no longer be converted efficiently to ATP, whereupon PA gives up its phosphate to maintain steady state levels of ATP. Conversely, the recovery period was defined by the time required for PA and Pi concentrations to reach their pre-exposure values.

The objectives of this study were to (1) compare the biochemical actions of PCP in red abalone exposed to PCP at three different salinities; (2) compare the actions with those of black abalone exposed under the same conditions; and (3) determine whether the biochemical actions were dependent upon differential PCP disposition in the two species.

Materials and Methods

Chemicals

PCP sodium salt (p*K*a, 5.3; water solubility at 20°C, 80 mg/l; Merck Index, 1983) and methylene diphosphonate (MDP) were purchased from Aldrich Chemical Co. (Milwaukee, WI) and methanol was obtained from Fisher Scientific, Inc. Natural, seawater was collected and filtered (0.2 μm) at the UCSC Long Marine Laboratory, stored at 12°C and used within 3 d. Instant Ocean (Aquarium Systems, Mentor, OH) was used to adjust seawater salinity to 45 $^{0}/_{00}$, while distilled water was used to dilute seawater to 25 $^{0}/_{00}$.

[U-^{14}C] PCP (10.6 mCi/mmol toluene) was obtained from Sigma Chemical Co. (St. Louis, MO). Scintiverse I liquid scintillation cocktail, Scintigest tissue solubilizer, and anhydrous sodium sulfate were purchased from Fisher Scientific, Inc. Amberlite XAD-4 resin was obtained from Rohm and Haas (Philadelphia, PA).

Animals

Red abalone (*H. rufescens*), averaging 3 y in age and 146 g, were provided by U.S. Abalone, Davenport, CA. Black abalone (*H. cracherodii*) averaging 3 y in age and 168 g, were collected at Ano Nuevo Island, San Mateo County, CA. They were maintained in flowing seawater, fed giant kelp (*Macrocystis pyrifera*), and acclimated to 14°C for at least one week prior to use.

Exposure System

The NMR flow-through exposure system has been previously described (Tjeerdema *et al.*, 1991a; 1991c). Briefly, abalone are placed in a Tedlar® fluoropolymer bag (Aeroenvironment, Monrovia, CA.), which was anchored into an acrylic chamber and sealed. While the bag provided an inert containment for organism exposure, the box provided a solid support for the bag and a secondary containment to insure against leakage. Aerated and temperature-controlled seawater was pumped from a 20-liter carboy with a peristaltic pump through silicon tubing. PCP was combined with the seawater flow using a second pump from a 6-liter collapsible fluoropolymer gas sampling bag. The same method of exposure was used for [U-^{14}C]PCP toxicokinetic experiments.

^{31}P NMR Spectroscopy

^{31}P NMR spectra were collected using a one-turn spiral copper wire surface coil (4-cm dia.) on a General Electric CSI-2 spectrometer equipped with a 2-Tesla 225-mm clear-bore horizontal magnet. Methylene diphosphonate (MDP, pH 9.5) in a capillary tube mounted on the probe served as an external standard. One-dimensional data sets

were obtained using a 50-55 μs pulse width, a ± 1500-Hz spectral width, a 2-s pulse delay, and 2048 sample points. Files averaged 300 transients (11.7 minutes of acquisition time). For relative quantitation peak intensities were normalized to MDP, and pHi was calculated from the differences in chemical shift between the Pi and PA resonances and a standard calibration curve (Tjeerdema *et al.*, 1991a). Results were compared using a one-tailed Students t-test (Mason *et al.*, 1988).

NMR Exposure

For each species three sets of three abalone were first acclimated to ambient salinity for 2 h prior to afternoon exposures. They were than separately exposed to high ($45^0/_{00}$), ambient ($35^0/_{00}$), or low ($25^0/_{00}$) salinity for another hour to determine whether salinity alone would cause a metabolic response. Controls were performed in the absence of PCP at all three salinities for no less than 8 h (n=2 for each species except black abalone at low salinity, n=6). Finally, each animal was exposed to 1.2 mg/l of PCP at the same unique salinity until the animals MEP was attained which is defined as the time required for the Pi peak height to reach one-half that of PA. PCP dosing was then terminated but the unique water salinity was continued until [PA] and [Pi] had returned to pre-exposure levels. The dosing solution was prepared by dissolving 42.6 mg of PCP in 1.0 ml methanol and adding to 5 liters seawater. The final exposure concentration of 1.2 mg/l was prepared by mixing a 6 ml/min flow from the dosing bag with a 42 ml/min flow from the 20-liter carboy.

PCP Disposition

Three sets of red (n=3) and three sets of black (n=3) abalone were separately exposed to 1.2 mg/l of [U-^{14}C] PCP in ambient, low, and high seawater salinities (14°C) for 3.5 h. Another three sets of black abalone (n=3 each) were separately exposed for

6.5 h at ambient, low and high seawater salinities. The 3.5 h and 6.5 h time intervals represented the lowest average MEPs found using NMR for red and black abalone, respectively. These intervals were therefore chosen as the exposure lengths for the abalone used in the disposition analyses as well (preliminary data revealed a 3.5 h MEP which was subsequently found to average 3.3 h). Controls (n=2 each) at all three salinities were performed for each species for 6.5 h. The dosing solution was prepared by adding 0.97 ml of [U-^{14}C] PCP and 4.6 mg of unlabeled PCP to 700 mls of filtered seawater in a 6-liter Tedlar® bag for the 3.5 hour runs. The final exposure concentration (1.2 mg/l) was prepared by mixing a 3 ml/min flow from the dosing bag to a 17 ml/min flow from the 20-liter carboy. The dosing volumes were doubled for the 6.5 hour runs.

Nine tissues were dissected from the abalone and weighed: foot muscle, shell muscle, gill, gonad, neural, viscera, stomach, mantle, and epipodium. The tissues were homogenized using a Biohomogenizer tissue homogenizer (Biospec Products, Bartlesville, OK), solubilized by incubation (50°C, 48 h) in tissue solubilizer (1 ml deionized water and 1 ml Scintigest® per 0.1 g tissue), and quantified for ^{14}C by liquid scintillation counting with a Packard Tri-Carb PL scintillation spectrometer optimized for the scintillation cocktail. Disposition was compared using a one-tailed Students t-test.(Mason *et al.*, 1988)

Results

^{31}P-NMR spectroscopy

Representative ^{31}P-NMR spectra for abalone foot muscle have previously been published (Burt *et al.*,1976; Higashi *et al.*, 1988; Tjeerdema et al., 1991a). Spectral assignments were guided by the database of muscle constituents observable *in vivo* by ^{31}P-NMR (Gadian, 1982) and spectra from standards of Pi, ATP, ADP, PA, phosphocreatine (PC), glucose-6-phosphate, $MgCl_2$, and NaCl (Tjeerdema *et al.*,1991a). Therefore the nucleoside phosphates (NPs) referred to as γ, α, and β-NP were attributed to

γATP + βADP, αATP + αADP + the phosphate moiety of NAD, and βATP, respectively. Also, the similarity between in vivo spectra represent foot muscle only, with negligible interference from surrounding tissues (Higashi *et al.*, 1988; Tjeerdema *et al.*, 1991a).

Interactions of PCP and ambient salinity on phosphagens

Figure 1 presents the time-course changes of constituents observed *in vivo* in foot muscle of a representative red abalone during and after exposure to 1.2 mg/l of PCP at 14°C and a salinity of 35 ‰. PCP produced a decrease of up to 50% in [PA], an increase of as much as 750% in [Pi], an acidification of pHi (from 7.5 to 7.2), and a decrease of up to 50% in [ATP] (represented by the β-NP spectral resonance); the MEP response was 3.3 ± 0.2 h. On exposure to clean seawater, all measurable physiological endpoints recovered to near-original conditions within 4 h.

Figure 2 presents the time-course changes of constituents observed *in vivo* in a representative black abalone under the same conditions. PCP produced a decrease of up to 55% in [PA], an increase of as much as 1100% in [Pi], an acidification of pHi (from 7.4 to 7.2), and a decrease of up to 30% in [ATP]. The MEP was 6.8 h ± 0.7 h, indicating a greater degree of tolerance by black abalone for PCP under these conditions. All endpoints returned to normal within 8 h after exposure to clean seawater and the MEPs for red versus black abalone under ambient salinity significantly differed ($p < 0.05$).

Interactions of PCP and high salinity on phosphagens

Figure 3 presents the time-course changes in constituents measured in foot muscle of red abalone from exposure to 1.2 mg/l of PCP at 45 ‰. PCP caused a decrease of up to 60% in [PA], an increase of as much 1500% in [Pi], an acidification of pHi (from 7.5 to 7.3), and a decrease of up to 50% in [ATP]. Furthermore, the MEP was attained in 5.3 ± 0.7 h, indicating the effects of PCP were significantly delayed in hypersaline vs ambient conditions ($p < 0.05$). On exposure to clean seawater, all endpoints recovered within 12h.

Figure 4 presents the time-course changes in black abalone under the same conditions. In general they show a decrease of up to 45% in [PA], an increase of as much as 1300% in [Pi], an acidification of pHi (from 7.4 to 7.1), and a decrease of up to 30% in [ATP]. The MEP was 7.1 ± 0.7 h, indicating that the effects of PCP were not significantly different in hypersaline versus ambient conditions for black abalone ($p > 0.05$). However, the MEPs for red versus black abalone under high salinity conditions were significantly different ($p < 0.05$). Furthermore, the time to metabolic endpoint for those red abalone exposed to high salinity was significantly different than the time to metabolic endpoint for those red abalone exposed to ambient salinity ($p < 0.05$). On exposure to clean seawater all MEPs for black abalone recovered within 11h.

Interactions of PCP and low salinity on phosphagens

Figure 5 presents the time-course changes in constituents measured in foot muscle of red abalone from exposure to 1.2 mg/l of PCP at 25 $^{0}/_{00}$. PCP caused a decrease of up to 60% in [PA], an increase in [Pi] of as much as 1300%, an acidification of pHi (from 7.4 to 7.2), and a decrease of up to 75% in [ATP]. The MEP was 4.0 ± 0.5 h, indicating effects not significantly different from those of ambient conditions ($p > 0.05$). All endpoints returned to pre-exposure conditions within 9 h.

In all controls performed on red abalone as well as high and ambient controls in black abalone, [PA], [Pi], [ATP], and pHi showed no change indicating that variances in salinity alone do not effect red and black abalone. Conversely, black abalone exposed to low salinity alone showed a marked increase in [Pi] and decrease in [PA].

Figure 6 presents the time course changes in constituents of black abalone exposed to 1.2 mg/l PCP at 25 $^{0}/_{00}$. PCP caused a decrease of up to 56% in [PA], an increase of as much as 375% in [Pi], an acidification of pHi (from 7.4 to 7.1), and a decrease of up to 30% in [ATP]. The MEP, similar to that of both ambient and high salinities, was

6.5 ± 1.0 h. None of the abalone fully recovered even after 12 h.

The MEPs for red versus black abalone under low salinity conditions were significantly different ($p < 0.05$), while that for red abalone exposed to PCP at low salinity was not significantly different from the MEP for red abalone exposed at ambient salinity ($p > 0.05$), but was significantly different than that for red abalone exposed at high salinity ($p < 0.05$). Finally, the MEP for black abalone exposed to PCP at low salinity was not significantly different than those for abalone exposed at either ambient or high salinity ($p > 0.05$). Figure 7 compares the MEPs between red and black abalone at all three salinities.

^{14}C-PCP Disposition in Red Versus Black Abalone

^{14}C-PCP disposition was measured at 3.5 and 6.5 h to determine if differences in the MEPs measured by NMR could be explained by differences in disposition. Since 3.5 h and 6.5 h represented the lowest average MEPs measured for red and black abalone, respectively, they were chosen for use. Mean ^{14}C system recovery was 93.6%, indicating negligible losses from volatilization or other causes.

Figure 8 compares the total concentration factors (TCFs) for red and black abalone exposed to PCP at all three salinities, and were calculated as follows: $TCF = Ct \div Cw$; where C_t and C_W represent PCP concentration in tissue and water, respectively (Tjeerdema and Crosby 1987, 1988). The TCFs for red abalone did not differ significantly regardless of salinity. For black abalone, the TCFs at high (66.8 at 3.5 h; 114.5 at 6.5 h) and ambient (61.9 at 3.5 h; 119.3 at 6.5 h) salinities did not differ significantly ($p > 0.05$); both differed significantly from those at low salinity ($p < 0.05$). In fact, black abalone exposed to PCP at low salinity for 6.5 h (TCF = 37.3) and 3.5 h (TCF = 18.9) had TCFs three times lower than those from exposure at ambient and high salinity for 6.5 and

3.5 h. Black abalone exposed to PCP for 6.5 h at either high or ambient salinity had TCFs similar to red abalone exposed for 3.5 h.

Tables 1 through 3 compare PCP disposition in red versus black abalone at 3.5 and 6.5 h at all three salinities. In red abalone, foot muscle retained the largest amount of total retained residue (37.1 to 44.4%), regardless of salinity, while the gonad and stomach retained the smallest amount of residue (1.0 to 3.8%) and (1.2 to 2.6%), respectively. Gill, epipodium, viscera, and mantle attained the highest tissue concentrations regardless of salinity or species, with gill attaining the highest concentrations (88 to 108 nmol/g). In black abalone, the foot muscle also retained the largest amount of residue regardless of salinity or exposure length, ranging from 18 to 29%, while neural tissue and stomach retained the least amount . As with red abalone, the gill also contained the highest overall tissue concentrations. At 3.5 h, gill concentration ranged from 30.5 nmol/g at 25 $^{0}/_{00}$ to 122 nmol/g at 45 $^{0}/_{00}$. This variation in tissue concentration between low and high salinity conditions was also found during the 6.5 h exposure period.

Discussion

Exposure of red and black abalone to PCP in either hypo- or hypersaline conditions produced effects in foot muscle qualitatively similar to those produced in ambient conditions: both [PA] and [ATP] declined, [Pi] concurrently increased, and pHi decreased. While PCP is believed to be an uncoupler of mitochondrial oxidative phosphorylation, the decline in [ATP] prior to the complete utilization of PA indicates that it may also inhibit arginine kinase, which catalyzes phosphate transfer from PA to ADP to produce new ATP. Also, neither hypo- nor hypersaline conditions alone produced measurable effects in red abalone, which differs from an earlier report in which both red and black abalone exposed to hypersaline conditions ($51^{0}/_{00}$) exhibited a marked decline in [PA] and [ATP] and increased [Pi] (Higashi *et al.*, 1988).

While this investigation found that high salinity alone produced no measurable effects in black abalone, repeated NMR runs revealed that black abalone are compromised when exposed to low salinity in the absence of PCP. Exposure to $25^{0}/_{00}$ seawater regularly caused an immediate increase in [Pi] and concurrent decrease in [PA]. However, when black abalone were exposed to low salinity conditions plus PCP the endpoints were similar to those for black abalone exposed to PCP at ambient or high salinity conditions, indicating a possible subadditive mechanism. In general, black abalone appear to have greater resistance to the combined effects of PCP and variations in salinity, as the MEP for PCP-exposed black abalone is significantly larger ($p < 0.05$) at all three salinities than those for red abalone. Deviation from ambient salinity increased the recovery times for both species. Both [PA] and [Pi] returned to pre-exposure concentrations nearly twice as quickly for abalone exposed to PCP at ambient salinity than for abalone exposed at either high or low salinity.

In red abalone, hypersalinity significantly delayed the onset of PCP's actions ($p < 0.05$). The potential increase in metabolic demand for osmotic regulation (possibly through elevated Na^{+}-K^{+} ATPase activity) was hypothesized to hasten the onset of toxicity. However, the results reveal that in hypersaline conditions red abalone are better able to withstand the sublethal actions of PCP, suggesting an antagonistic or subadditive relationship. Although PCP is a weak acid, using ^{14}C PCP it was found that elevated salinity had an insignificant influence on either seawater pH (8.0) or the solubility of PCP at the concentration used ($p > 0.05$). Therefore, bioavailability of PCP would have remained unchanged and should not have been a factor.

Hyperosmotic shock has been shown to decrease oxidation activity in *Eriocheir sinensis* which accounts for an increase in intracellular amino acid concentration (Gilles, 1974a). A decrease in oxidation activity by abalone exposed to high salinity might explain

the increase in time taken for those animals to reach their MEP. The onset of PCP's actions in red abalone exposed to hyposaline conditions did not differ significantly from those exposed to PCP in ambient seawater ($p > 0.05$). The decline in metabolic demand for osmotic regulation in ambient salinity was hypothesized to delay the onset of effects.

Whereas hyposalinity did not effect red abalone, black abalone were consistently adversely effected. Tissue oxygen consumption and mitochondrial respiration rate have been shown to increase under hypoosmotic stress in many invertebrates (Hoffman and Simonsen, 1989). Furthermore, elevated excretion of free amino acids accumulated as solute for osmotic adjustment may represent a significant loss of endogenous protein nitrogen during hypoosmotic change (Hawkins and Hilbish, 1992). These losses represent a major component cost of cell volume regulation, possibly explaining stress and even mortality after even small fluctuations in salinity. This may help to elucidate the adverse impact of low salinity alone on black abalone. The magnitude and duration of the amino acid efflux depends upon external divalent cation concentration and intracellular ATP synthesis (Pierce and Greenburg, 1976). It was hypothesized that the disruption of ATP synthesis by an uncoupler such as PCP would impair this process and potentiate the effect of hyposalinity on the abalone. This was not found to be the case, as the MEP at all three salinities were not significantly different ($p < 0.05$).

Osmotic stress is important to the initiation of phosphorylation of membrane proteins, thereby regulating osmolyte permeability. The mechanism of passive permeability control, and thereby cell volume regulation, during salinity stress in molluscs has been shown to rest with a membrane-bound divalent ATPase (Pierce and Greenberg, 1976). Recovery of amino acid permeability is evidently dependent on both membrane potential and ATP production (Pierce and Greenberg, 1976). This was further demonstrated when Watts and Pierce (1978) found that both 2,4-DNP and cyanide potentiate the

hypoosmotically-induced efflux of amino acids in the ribbed mussel *Modiolus demissus demissus*. Inhibition of the ATPase controlling the recovery of normal amino acid permeability would produce a greater efflux and, conversely, if ATPase activity is enhanced, a reduced efflux would result. Whereas cyanide depresses cellular respiration by combining with and inactivating mitochondrial cytochrome oxidase, and 2,4-DNP uncouples oxidative phosphorylation, both compounds, like PCP, inhibit ATP synthesis. Therefore, it was noteworthy that the hypothesized potentiation of the MEP was not observed when black abalone were exposed to PCP at low salinity but in fact a subadditive effect was observed.

The enzymes of some euryhaline invertebrates may not be specially adapted to tolerate wide salinity ranges. It has been previously found that at reduced salinities some mitochondrial enzymes have reduced activities. Studies of gill tissue of the oyster (*Crassostrea virginica)* indicate reduced enzyme activities occurring at low salinities, probably in response to low intracellular ionic strength (Ballantyne and Berges, 1991). If ATPase activity were reduced during hyposaline conditions, there would be a concurrent reduction in ATP utilization, and the MEPs at low salinity would not vary measurably from those at ambient and high salinities which is consistent with the results found for black abalone. The effectiveness of these toxic agents in augmenting the efflux suggests that the mechanism proposed to *restore* the normal permeability of the membrane to amino acids requires ATP and may therefore explain the additional time required for animals exposed to hyposaline seawater to recover.

Due to the qualitative nature of NMR, the intensity and duration of effects is more difficult to define. Although the red abalone were cultured from larvae, black abalone were collected from the wild and tend to have a greater degree of genetic variation. However, in red abalone, the average intensity of response to PCP, and the time required

for full recovery, were increased in individuals exposed to the biocide in either hypo- or hypersaline water. Hypersaline conditions did not produce a significant difference in recovery times for black abalone, but those exposed to low salinity were not able to *fully* recover even after 12 h, although both [PA] and [Pi] began to return to pre-exposure levels. As suggested, this may be due to the effects of low salinity on ATPase activity in combination with the PCP induced inhibition of ATP production impairing the return to normal cell volume regulation.

The comparative disposition of PCP has been described in several species of molluscs including oysters and abalone (Shofer and Tjeerdema, 1993). Compared to many marine organisms both green abalone (*H. fulgens*) and red abalone are particularly sensitive to PCP, as demonstrated by the 5-h LC_{50} of 1.4 mg/l and 1.6 mg/l, respectively (Shofer and Tjeerdema, 1993). Despite the similarity in their LC_{50}'s, green abalone are not only less adept at depurating PCP than red abalone, but they also seem to bioconcentrate PCP more readily as the 5 h TCF for green abalone was more than twice that of red abalone (Tjeerdema and Crosby, 1992). A comparison among the three species of abalone (red, green, and black) demonstrates that the same tissue types tend to concentrate PCP, with the epipodium, gill, viscera and mantle having the highest concentrations. Although metabolism of PCP in black abalone has not been examined, the fact that significant differences exist among other species of abalone indicates that metabolic variations may contribute to the differences found in the TCFs.

One of the most significant aspects of this investigation is that the TCFs measured directly correlate with the MEPs determined using NMR spectroscopy. It seems that red abalone take up PCP more quickly than black abalone in that similar TCFs were achieved even when red abalone were exposed for only half as long (3.5 h versus 6.5 h). Similarly, the MEP demonstrated by NMR was 1.4 - 2 times faster in red abalone reflecting a greater

short term exposure to PCP by red abalone. Low salinity appears to discourage the uptake of PCP in black abalone as demonstrated by the TCFs at $25^{0}/_{00}$ for both the 3.5 h and 6.5 h runs as they are three times lower than the TCFs at $35^{0}/_{00}$ and $45^{0}/_{00}$.

Abalone, like limpets, may draw their shells down in hyposaline environments thereby reducing gill surface area exposed to hyposaline water and enhancing survival. (Marshall and McQuaid, 1993). Gas exchange may be limited during these conditions causing an overshoot in heart rate. Elevation of the respiratory rate is probably stimulated by the need to repay oxygen debt and regenerate phosphagens and ATP (Marshall and McQuaid, 1993). Since the gill is the primary organ responsible for uptake of oxygen *and* PCP, it does not seem unreasonable that the TCFs for black abalone exposed at low salinity are lower than the TCFs at ambient and high salinities. The subadditive effect observed seems to be a byproduct of the fact that less PCP is being absorbed from the seawater. The fact that this was not observed for red abalone leaves room for speculation as to species variation. On the other hand, the oxygen debt that is incurred may contribute to the extensive recovery times demonstrated in both species. Furthermore, it appears that regardless of the exposure time, salinity or species the same organs will take up the majority of PCP.

This report describes the influence of seawater salinity, and toxicokinetics, on the sublethal effects of PCP in the red and black abalone. *In vivo* ^{31}P NMR provides a sensitive approach for investigation of toxic actions in intact organisms by measuring important biochemical actions as they occur.

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Figs. 1-6. Comparison of time-course for (a) phosphoarginine (PA) and inorganic phosphate (Pi); (b) phosphoarginine (PA) and intracellular pH (pHi) and (c) nucleoside phosphates (Nps) in representative samples of red and black abalone during and after exposure to 1.2 mg/L of PCP in 25, 35, and 45 $^{0/}00$ seawater salinity. Points indicate changes in peak areas as normalized to methylene diphosphonate (MDP), and each represents 300-600 spectra averaged over 11.7 to 23.4 min, respectively

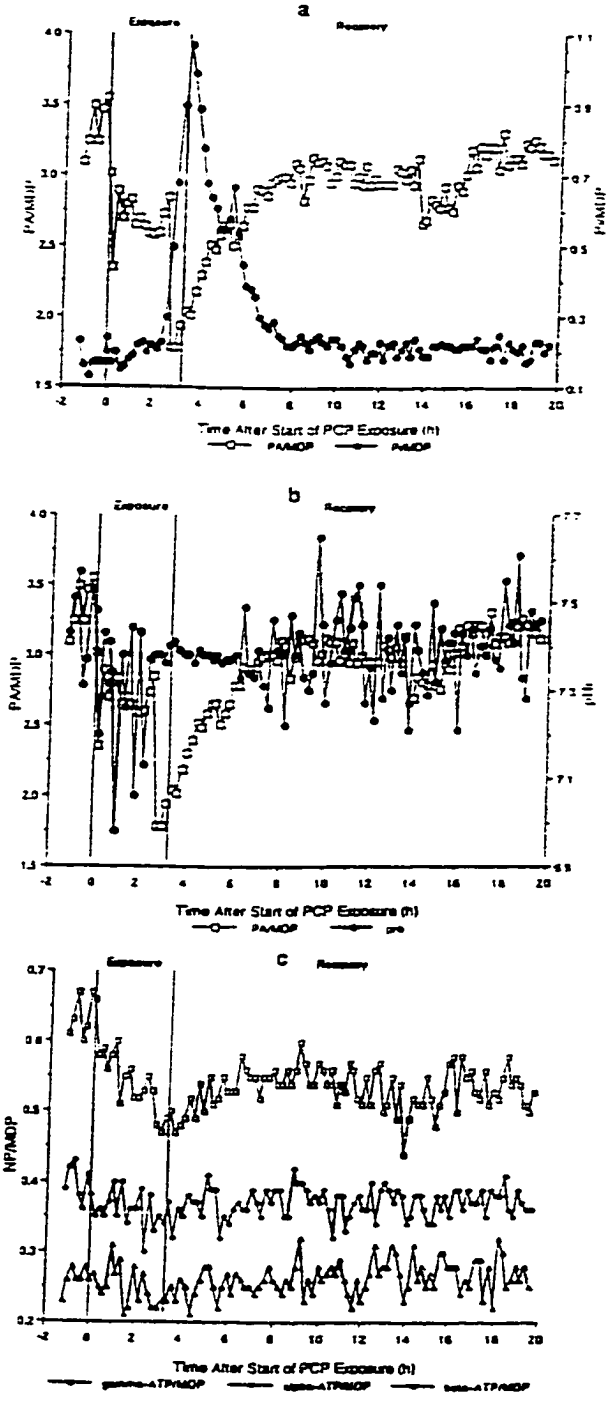


Fig. 1. Comparison of the time-course for (a) phosphoarginine (PA) and inorganic phosphate (Pi); (b) phosphoarginine (PA) and intracellular pH (pHi) and (c) nucleoside phosphates (NPs) in a representative red abalone during and after exposure to 1.2 mg l^{-1} of PCP in 35 ‰ seawater. Points indicate changes in peak areas as normalized to methylene diphosphonate (MDP), and each represents 300–600 spectra averaged over 11.7 to 23.4 min, respectively.

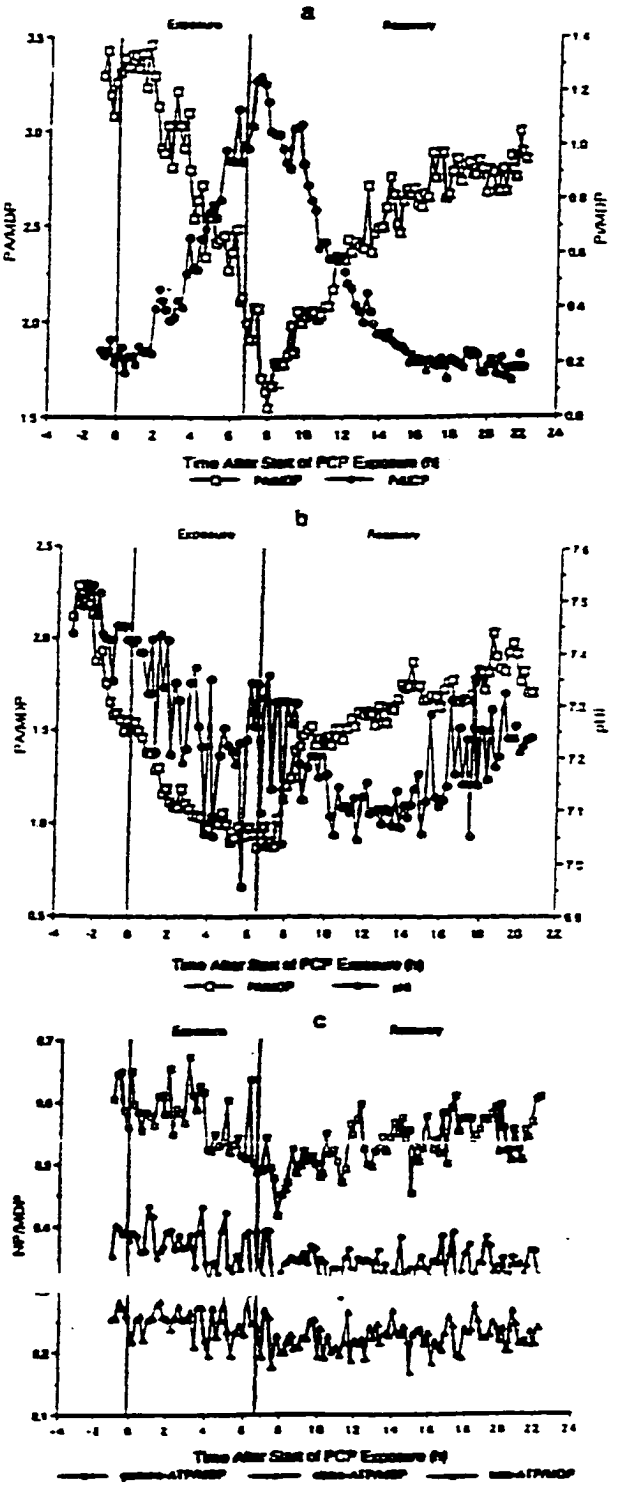


Fig. 2. Comparison of the time-course for (a) phosphoarginine (PA) and inorganic phosphate (Pi); (b) phosphoarginine (PA) and intracellular pH (pHi) and (c) nucleoside phosphates (NPs) in a representative black abalone during and after exposure to 1.2 mg l^{-1} of PCP in 35 ‰ seawater. Points indicate changes in peak areas as normalized to methylene diphosphonate (MDP), and each represents 300–600 spectra averaged over 11.7 to 23.4 min, respectively.

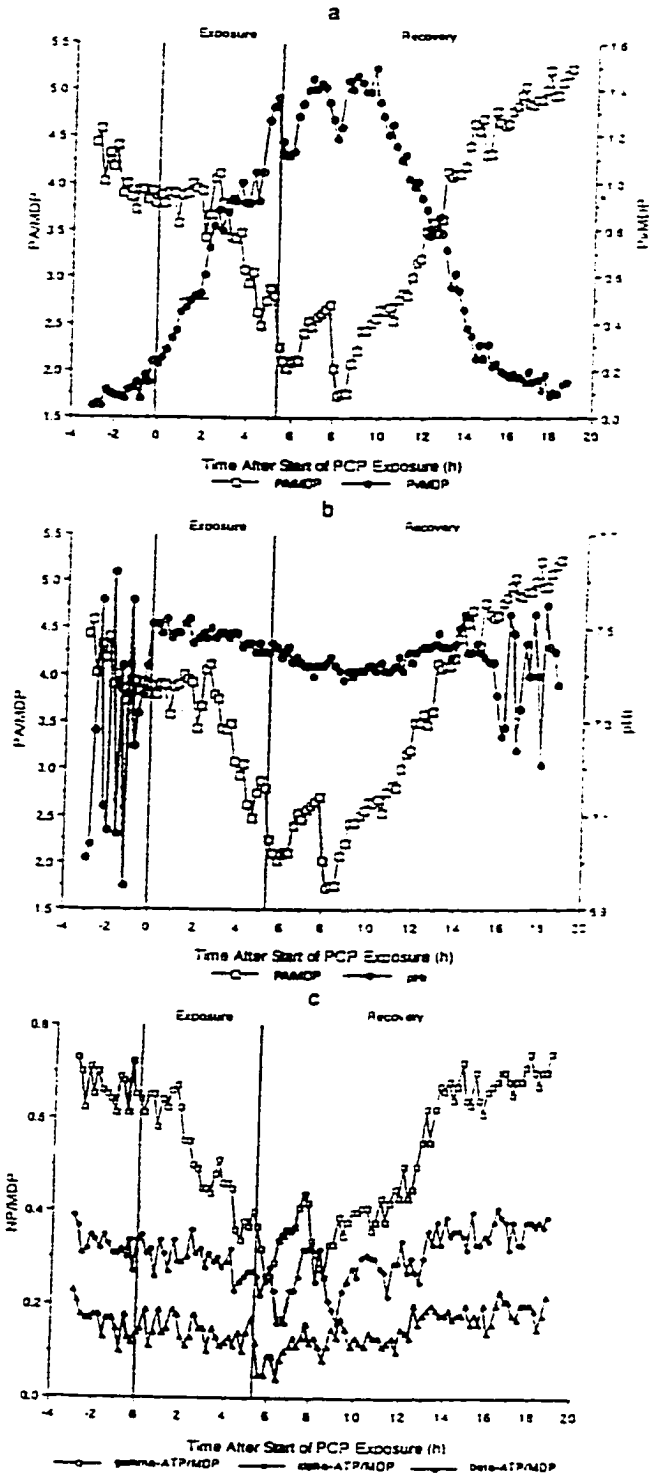


Fig. 3. Comparison of the time-course for (a) phosphoarginine (PA) and inorganic phosphate (Pi); (b) phosphoarginine (PA) and intracellular pH (pHi) and (c) nucleoside phosphates (NPs) in a representative red abalone during and after exposure to 1.2 mg l^{-1} of PCP in 45 ‰ seawater. Points indicate changes in peak areas as normalized to methylene diphosphonate (MDP) and each represents 300–600 spectra averaged over 11.7 to 23.4 min, respectively.

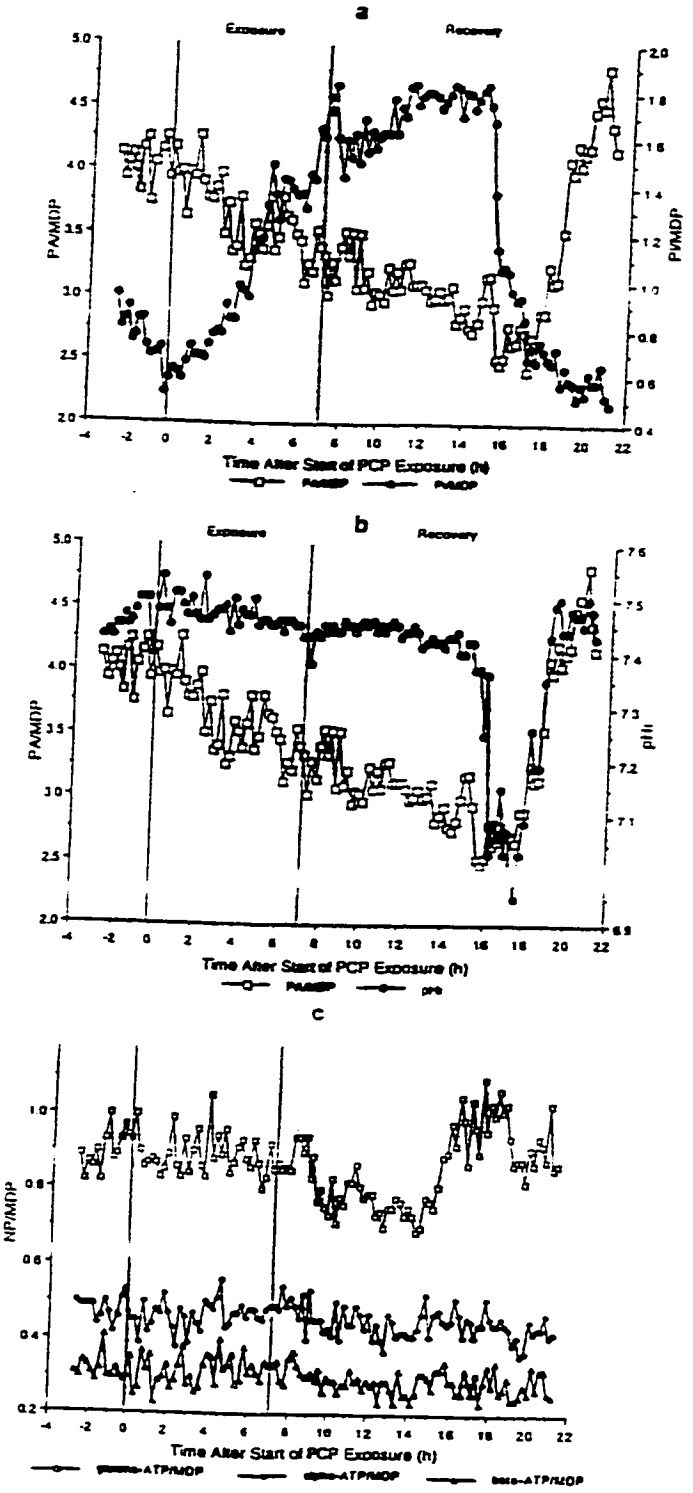


Fig. 4. Comparison of the time-course for (a) phosphoarginine (PA) and inorganic phosphate (Pi); (b) phosphoarginine (PA) and intracellular pH (pHi) and (c) nucleoside phosphates (NPs) in a representative black abalone during and after exposure to 1.2 mg l^{-1} of PCP in 45 ‰ seawater. Points indicate changes in peak areas as normalized to methylene diphosphonate (MDP) and each represents 300–600 spectra averaged over 11.7 to 23.4 min, respectively.

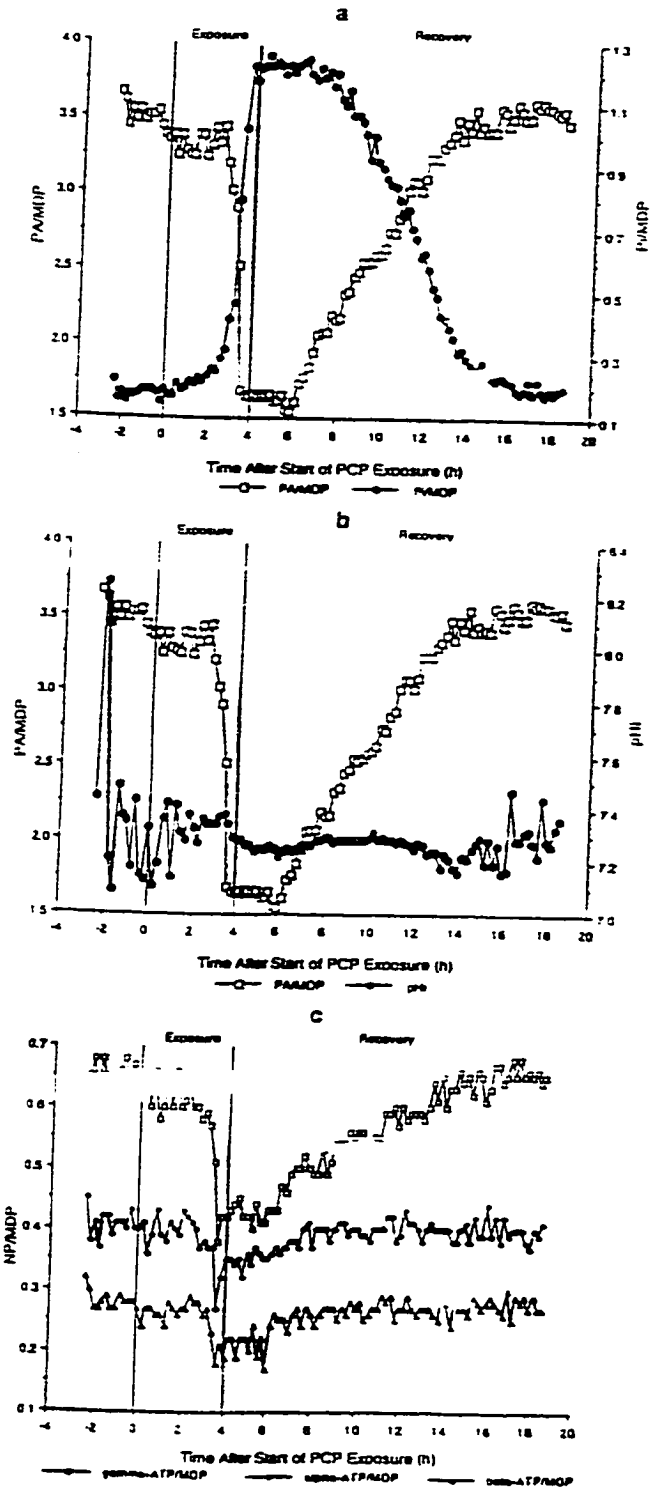


Fig. 5. Comparison of the time-course for (a) phosphoarginine (PA) and inorganic phosphate (Pi); (b) phosphoarginine (PA) and intracellular pH (pHi) and (c) nucleoside phosphates (NPs) in a representative red abalone during and after exposure to 1.2 mg l^{-1} of PCP in 25 ‰ seawater. Points indicate changes in peak areas as normalized to methylene diphosphonate (MDP) and each represents 300–600 spectra averaged over 11.7 to 23.4 min, respectively.

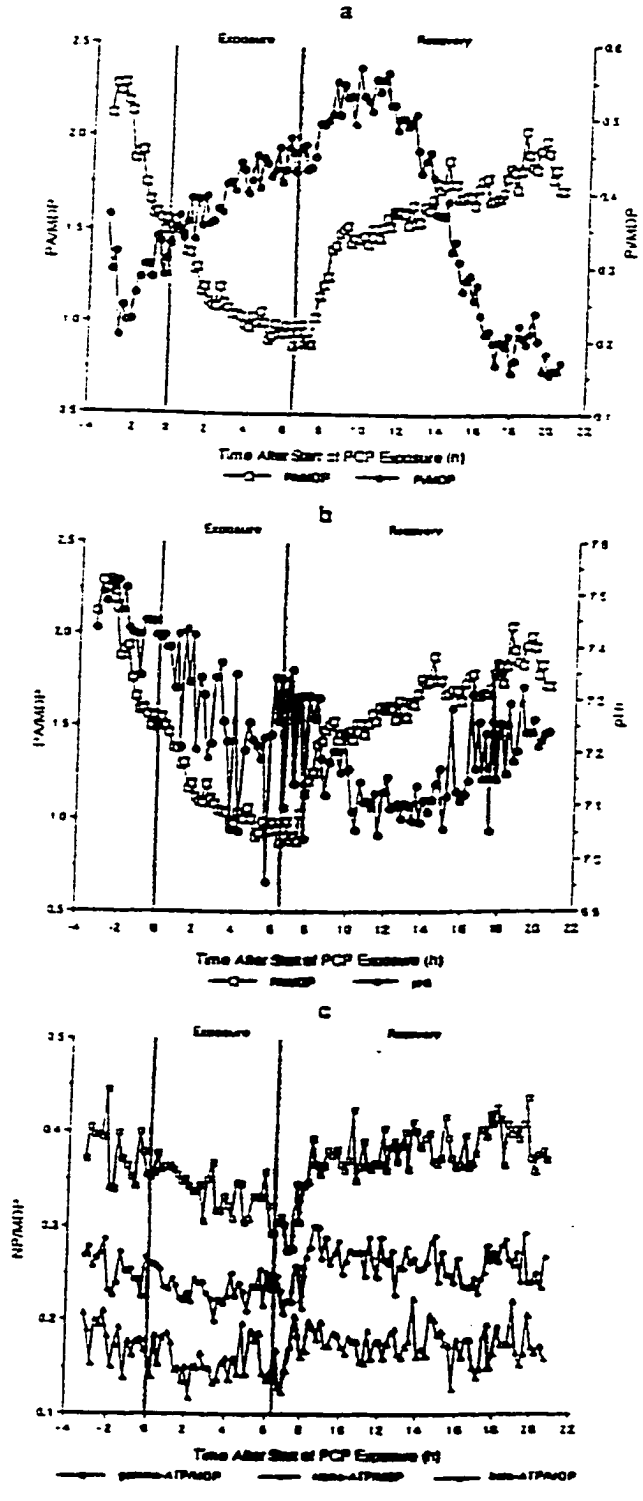


Fig. 6. Comparison of the time-course for (a) phosphoarginine (PA) and inorganic phosphate (Pi); (b) phosphoarginine (PA) and intracellular pH (pHi) and (c) nucleoside phosphates (NPs) in a representative black abalone during and after exposure to 1.2 mg l^{-1} of PCP in 25 ‰ seawater. Points indicate changes in peak areas as normalized to methylene diphosphonate (MDP) and each represents 300–600 spectra averaged over 11.7 to 23.4 min, respectively.

Fig 7. Comparison of metabolic endpoints (MEPS) between red and black abalone at three water salinities.

Student T-tests were used to analyze the data. Error bars represent the standard error of the means.

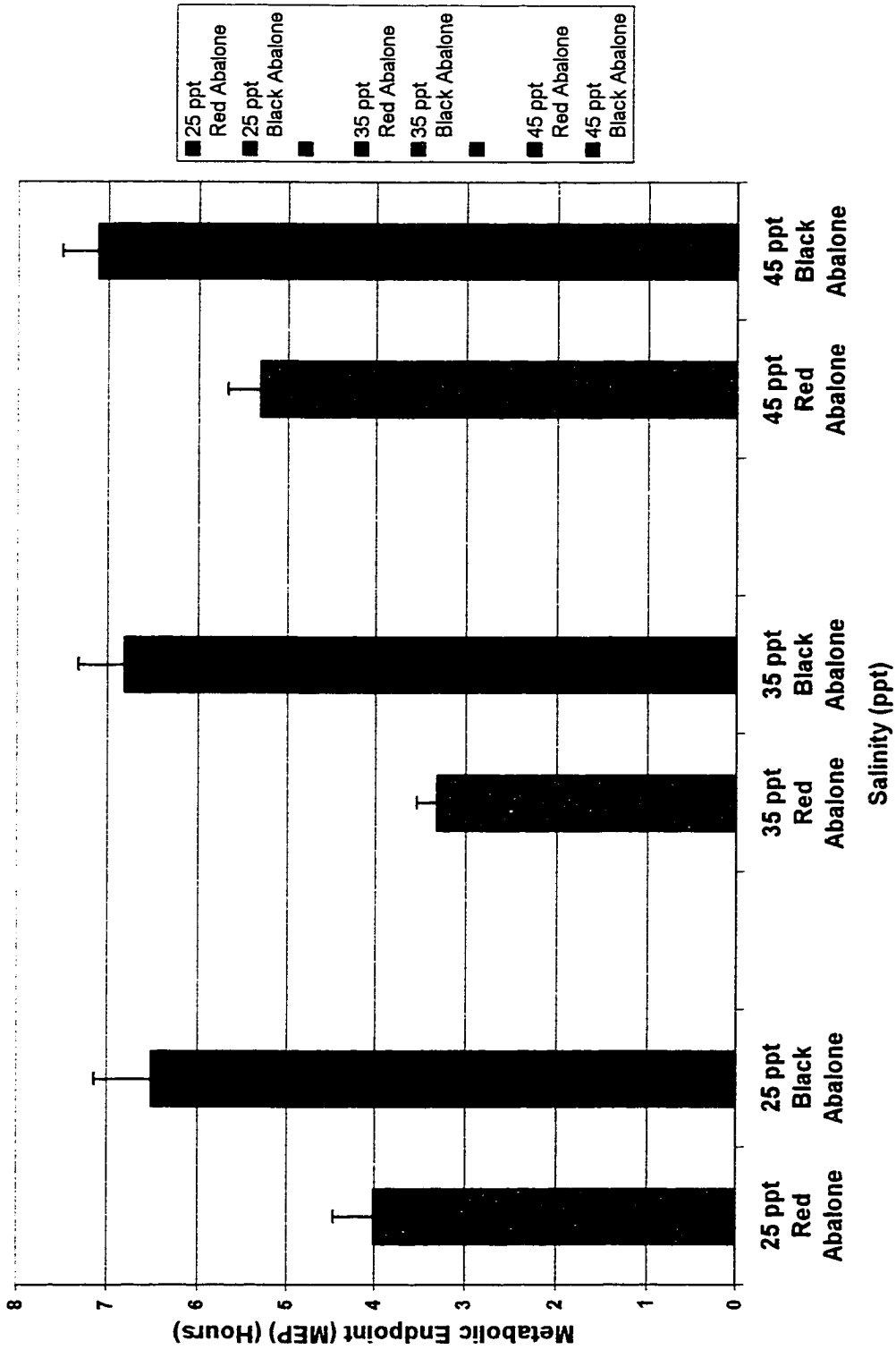
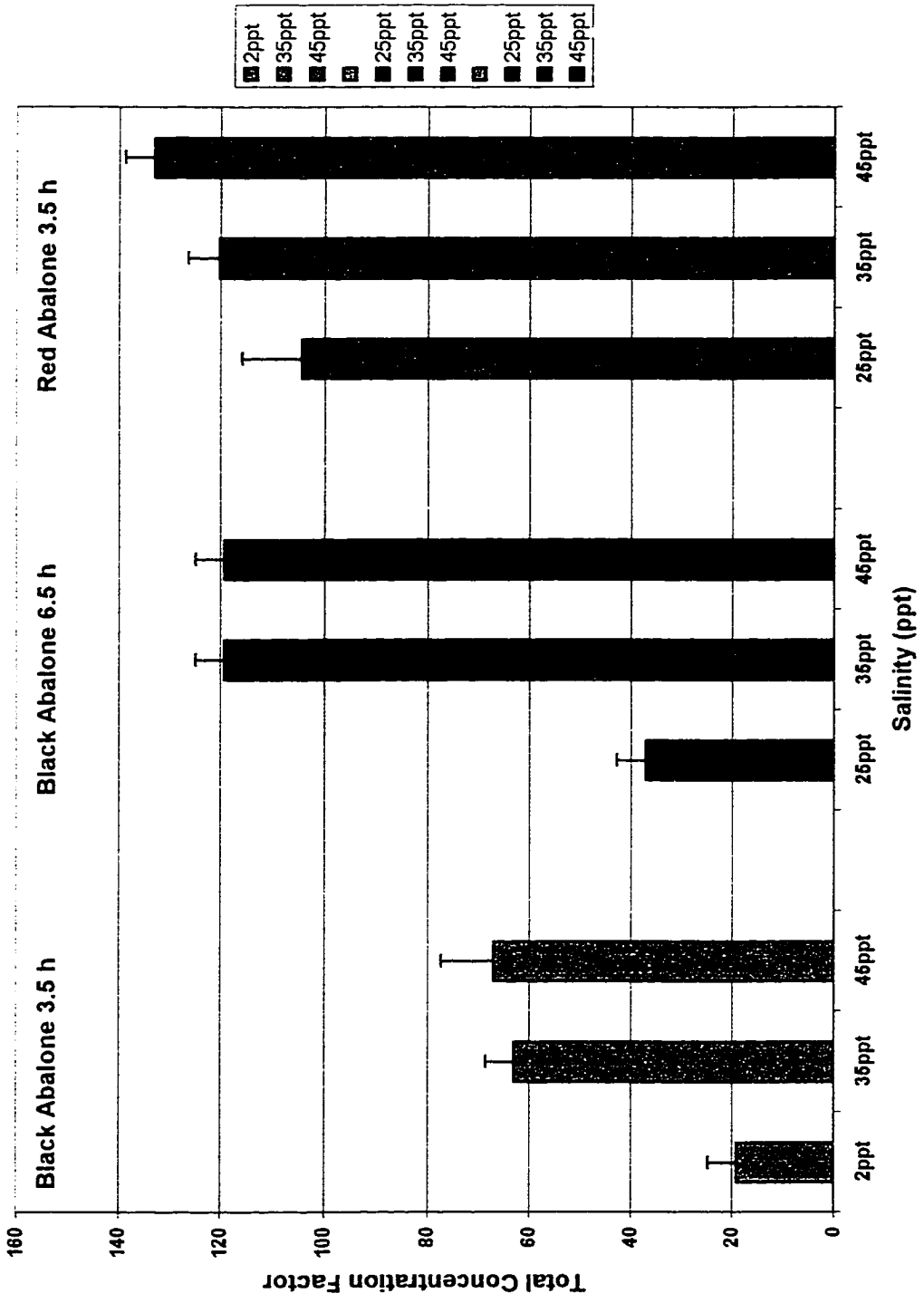


Fig 8. Comparison of total concentration factors (TCFs) between red and black abalone after exposure to PCP for 3.5 or 6.5h at three water salinities.

Student T-tests were used to analyze the data. Error bars represent the standard error of the means.



Tables 1-3. Residue disposition in red and black abalone after 3.5h or 6.5h exposures to 1.2mg/L pentachlorophenol in nine tissues.

Data were analyzed as an ANOVA. Error bars represent the standard error of the mean (SEM).

Table 1
Residue disposition in red abalone[a]

Red abalone 3433.5 h runs	35 ppt			25 ppt			45 ppt		
Organ	Total mass (μmol)	Percentage	Concentration (nmol g^{-1})	Total mass (μmol)	Percentage	Concentration (nmol g^{-1})	Total mass (μmol)	Percentage	Concentration (nmol g^{-1})
Foot muscle	0.99 (0.22)	42.9	56.8 (13.0)	0.76 (0.14)	37.1	39.98 (12.7)	1.12 (0.17)	44.4	76.0 (6.2)
Viscera	0.11 (0.01)	4.7	90.9 (35.7)	0.19 (0.05)	9.2	84.58 (25.0)	0.23 (0.09)	9.2	78.2 (29.0)
Shell muscle	0.19 (0.05)	8.6	31.6 (14.3)	0.11 (0.06)	5.3	15.67 (3.56)	0.24 (0.05)	9.3	36.7 (13.2)
Epipodium	0.27 (0.13)	11.6	86.2 (11.5)	0.43 (0.09)	20.7	97.53 (17.0)	0.18 (0.07)	7.2	97.7 (21.0)
Neural	0.19 (0.07)	8.4	62.1 (16.5)	0.13 (0.03)	6.4	49.04 (11.6)	0.14 (0.08)	5.5	68.9 (18.5)
Gonad	0.09 (0.03)	3.8	21.4 (1.4)	0.02 (0.01)	1.0	9.29 (2.1)	0.05 (0.07)	1.9	11.9 (2.1)
Gill	0.27 (0.10)	11.8	107.8 (33.0)	0.26 (0.11)	12.7	88.09 (29.0)	0.24 (0.15)	9.8	101.6 (46.0)
Mantle	0.13 (0.08)	5.6	60.7 (6.2)	0.12 (0.09)	5.7	71.54 (3.1)	0.28 (0.18)	11.5	108.2 (14.7)
Stomach	0.06 (0.03)	2.6	23.7 (2.5)	0.04 (0.01)	1.9	15.08 (1.7)	0.03 (0.01)	1.2	19.4 (5.9)
	2.30 (0.72)	100.0	60.13 (14.9)	2.06 (0.59)	100.0	52.31 (11.8)	2.51 (0.87)	100.0	66.51 (17.4)

[a] After 3.5 h exposure to 1.2 mg l^{-1} pentachlorophenol in nine tissues.

Table 2
Residue disposition in black abalone[a]

Black abalone 3.5 h runs	35 ppt			25 ppt			45 ppt		
Organ	Total mass (μmol)	Percentage	Concentration (nmol g^{-1})	Total mass (μmol)	Percentage	Concentration (nmol g^{-1})	Total mass (μmol)	Percentage	Concentration (nmol g^{-1})
Pool muscle	0.52 (0.42)	30.9	10.9 (3.1)	0.19 (0.20)	22.7	3.2 (2.8)	0.55 (0.29)	18.5	12.0 (3.0)
Viscera	0.13 (0.02)	7.8	31.3 (6.4)	0.03 (0.01)	3.3	11.0 (7.9)	0.16 (0.01)	5.3	35.7 (2.3)
Shell muscle	0.11 (0.08)	6.4	5.2 (2.1)	0.16 (0.06)	18.7	1.6 (1.1)	0.45 (0.39)	15.2	8.3 (2.9)
Epipodium	0.26 (0.12)	15.5	34.1 (4.8)	0.20 (0.08)	24.2	14.9 (6.3)	0.41 (0.04)	13.8	33.2 (4.0)
Newal	0.04 (0.01)	2.6	11.9 (7.2)	0.02 (0.01)	1.9	3.4 (1.4)	0.10 (0.03)	3.3	17.2 (2.9)
Gonad	0.17 (0.12)	10.0	9.8 (3.5)	0.04 (0.01)	5.1	1.2 (0.9)	0.28 (0.11)	9.5	12.9 (8.9)
Gill	0.18 (0.01)	10.6	111.4 (25.9)	0.09 (0.03)	11.0	30.5 (3.6)	0.51 (0.25)	17.1	122.9 (9.8)
Mantle	0.20 (0.19)	12.0	44.8 (10.2)	0.09 (0.03)	10.7	14.8 (9.9)	0.29 (0.19)	9.9	33.6 (3.6)
Stomach	0.07 (0.01)	4.2	19.8 (1.0)	0.02 (0.01)	2.4	3.9 (2.7)	0.22 (0.09)	7.4	25.1 (4.1)
	1.68 (0.98)	100.0	32.11 (7.1)	0.84 (0.443)	100.0	9.39 (4.1)	2.97 (1.4)	100.0	33.43 (4.6)

[a] After 3.5 h exposure to 1.2 mg l^{-1} pentachlorophenol in nine tissues.

Table 3
Residue disposition in black abalone[a]

Black abalone 6.5 3 h runs	35 ppt			25 ppt			45 ppt		
Organ	Total mass (μmol)	Percentage	Concentration (nmol g^{-1})	Total mass (μmol)	Percentage	Concentration (nmol g^{-1})	Total mass (μmol)	Percentage	Concentration (nmol g^{-1})
Foot muscle	1.19 (0.22)	29.3	30.9 (4.0)	0.49 (0.01)	29.2	7.5 (1.9)	1.76 (1.2)	25.8	40.4 (15.3)
Viscera	0.35 (0.06)	8.5	56.2 (11.0)	0.12 (0.10)	7.3	27.2 (26.7)	0.12 (0.02)	1.7	60.9 (5.7)
Shell muscle	0.42 (0.06)	10.4	6.9 (3.7)	0.29 (0.07)	16.9	2.4 (1.4)	0.94 (0.20)	13.8	32.4 (12.5)
Epipodium	0.39 (0.07)	9.7	122.9 (9.8)	0.33 (0.23)	19.7	21.5 (8.8)	1.30 (0.23)	19.0	109.8 (2.3)
Neural	0.26 (0.04)	6.4	63.8 (7.9)	0.03 (0.02)	1.9	6.2 (2.1)	0.18 (0.01)	2.6	30.7 (0.6)
Gonad	0.20 (0.09)	4.9	9.9 (5.0)	0.08 (0.03)	4.7	4.7 (2.0)	0.73 (0.22)	10.7	39.6 (8.8)
Gill	0.56 (0.09)	13.8	109.8 (14.5)	0.17 (0.07)	9.9	48.2 (21.4)	0.72 (0.17)	10.6	96.8 (40.5)
Mantle	0.46 (0.07)	11.3	89.4 (5.9)	0.14 (0.14)	8.2	43.8 (17.9)	0.61 (0.07)	8.9	55.7 (5.9)
Stomach	0.23 (0.04)	5.7	47.2 (3.7)	0.04 (0.01)	2.2	6.5 (2.0)	0.47 (0.17)	6.9	50.6 (2.39)
	4.06 (1.9)	100.0	59.67 (7.3)	1.69 (0.68)	100.0	18.67 (9.3)	6.83 (2.29)	100.0	57.43 (10.4)

[a] After 6.5 h exposure to 1.2 mg l^{-1} of pentachlorophenol in nine tissues.

Chapter 3

The Combined Effects of Chemical and Natural Stressors on Nonspecific Immunity in Two Species of Abalone

Introduction

A variety of studies have linked stress with the inability of aquatic organisms to resist disease as a result of modulation of the immune system (Snieszko, 1973; Sindermann, 1979; Chu and Hale, 1984; Anderson, 1981a, 1988a). Stress can be induced by a number of factors including temperature, salinity, pathogens or toxic chemicals in the environment. Because phagocytic cells of an organism play a critical role in defense against invading microorganisms, any fluctuation in the immune response could potentially effect resistance to disease and parasitism.

As osmoconformers, marine gastropods readily assume the salinity of the external environment (Shumway, 1977; Gilles, 1979). Therefore, hemocytes circulating in hemolymph and tissue sinuses are readily exposed to environmental salinity variations. Exposure to extremes can adversely affect organism health. Fisher and Newell (1986) found that elevated salinity not only caused hemocytes to spread more slowly (an indication of the response-time of the hemocytes), but also reduced the rate of hemocyte locomotion towards an antigen. This suggests that intracellular volume regulation may be linked to hemocyte spreading. Alterations in salinity may, therefore, affect both hemocyte locomotion as well as foreign particle binding. Locomotion is a critical aspect of invertebrate immunity, as it is required for endocytosis, wound healing, inflammation and encapsulation (Fisher and Tamplin, 1988).

Eight North American abalone species are found along the coast of California, where declines of over 70% in the fisheries have been observed over the last 20 years (Haaker *et al.*, 1986). Around the Channel Islands the once abundant black abalone,

Haliotis cracherodii, began to die in large numbers in 1985 (Haaker *et al.*, 1992). This epidemic, now known as withering syndrome (WS), can be observed as far north as Pacifica (Friedman *et al.*, unpublished data). Gardner *et al.* (1995) demonstrated a pathogen-disease association in WS, implicating a rod-shaped, gram negative, intracellular prokaryote consistent with the order Rickettsiales. This progressive wasting disease is characterized by a shrunken appearance of the foot muscle, retracted visceral tissues, and the inability to tightly adhere to the substrate (Haaker *et al.*, 1992). Although still most pervasive in black abalone, WS has now been found in red, pink and green abalone as well (Haaker *et al.*, 1995).

Natural stresses combined with those of overharvesting and pollution may assist in the ability of microorganisms to act opportunistically. Although the influence of pollution on the abalone decline has not been observed directly, chemicals may indirectly synergize infectious organisms by increasing their virulence or reducing host resistance. Several previous studies have reported that bacterial resistance may be compromised by exposure to xenobiotics in a number of molluscs (Anderson *et al.*, 1981; Nimmo *et al.*, 1978; Jeffries, 1972; Fries & Tripp, 1980; Cheng, 1988a; Alvarez *et al.*, 1991, Anderson *et al.*, 1981; Sami *et al.*, 1990, Chu and Hale, 1994). Chu and Hale (1994) found that pollutant exposure enhanced *Perkinsus* infection in the oyster *Crassostrea virginica* and increased their susceptibility to experimentally induced infection, in a dose-dependent manner. Furthermore, the virulence of *Perkinsus* in the field was correlated with high salinity and temperature. Roszell and Anderson (1994) found a dose-dependent suppression of phagocytosis in response to PCP treatment in the

marine teleost *Fundulus heteroclitus*. Moreover, exposure of *Mercenaria mercenaria* to phenol was shown to damage gill and digestive tract epithelia, which may render the clams more susceptible to microbial infection and disease (Fries and Tripp, 1980).

The purpose of this investigation was to examine the effects of PCP on several aspects of nonspecific immunity mediated by phagocytic hemocytes of red and black abalone. PCP is an EPA priority pollutant that is a potent fungicide, herbicide, bactericide, and algaecide and is introduced into California coastal waters from pressure treated wood pilings, anti-fouling paints and urban runoff sources. Previous studies in our laboratory have demonstrated that exposure of abalone to PCP effectively reduces the intracellular phosphagen pool by 50% within 3.5 h for red abalone and 6.5 h for black abalone. These intervals have been defined as metabolic endpoints (MEPs). Diminished energy production may reduce an organism's defenses against microorganisms. The objective of this study was to examine whether in vivo exposure of red and black abalone to both natural and chemical stressors can compromise immune function namely phagocytosis, chemotaxis, and cytoskeletal organization. Furthermore, this study was designed to assess the validity of the MEP defined using NMR as it relates to immune function (Martello *et al.*, 1998).

Materials and Methods

Chemicals

PCP sodium salt (p*Ka*, 5.3; water solubility at 20 °C, 80 mg/L; Merck Index, 1996) were purchased from Aldrich Chemical Co. (Milwaukee, WI) and methanol was purchased from Fisher Scientific, Inc. (Pittsburgh, PA). Natural seawater was collected and filtered (0.2 μm) at the UCSC Long Marine Laboratory, stored at 14 °C, and used within 3 d. Instant Ocean (Aquarium Systems, Mentor, OH) was used to adjust water salinity to 45$^{0}/_{00}$, while distilled water was used to dilute seawater to 25$^{0}/_{00}$.

Animals

Red abalone (*H. rufescens*), averaging 9-14 cm, were provided by U.S. Abalone, Davenport, CA. Black abalone (*H. cracherodii*) averaging 10-16 cm, were obtained from Ano Nuevo Island, San Mateo County, CA. They were maintained in flowing seawater, fed giant kelp (*Macrocystis pyrifera*), and acclimated to 14°C for at least two weeks prior to use. Animals were measured and weighed prior to exposure to insure uniformity.

Exposure System

PCP exposures were conducted according to Martello *et al.*, 1998. Briefly, abalone were placed in a Tedlar® fluoropolymer bag (Aeroenvironment, Monrovia, CA.), which was anchored into an acrylic chamber and sealed. Aerated and

temperature-controlled seawater was pumped from a 20-liter carboy with a peristaltic pump through silicon tubing. PCP was combined with the seawater flow using a second pump from a 6-liter collapsible fluoropolymer gas sampling bag.

Exposures

Based on PCP toxicity range-finding tests performed on juvenile red abalone (6-h LC_{50} 1.6 mg/l; 6-h NOEL=0.8 mg/l), animals were exposed in flowing seawater (14°C) to a sublethal PCP concentration of 1.2 mg/l and a water salinity of either 25, 35, or 45 $^{0}/_{00}$. Red (n=6) and black (n=6) abalone were individually exposed to high ($45^{0}/_{00}$), ambient ($35^{0}/_{00}$), or low ($25^{0}/_{00}$) salinities for 3.5 and 6.5 h, respectively, not only to serve as controls but to determine whether salinity alone would cause an adverse response. Three sets of red (n=6) and three sets of black (n=6) abalone were then separately exposed to 1.2 mg/L PCP in ambient, low, and high seawater salinities at 14°C for 3.5 h. Another three sets of black abalone (n=6) were individually exposed to 1.2 mg/L PCP for 6.5 h at ambient, low and high seawater salinities. The 3.5 h and 6.5 h time intervals represented the lowest average metabolic endpoints (MEPs) found using surface-probe localized ^{31}P NMR spectroscopy for red and black abalone, respectively, and were therefore chosen as the exposure periods for the following experiments (Tjeerdema *et al.*, 1996, Martello *et al.*, 1998). Moreover, the exposure periods represent the time within which animals were consistently able to recover. The dosing solution was prepared by adding 5.6 mg of PCP to 700 mL of filtered seawater in a 6-LTedlar bag for the 3.5-h periods. The final exposure concentration of 1.2 mg/L was

prepared by mixing a 3 ml/min flow from the dosing bag to a 17 ml/min flow from the 20-liter seawater carboy. The volumes were doubled for the 6.5-h exposures.

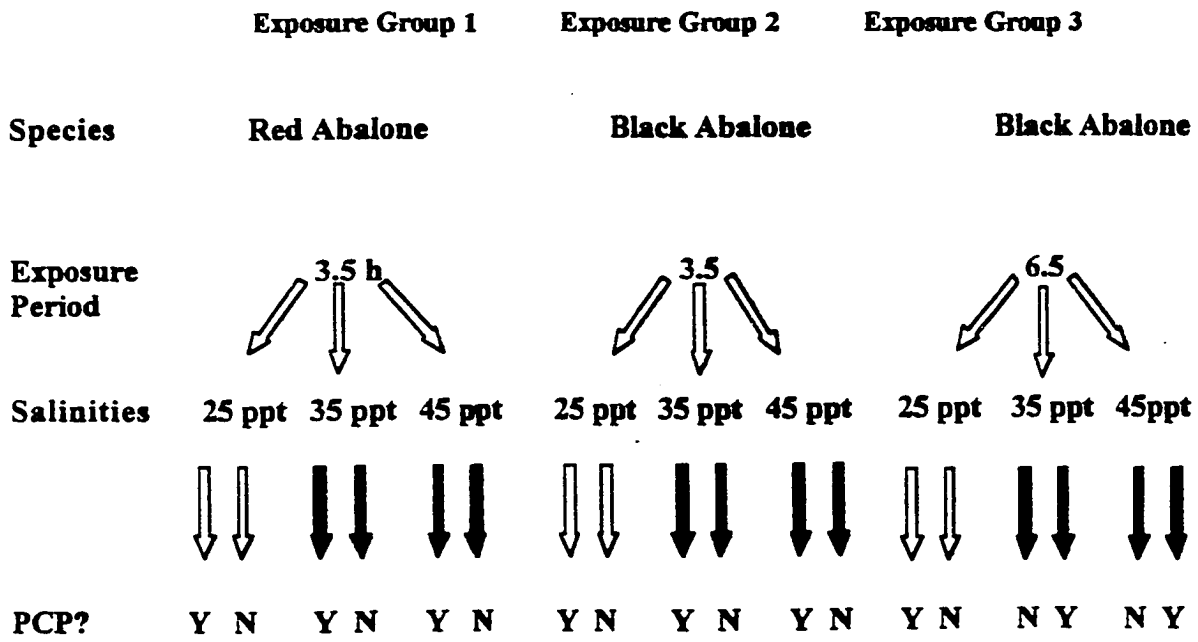
Comparisons were made on a time equivalent basis (red abalone 3.5 h exposure to black abalone 3.5 h exposure) as well as a metabolic equivalent basis. These were established from the results of the ^{31}P NMR spectroscopy study (Chapter 2) that indicated that PCP manifests its effects in red abalone after 3.5 h and in black abalone after 6.5 h. Therefore, red abalone after 3.5 h of exposure were also compared to black abalone after 6.5 h of exposure.

Assignments:

Exposure group 1: Red abalone exposed to PCP/salinity for 3.5 h

Exposure group 2: Black abalone exposed to PCP/salinity for 3.5 h,

Exposure Group 3: Black abalone exposed to PCP/salinity for 6.5 h



Y = 1.2 mg/L PCP

N = 0 PCP

Objectives:

-Determine the effect of *in vivo* exposure to PCP on the generation of reactive oxygen species (ROIs) among abalone.
-Determine the interactive effects of PCP combined with salinity stress on the animals ability to generate ROIs.
-Determine the inherent differences in ROI generating abilities in red versus black abalone after exposure to salinity and PCP stress.
-Determine whether the "metabolic endpoint" (MEP) is a viable tool for use in exposure studies that examine physiological mechanisms of toxicity.

Differential Counts and Apoptosis

Hemolymph samples were collected from the pallial sinus after each treatment and differential hemocyte counts were made on slides stained with Giemsa stain (Sigma St. Loius, MO). In order to assess cell viability (live versus apoptotic cells), a 25 µl cell suspension (1 X 10^6 cells/ml) was incubated with 1 µl of acridine orange (AO)/ethidium bromide (EB) solution (1 part 100 µl/ml AO in PBS; 1 part 100 µl/ml EB in PBS) to determine apoptotic cells. Cell suspensions from each abalone were placed on glass slides and at least 300 cells were examined using fluorescence microscopy with a fluorescein filter and a 60X objective. Nuclear morphology and chromatin condensation was assessed immediately after addition of AO/EB mixture.

In vitro Phagocytosis

Phagocytosis was measured using a modification of R.S. Anderson's Microtiter Plate Assay (Anderson and Mora, 1995; Friedman *et al.*, in press). Approximately 500 µL hemolymph was withdrawn from the pallial sinus using a 1-cc syringe with a 23-gauge needle. Cells were counted with a hemocytometer (Fisher Scientific, Inc) and

cell density was adjusted to 1 x 10^6 cells/ ml. A 50 µL aliquot of the cell suspension was placed on a glass slide and allowed to incubate for 20 minutes at 21°C, allowing hemocytes adhere and spread. A 50 µL suspension of 1 x 10^7 FITC-labeled yeast/ml in 50% serum was then added to the cells and the suspension was incubated for 45 min at 21°C. Fluorescence was quenched with 50 µL of 10% Trypan Blue in sodium citrate buffer (pH 4.5) for 8 min. Only those yeast particles that have been engulfed retain their fluorescence. Number of yeast/hemocyte (phagocytic index) and the number of hemocytes with engulfed yeast (% phagocytosis) was quantified by the random counting of 200 hemocytes.

In vitro Chemotaxis

Chemotaxis was measured using a modification of the Boyden microscopic filter technique (Boyden, 1962). A blind-well, double chamber apparatus (Corning Costar, Acton, MA) separated by a 5 µm millipore membrane allowed chemotactically-stimulated hemocytes to migrate from the upper chamber to the lower chamber. The lower chamber contained a 200 µL suspension of live *Pseudomonas stutzeri* in 50% serum (1 x 10^8 bacteria in 0.4 mL) while the upper chamber contained an equal volume of 1 x 10^5 cell/mL hemocyte suspension in 50% serum. Chambers were incubated for 1.5 h at 21°C to allow migration of chemotactically stimulated cells through the filter pores toward the bacteria. Membranes were removed and stained with Camco Differential Stains (Sigma, St Louis, Mo). Chemotaxis was quantified microscopically by counting 5 separate fields. The ratio of hemocytes that migrated to the lower surface

of the membrane to the total number of hemocytes in the field is a representation of percent chemotaxis. Controls containing hemocytes in 50% serum and augmented Hanks Balanced Salt Solution in the upper chamber and homologous buffer alone in the bottom well were run for each trial in order to quantify random migration.

Cytoskeletal Organization

The hemocytes from exposed and unexposed abalone were examined using phase contrast and fluorescence microscopy. After hemolymph was withdrawn from abalone, cells were allowed to settle onto glass slides for 20 minutes. They were then rinsed with PBS and covered with 1% fresh phosphate buffered glutaraldehyde and incubated for 10 minutes. The slides were rinsed and covered with 1% Triton X100 for 5 minutes at room temperature with the lights dimmed. The slides were rinsed in PBS and covered with 50 µL 4,6-Diamidino-2-phenylindole (DAPI) and 50 µl FITC-Phalloidin (Sigma, St. Louis, MI), mounted with aqueous mounting solution, covered, and allowed to dry before viewing. Cell morphology was assessed qualitatively according to the extent of pseudopods extended per cell. Controls for both exposed and unexposed hemocytes were prepared and incubated in PBS only.

Statistical Analysis

Statistical analysis was conducted using the general linear model procedure of SAS version 6.12 (1989). Data were analyzed as an ANOVA with a 3x2x2 factorial arrangement of treatments. If the F-test was significant then differences among

individual means were tested using the PDIFF option. Differences were considered significant if the P-value was < 0.05.

Two types of comparisons were performed. The first comparison assessed the differences among dependent variables based on their time equivalent. This comparison included red abalone at 3.5 h of exposure and black abalone at 3.5 h of exposure. Next, a comparison based on the species individual metabolic equivalents was performed. This included comparing red abalone at 3.5 h with black abalone at 6.5 h which are the respective metabolic endpoints derived from the initial ^{31}P NMR spectroscopy experiments.

Results

No significant differences were found in total hemocyte counts or percentage Type II hyalinocytes (Shields et al., 1996, 1997) between control and exposed abalone. Cellular viability was assessed immediately after *in vivo* exposure as well as just prior to analysis and was consistently over 92% in each application. Furthermore, using ^{14}C PCP it was determined that salinity has an insignificant influence on both seawater pH (8.0) and the solubility of PCP at the concentration used ($p > 0.05$) (Martello *et al.*, 1998). All of the PDIFFs in the following analyses have been corrected using the Bonferoni correction in order to insure conservatism.

Phagocytosis

Table 1

Effects in Model	F-Test P-Values from ANOVA
Exposure Group	0.0003
Salinity	0.0030
PCP	0.0320
Exposure Group * Salinity	0.1389
Exposure Group * PCP	0.6308
Salinity * PCP	0.5068
Exposure Group * Salinity * PCP	0.7878

Overall, there was a highly significant effect of exposure group ($p = 0.0003$, ANOVA) for % phagocytosis (% P) (Table 1). This effect occurred because %P among exposure group 1 was significantly lower than exposure groups 2 and 3 ($p < 0.0022$, Bonferroni adjusted PDIFF/SAS) suggesting that there are inherent differences in phagocytic ability between species. Furthermore, there was an significant effect of salinity ($p = 0.003$, ANOVA), as high salinity was found to cause a significant decline in %P versus ambient salinity (35 ppt) or low salinity (25 ppt; $p < 0.0337$, Bonferroni adjusted PDIFF/SAS). Finally, there was an effect of PCP ($p = 0.0320$, ANOVA) as PCP exposure caused an overall reduction in %P. There were no interactions among these effects as demonstrated by the lack of significance according to the ANOVA (Table 1). This suggests that the effects of treatment, salinity and PCP were not influenced by each other.

Pair-wise Comparison of Means

Relevant pair-wise comparisons of means were performed using the SAS multiple comparison PDIFF procedure with a Bonferroni adjustment. Figure1 presents the influence of salinity on PCP exposure and %P in red versus black abalone. However, there is a high probability that a type I error will occur among these comparisons because the F-test from the ANOVA indicate that there is no significant three-way interaction. There is an apparent trend, though not significant, of decreasing %P with increasing salinity among red abalone both in the presence and in the absence of PCP. Salinity variations had no impact on black abalone at either 3.5 or 6.5 h. Furthermore, although phagocytic ability was comparable between red and black abalone at low salinity, the %P among red abalone in high salinity is substantially lower, though not significantly, than black abalone at any salinity.

PCP seemed to cause the same slight reductions in %P among red abalone at all salinities although none were significant. Finally, a consistent trend throughout these data suggests that high salinity in combination with PCP has a subadditive effect on red abalone while more of a potentiating effect among black abalone.

Phagocytic Index

Table 2

Effects in Model	F-Test P-Values from ANOVA
Exposure Group	0.0001
Salinity	0.0007
PCP	0.0001
Exposure Group * Salinity	0.0234
Exposure Group * PCP	0.0726
Salinity * PCP	0.2992
Exposure Group * Salinity * PCP	0.5064

Overall, there was a highly significant effect of exposure group ($p = 0.0001$, ANOVA) for the measure of phagocytic index (PI; Table 2). This effect occurred because PI among red abalone exposed for 3.5 h was significantly lower than black abalone exposed for 3.5 h and black abalone exposed for 6.5 h ($p < 0.0001$, Bonferroni adjusted PDIFF/SAS). Again this suggests that species have different abilities to perform this particular measure. Furthermore, there was an effect of salinity ($p = 0.0007$, ANOVA), as the effect of high salinity was found to cause significantly greater reduction in PI than either ambient salinity or low salinity ($p < 0.01$, Bonferroni adjusted PDIFF/SAS). Figure 2 presents the effect of salinity on phagocytic index (PI) across the three exposure groups. While there was no effect of salinity among red abalone after 3.5 h, there was a significant reduction in PI due to salinity variations among black abalone after 6.5 h only ($p = 0.0234$, ANOVA). Furthermore, there was a significant PCP effect ($p = 0.0001$, ANOVA) as PCP caused a significant reduction in PI overall.

Relevant pair-wise comparisons of means were performed using the SAS multiple comparison PDIFF procedure with a Bonferroni adjustment. Figure 3 presents the influence of salinity on PCP exposure and PI in red versus black abalone. However, there is a high probability that a type I error will occur among these comparisons because the F-test from the ANOVA indicate that there is no significant three-way interaction.

According to the pair-wise comparison, black abalone (6.5 h) demonstrated greater phagocytic indices than red abalone (3.5 h) at high salinity ($p < 0.05$) in the absence of PCP. Although nonsignificant, there was a trend of decreasing PI with increasing salinity much like that for %P among red abalone. Only subtle PCP effects were observed at each salinity among red abalone and were not significant. Although nonsignificant, black abalone exposed for 6.5 h to either low or high salinity plus PCP displayed a substantial decline in PI compared to low and high salinity alone. Again, it appears that the effect of PCP is potentiated by salinity variations among black abalone (after 6.5 h) and is subadditive among red abalone (3.5 h). No significant effect was demonstrated for black abalone exposed for 6.5 h to ambient seawater plus PCP and no significant salinity or PCP effects were demonstrated among black abalone after 3.5 h of exposure.

Chemotaxis

Table 3

Effects in Model	F-Test P-Values from ANOVA
Exposure Group	0.2931
Salinity	0.0232
PCP	0.1312
Exposure Group * Salinity	0.0910
Exposure Group * PCP	0.9500
Salinity * PCP	0.9812
Exposure Group * Salinity * PCP	0.5649

The effect of salinity was the only significant main effect demonstrated for % chemotaxis (%C; $p = 0.0374$, ANOVA; Table 3). This effect occurred because high salinity caused a reduction in %C when compared to ambient and low salinity ($p < 0.0374$, Bonferroni adjusted PDIFF/SAS). There were no interactions among the varying effects as demonstrated by the lack of significance according to the ANOVA (Table 3). This suggests that the effects of treatment, salinity and PCP were not influenced by each other.

Pair-wise Comparison of Means

Relevant pair-wise comparisons of means were performed using the SAS multiple comparison PDIFF procedure with a Bonferroni adjustment. Figure 4 presents the influence of salinity on PCP exposure and % Chemotaxis in red versus black abalone. Interestingly, in contrast to the previous two assays, the chemotactic ability *between* species at ambient salinity is very similar. Once again, high salinity in

combination with PCP appears to have a subadditive effect among red abalone which does not appear to be the case among black abalone at high salinity.

Cytoskeletal Organization

Within 15 min of extraction, the unexposed hemocytes became fully spread and ameboid, measuring approximately 20 μm across the longest axis. The majority of cells became completely flattened, with the nucleus eccentrically positioned and most cells extended numerous pseudopods. Conversely, over 30% of cells of both red and black abalone exposed to high salinity did not aggregate and remained completely rounded and unattached to the glass slide. The rounded cells measured less than 10 μm across and, although it was possible to fix the cells onto glass slides, the cells did not demonstrate the characteristic spreading of unexposed cells. Pseudopod extensions were negligible and the nucleus and various organelles were not discernable. A cells inability to adhere to and spread on glass is symptomatic of a reduction in phagocytic ability as these are prerequisites for a functioning cytoskeleton.

Table 4

Effects in Model	F-Test P-Values from ANOVA
Exposure Group	0.0009
Salinity	0.0001
PCP	0.0704
Exposure Group * Salinity	0.1354
Exposure Group * PCP	0.8325
Salinity * PCP	0.2281
Exposure Group * Salinity * PCP	0.5260

ANOVA results

A significant main effect of exposure group was demonstrated for the assessment of cell morphology ($p = 0.0009$, ANOVA; Table 4). This effect occurred because red abalone 3.5 h (exposure group 1) had significantly higher percentages of cells that retained a rounded conformation than black abalone after 3.5 h (exposure group 2) and black abalone after 6.5 h (exposure group 3; $p < 0.0006$, Bonferroni adjusted PDIFF/SAS). Furthermore, there was an effect of salinity ($p = 0.0001$, ANOVA), as high salinity was responsible for causing more cells to retain a rounded conformation than either ambient salinity or low salinity ($p < 0.0001$, Bonferroni adjusted PDIFF/SAS). There were no interactions among these effects as demonstrated by the lack of significance according to the ANOVA (Table 4) suggesting that the effects of treatment, salinity and PCP were not influenced by each other.

Relevant pair-wise comparisons of means were performed using the SAS multiple comparison PDIFF procedure with a Bonferroni adjustment. Figure 5 presents the influence of salinity on PCP exposure and cell morphology in red versus black abalone. Low salinity did not have a significant effect on the percent of cells that retained a rounded conformation in either red or black abalone relative to controls in the presence or absence of PCP. Conversely, although not significant, high salinity caused an apparent increase in the percentage of cells that assumed a rounded conformation in red abalone exposed for 3.5 h both in the presence and absence of PCP. Furthermore, high salinity potentiated the effect of PCP among black abalone when compared with ambient salinity plus PCP ($p < 0.0463$).

Discussion

Disease outbreaks in marine animals depends on the presence of infectious pathogens, environmental conditions as well as the physiological state of the host. Exposure to stress may enhance host susceptibility. Overall, we were surprised to find that black abalone had a greater ability to perform phagocytic functions (%P and PI) than red. The hypothesis suggested that perhaps the reason Withering Syndrome (WS) has targeted black abalone is because their phagocytic ability is not as robust as that for red abalone. Not only were both %P and PI greater among black abalone but the other two measures (%C and cell morphology) were comparable between species. This suggests that there is no reason to believe that the parameters used to test locomotory aspects of the immune response is any less robust for black abalone than it is for red abalone. In fact, it generally appears more robust among black abalone. What was not surprising was that the effect of salinity was more pronounced among red abalone than black abalone which was particularly evident when measuring PI whereupon a significant exposure group by salinity interaction was exhibited. Furthermore, black abalone did not demonstrate any adverse effects of salinity changes after either 3.5 or 6.5 h. Red abalone, on the otherhand, demonstrated a consistent trend of decreased phagocytic response (%P and PI) with increased salinity. While black abalone inhabit the intertidal zone, red abalone reside deeper in the subtidal areas and are therefore exposed to fewer natural environmental variations such as temperature and salinity changes. Black abalone may be particularly well adapted to lower salinities as it is not

uncommon for tide pools to assume salinities of 25 ppt after significant rainfall events in the winter months (John Pearse, person. commun.).

It may be important to keep in mind that the black abalone collected for these experiments are from a population of black abalone that reside in a location that serves as a rookery for Elephant Seals and California Sea Lions (Ano Nuevo Island , CA). The waters surrounding this island (as well as the Channel Islands and Farallon Islands, CA) undoubtedly contain high concentrations of microorganisms such as *ecoli* from animal waste. It is plausible that black abalone have adapted to this increase in microbial exposure by having a (hyper) sensitized phagocytic response. This may be a necessary adaptation that farmed populations of red abalone, in particular, may lack.

The data presented here suggests that *in vivo* exposure of abalone to PCP causes modulation of phagocytic measures (%P and PI) while having limited effects on chemotactic ability as well as cell morphology. Surprisingly however, variations in salinity did not exacerbate the PCP effect for any of the four measures tested according to the ANOVAs. Moreover, salinity in the absence of PCP had virtually no effect on black abalone whatsoever making it possible to directly compare red abalone after 3.5 h of exposure to black abalone after 6.5 h of exposure in many circumstances. While red abalone demonstrate effects of PCP after 3.5 h it was not until 6.5 h that black abalone manifested any PCP effects. These intervals reflect the respective metabolic endpoint for each species as determined by ^{31}P NMR spectroscopy (Martello *et al.*, 1998). The MEP may therefore prove to be a useful framework for physiological comparisons between species. This method can be used in conjunction with conventional studies that

use identical exposure periods in an effort to keep adverse effects from being overlooked.

One of PCPs mechanisms of action is the uncoupling of oxidative phosphorylation, leading to a decrease in ATP available to the cell (Weinbach & Garbus, 1965). Both phosphagen concentrations and arginine kinase activity have been shown to be effected by PCP exposure (Martello *et al.*, 1998; Tjeerdema *et al.*, 1991a, b, 1993; Shofer *et al.*, 1996, 1997). Phagocytosis is an energy requiring processes. It is therefore likely that the PCP induced suppression of immune function seen in these experiments, as well as previous experiments, (Anderson & Brubacher, 1992, 1993; Roszell & Anderson, 1993) result in part from a decrease in the energy available to the hemocytes. Supporting this argument are previous reports that another uncoupler of oxidative phosphorylation, 2-4 dinitrophenol, inhibits superoxide release by rat alveolar macrophages (Castranova *et al.*, 1987). An interesting observation was that although there was no overall interaction of salinity variations on the magnitude of PCP effects, high salinity seemed to potentiate the effect of PCP among black abalone and have a subadditive effect at high salinity among red abalone. Furthermore, whereas red abalone seemed to demonstrate a slight resistance to PCP at low salinity, black abalone appeared more resistant to PCP at both ambient and low salinity. This observation suggests that hypersaline conditions may be more stressful and perhaps reduce an individuals tolerance to PCP. These data are in direct contrast to the observations of Kim (1997) who observed increasing phenol toxicity to juvenile Japanese abalone (*Haliotis discus hannai*) with decreasing salinity. Phenols lethality to these juveniles may be due to its

role in damaging gill tissue as well as its capacity to denature proteins (Fries and Tripp, 1980). Black abalone inhabit the intertidal and shallow subtidal zones, while red and Japanese abalone are subtidal (Haaker et al., 1986, Tom McCormick, Proteus Seafarms, Pers. Comm.). That animals in the intertidal zone are routinely exposed to salinity fluctuations, especially hyposaline conditions in winter and spring, may explain the resistance of black abalone to changes in salinity alone and their greater resistance to PCP at varying salinities than the subtidal red abalone. The young life stage combined with habitat preference of the Japanese abalone may account for the apparent difference in tolerance to phenolic compounds at varying salinities.

Both salinity and temperature variations have been previously found to influence the intensity of response to PCP *in vivo* (Tjeerdema *et al.*, 1993, 1996; Martello *et al.*, 1998). The 3.5 h total concentration factor (TCF) for red abalone exposed to PCP at all salinities was similar to the 6.5 h TCF for black abalone (Martello *et al.*, 1998). This suggests that black abalone possess greater resistance to PCP with salinity variation due to slower uptake. Furthermore, the 6.5-h TCF for black abalone at $25^{0}/_{00}$ was 30% lower than at ambient and high salinity. It is, therefore, surprising that the effect of PCP at low salinity is not notably different than at high salinity among black.

Marine mollusc hemolymph follows the osmotic strength and ionic composition of ambient water (Shumway, 1977; Gilles, 1979). Fisher & Newell (1986) found that increased salinity retards hemocyte activity in *C. virginica* in both acute and acclimated conditions. Increased salinity not only caused hemocytes to spread more slowly but also reduced the rate of hemocyte locomotion towards antigens, thereby preventing the cells

from reacting to a pathogen in a timely manner (Fisher & Newell, 1986). Phagocytosis and chemotaxis are influenced by both response time and locomotory ability. The results presented here show reduced activity with increases in salinity among both red and black abalone. As Fisher *et al.* (1987) postulated, it is possible that hemocytes were adjusting intracellular osmotic pressure and may need to be iso- or hyperosmotic relative to their environment in order to become ameboid. While the authors found that acute increases in salinity retard the locomotion of *C. virginica* hemocytes, apparently acute salinity reductions enhance hemocyte locomotion, implying that it is more time-consuming for oysters to adjust to higher salinities than to lower salinities (Fisher and Newell, 1986). In addition, several investigators have found that isolated molluscan cells respond more rapidly to resist swelling (hypo-osmotic conditions) than to resist shrinking (Davenport, 1982; Newell *et al.*, 1986). Red abalone do indeed demonstrate increased chemotactic ability at low salinity but this was not observed among black abalone. Furthermore, the overall effects of PCP are less severe when combined with low salinity among red abalone. Chu *et al.* (1993) found that the prevalence and intensity of *Perkinsus marinus* infections were positively correlated with salinity. In fact, low salinity treatment of *P. marinus* infected oysters was found to significantly reduce oyster mortality while not eliminating the disease (Scott *et al.*, 1985). This may be a consequence of reduced activity of the pathogen, a more competent oyster defense system at low salinities, or both (Chu *et al.*, 1993). Because hemocyte spreading and locomotion is linked to intracellular volume regulation, exposure to high salinity may delay phagocytosis enough to corroborate the effects seen. Like black abalone, oysters

generally occupy the intertidal zone and are accustomed to fluctuations in salinity and hyposaline conditions (Galtsoff, 1964). Indeed, low salinity offset the effects of PCP for red but not black abalone. Based on the information presented here, red abalone seem to respond with greater sensitivity to salinity variations than do black abalone which is consistent with the environment the two species inhabit.

Although no changes in total or differential hemocyte counts were detected here, Fries and Tripp (1980) found a reduction in granulocyte number in a dose-dependent manner in *M. mercenaria* upon exposure to phenol. Furthermore, no apoptotic or necrotic deterioration was detected which would reduce their efficacy. We observed inherent differences between the phagocytic ability of red and black abalone. The response of black abalone is greater than that of red abalone in both ambient and high salinity but is comparable at low salinity suggesting that while low salinity augments phagocytic ability in red abalone it does not do so among black abalone. This suggests that salinity changes may have a lesser impact on black abalone than red in that inhibition as well as stimulation of phagocytosis may be due to cellular damage not detected by the apoptosis assay.

PCP exposure had the greatest impact on PI suggesting that the PCP induced reduction of intracellular ATP impairs repeated engulfment or perhaps the intracellular signaling mechanism that initiates phagocytosis is affected. Moreover, additional foreign particles per cell would necessitate increased production of reactive oxygen intermediates which is energetically exhaustive. Peirce and Greenberg (1976) found that the metabolic inhibitors cyanide and 2,4-dinitrophenol in 50% seawater augmented the

amino acid efflux, the mechanism behind volume regulation, versus exposure to 50% seawater alone. Evidently, the control mechanisms behind amino acid release from molluscan cells stressed by hypo-osmotic conditions depend on external divalent cation concentration, membrane permeability, and ATP production. Both of these metabolic inhibitors impair ATP synthesis as does PCP (Goodman and Gilman, 1996) indicating that the regulation of cellular permeability may be impaired following exposure to this class of compounds.

According to Karnovsky (1962, 1970) the total quantity of intracellular ATP does not change during phagocytosis but the rate of ATP turnover increases dramatically. Mammalian macrophages contain large quantities of creatine phosphate, similar to phospharginine in invertebrate hemocytes. According to Densen (1978) the levels of these phosphate storage molecules fall dramatically during phagocytosis. These results strongly suggest that ATP is, in fact, the energy source for phagocytosis by macrophages as well as hemocytes and that intracellular levels of ATP are preserved during phagocytosis by the phosphorylation of adenosine diphosphate (ADP) by creatine phosphate/phosphoarginine. Continuous phagocytic events may deplete these reserves sufficiently to cause the demonstrated declines in PI.

Fries and Tripp (1976) also observed that exposure of the clam (*M. mercenaria*) to > 1 ppb phenol produced damage to gill and digestive tract epithelia, which may increase susceptibility to microbial infection and disease. Subsequently they found that exposure to 10 ppb phenol reduced the number of yeast cells ingested per hemocyte (PI). PCP exposure did result in significant reductions in PI for both red and black abalone

but only in combination with salinity stress among black abalone. It is possible that the combined stress of intracellular volume regulation and energy depleting PCP exposure potentiated the effect on black abalone after 6.5 h. Trends demonstrated during phagocytosis were accentuated for PI among both red and black abalone. Anderson (1981a) found no significant reductions in phagocytosis of yeast in *Mercenaria* granulocytes after PCP exposure but found reductions in uptake and killing of bacteria by *Mercenaria* hemocytes after PCP exposure suggesting that species may be sensitive to the type of target particle itself (Anderson 1988a).

Preliminary chemotaxis studies using yeast as the target particle demonstrated limited chemotaxis as well as high variability for both species making pattern determinations difficult. The use of the live marine bacteria *Pseudomonas stutzeri* augmented the signal suggesting again that hemocyte response may vary according to the target particle used. Hemolymph in the medium may have increased the signal as it may contain opsonizing material in the form of lectins and/or agglutinins thereby augmenting chemotaxis as well as phagocytosis. Although red and black abalone demonstrate similar chemotactic ability, low salinity augmented chemotaxis in red but not black abalone.

Cytoskeletal architecture was shown to be effected primarily by high salinity among red abalone and high salinity plus PCP among black abalone. Although not as well documented as in vertebrate cells, cytoskeletal elements also contribute to the motility and phagocytic capability of invertebrate hemocytes (Alvarez *et al.*, 1989). The altered morphology demonstrated at high salinity may impair the hemocytes ability to

adhere to glass, spread and phagocytose nonself material. Svitkina *et al.* (1986) found that the metabolic inhibitors sodium azide and dinitrophenol act on actin structures by the reduction of the ATP pool. They found a strong correlation between the extent of cytoskeletal destruction and ATP depletion by these compounds within just one hour of *in vitro* exposure (Svitkina *et al.*, 1986). A reduction in ATP can impair the processes involved in the polymerization of actin as demonstrated by *in vitro* studies (Lal *et al.*, 1984; Pollard, 1984). Furthermore, the researchers suggested that several mechanisms, including specific ionic conditions, may influence the process of actin meshwork assembly particularly at the cell perimeter (Svitkina *et al.*, 1986).

The information presented here suggests that acute environmental variations combined with toxicant exposure may compromise the ability of *H. rufescens* and *H. cracherodii* to defend themselves against pathogenic infection. The species variation demonstrated may elucidate why specific populations are targeted during certain epizootics. There is unequivocal evidence to support the fact that pollutants present in the environment are capable of increasing susceptibility to infectious disease in marine organisms. As a consequence, environmental degradation may contribute to some epizootics which may be exacerbated by the addition of temperature and salinity variations. It would be interesting to investigate the pollutant/stress threshold necessary to propagate these effects.

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Fig 1. Comparison of phagocytic ability in red (3.5h) vs black (3.5h and 6.5h) after exposure to 25, 35, and 45 ^{0}/00 salinity plus 1.2mg/L of pentachlorophenol.

Data were analyzed as an ANOVA. Error bars represent the standard error of the mean (SEM).

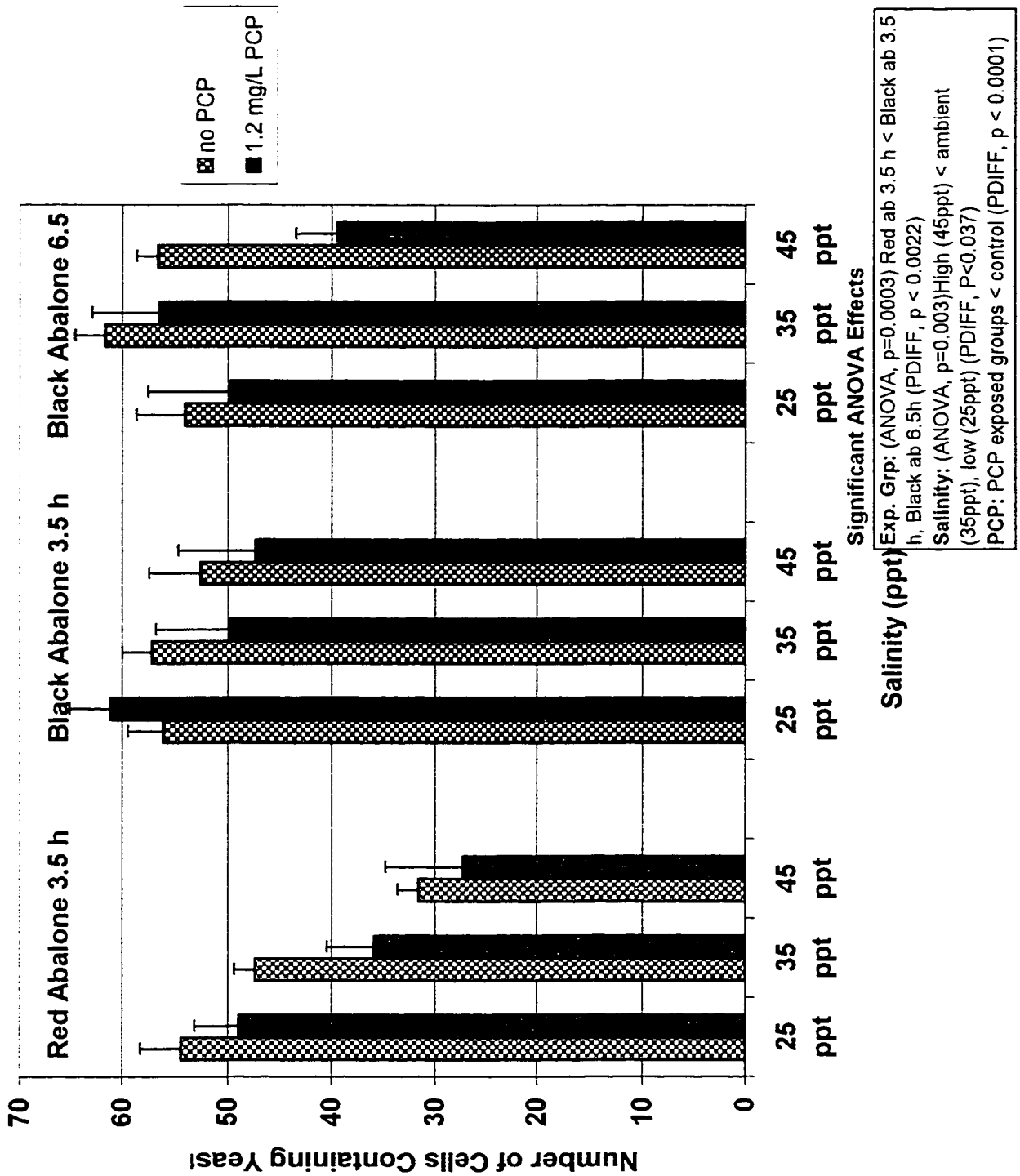


Fig 2. Two-way interaction of exposure group by salinity in red (3.5h) vs black abalone(3.5h and 6.5h) after exposure to 25, 35, and 45 ^{0}/00 salinity plus 1.2mg/L of pentachlorophenol.

Data were analyzed as an ANOVA. Error bars represent the standard error of the mean (SEM).

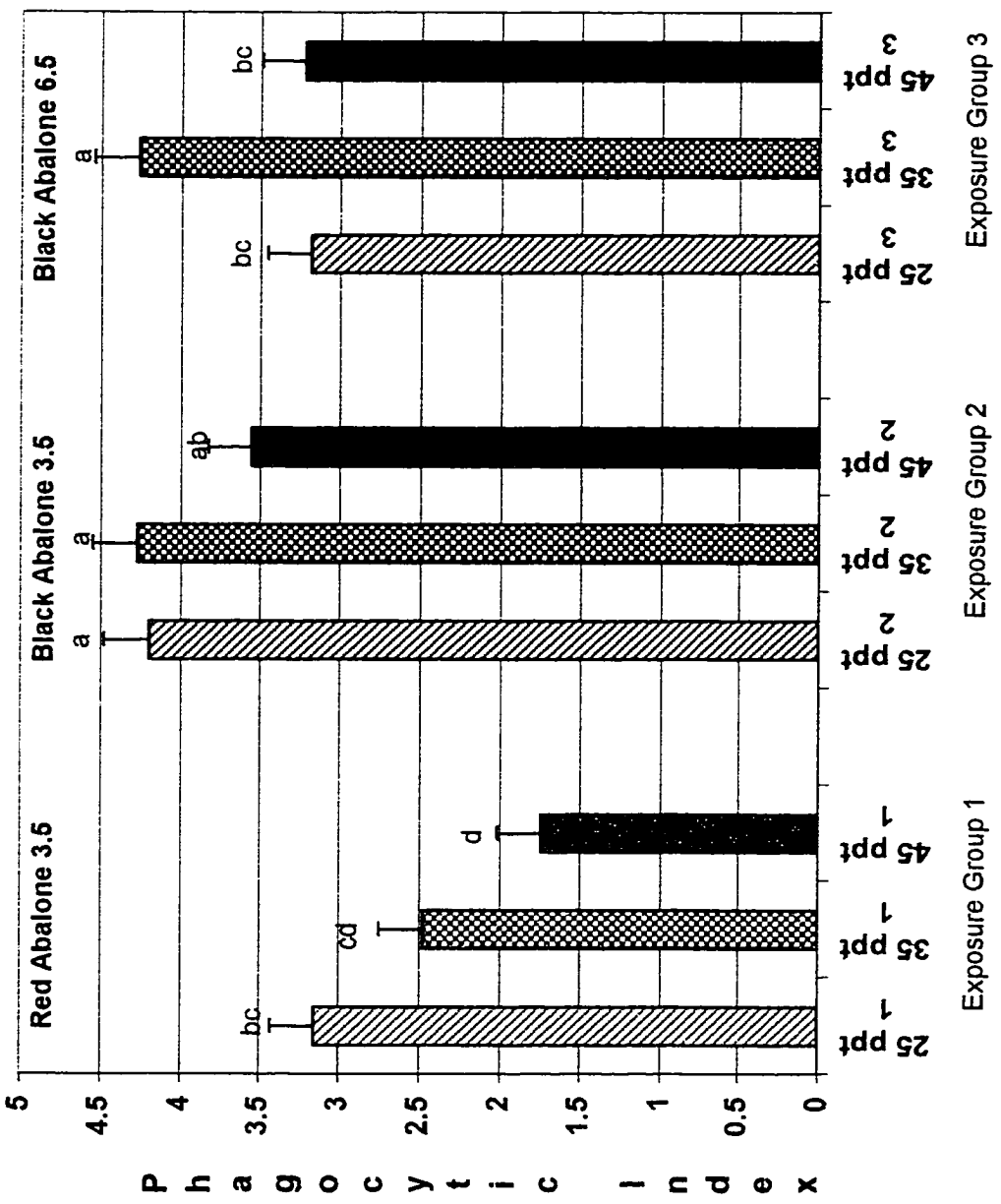


Fig 3. Comparison of phagocytic indices in red (3.5h) vs black (3.5h and 6.5h) after exposure to 25, 35, and 45 $^{0}/_{00}$ salinity plus 1.2mg/L of pentachlorophenol.

Data were analyzed as an ANOVA. Error bars represent the standard error of the mean (SEM).

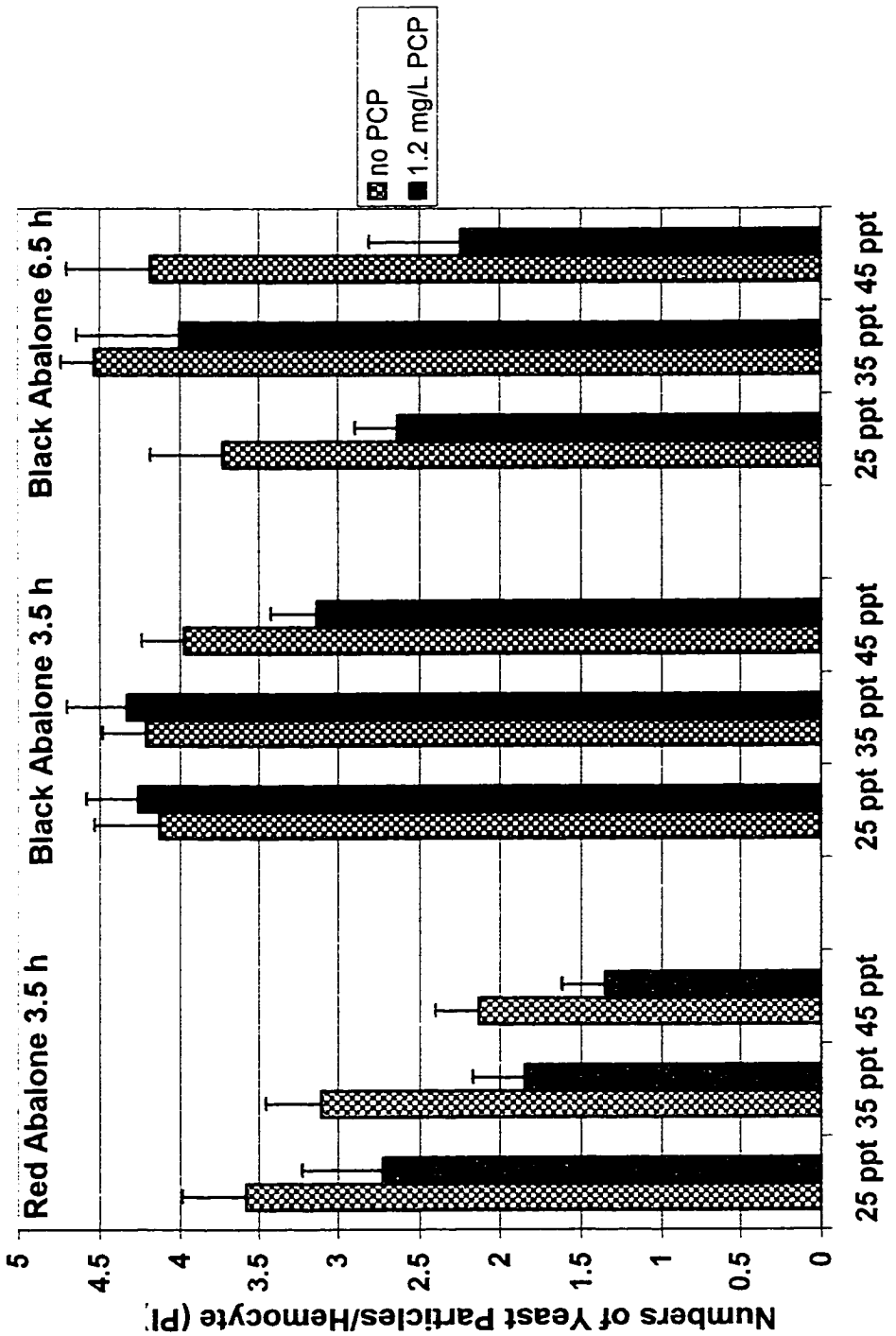


Fig 4. Comparison of Chemotactic ability in red (3.5h) vs black (3.5h and 6.5h) after exposure to 25, 35, and 45 ^{0}/00 salinity plus 1.2mg/L of pentachlorophenol.

Data were analyzed as an ANOVA. Error bars represent the standard error of the means.

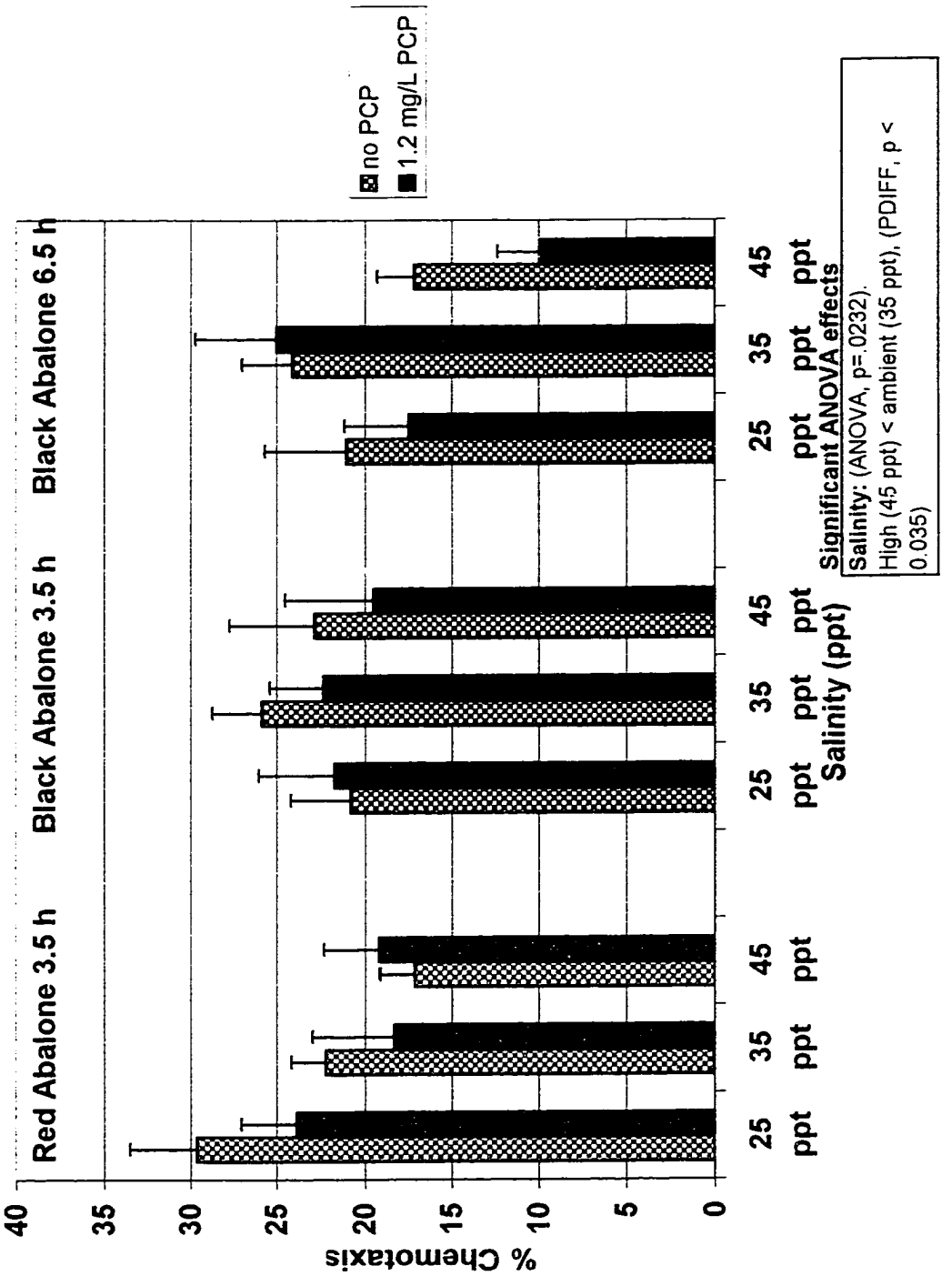
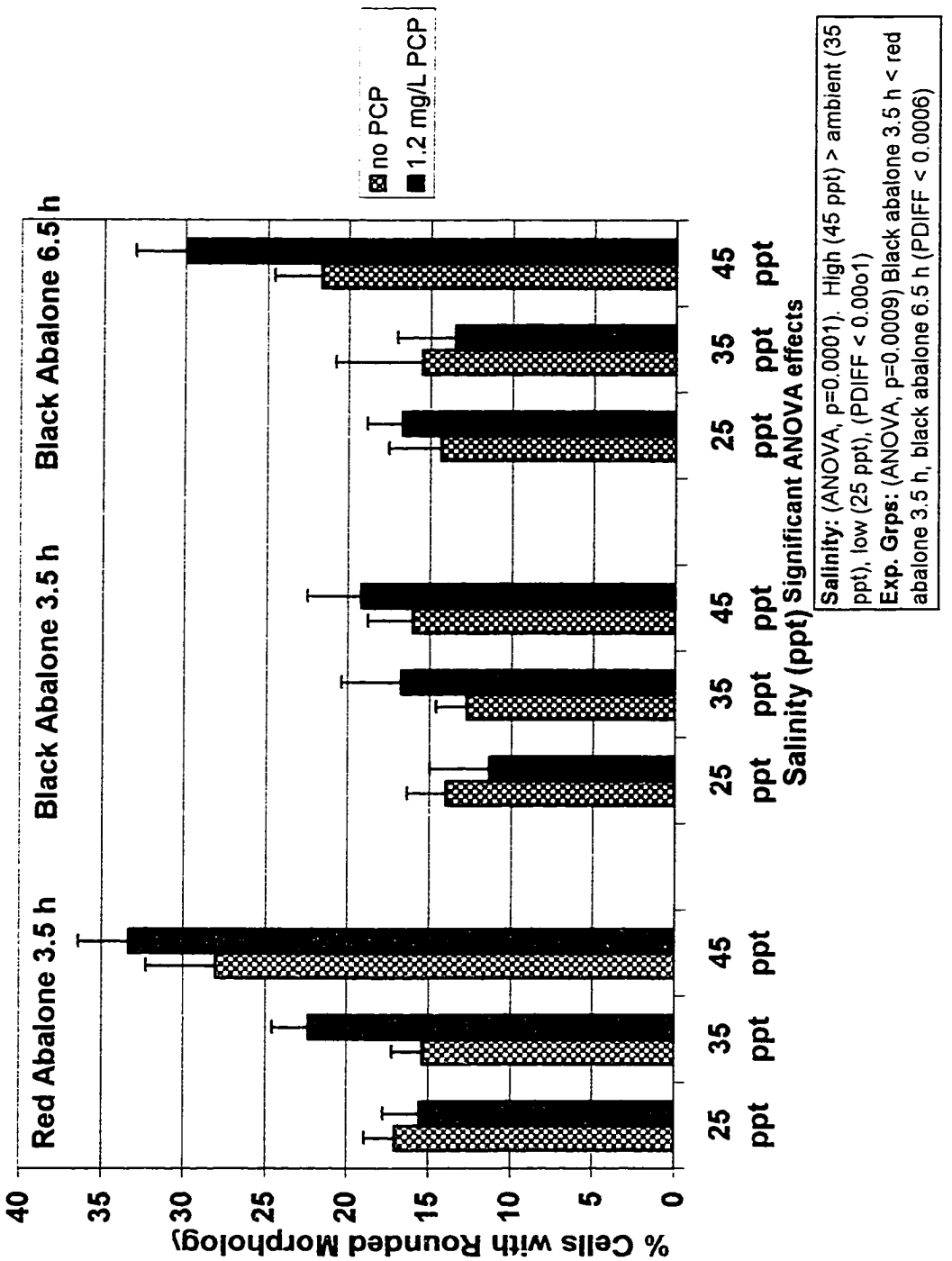


Fig 5. Comparison of cell morphology in red (3.5h) vs black (3.5h and 6.5h) after exposure to 25, 35, and 45 $^{0}/_{00}$ salinity plus 1.2mg/L of pentachlorophenol.

Date were analyzed as an ANOVA. Error bars represent the standard error of the mean (SEM).



Chapter 4

The Combined Effects of Chemical and Natural Stressors on Hemocyte Microbicidal Activity in Two Species of Abalone

Introduction

Phagocytic processes in both vertebrates and invertebrates are linked to microbicidal activities which rely on both oxygen-independent systems (Spitznagel, 1984) and oxidative metabolism (Thomas *et al.*, 1988). Oxidative metabolic events, known as the respiratory burst, are characterized by the production of reactive oxygen intermediates (ROIs; Halliwell & Guteridge, 1984). Contact with nonself leads to increased oxygen consumption due to NADPH-oxidase activity in the plasma membrane; the key enzyme in the generation of ROIs in molluscan hemocytes (Chagot, 1989). These oxidants are potent tumoricidal and antimicrobial agents that react with internalized particles in processes associated with photon-generating oxidation-reduction reactions. The light emitted can be detected by chemiluminescence (CL) which has been used to study phagocytosis and cell mediated cytotoxicity in both vertebrate (Allen *et al*, 1972., Trush *et al.*, 1978) and invertebrate (Bachere et al, 1991) blood cells. The suppression of ROI production after *in vitro* exposure to toxicants has been used as a sensitive biomarker for immunomodulation (Tam and Hinsdill, 1990). However, there is considerably less information regarding these endpoints with respect to *in vivo* exposure.

Oxidative metabolism represents one of two antimicrobial mechanisms employed during phagocytosis, the other being enzymatic processes (Bachere & Mialhe, 1991; Thomas *et al.*, 1988). Lysozyme has been detected in a variety of molluscs (Cheng and Roderick, 1974, 1975; Cheng *et al.*, 1975; McHenry *et al.*, 1979, 1986) and its biological role is believed to be involved with internal defense mechanisms and digestion (Cheng *et al.*, 1975; Cheng 1981, 1983a,b; McHenry *et al.*, 1979, 1986). However, detection and

quantification of lysozyme among abalone has yet to be established. Serum lysozyme was shown to demonstrate seasonal variation in a variety of molluscs indicating that temperature and/or salinity may effect lysozyme concentration (Chu et al., 1993; Feng and Canzonier, 1970). Furthermore, exposure of dab (*Limanda limanda*) to oil-contaminated sediments reduced lysozyme levels (Tahir *et al.*, 1993) and exposure of the clam (*Mercenaria mercenaria*) to pentachlorophenol (PCP), benzo[a]pyrene and hexachlorobenzene reduced its ability to clear a marine bacterium despite the fact that lysozyme levels were elevated (Anderson *et al.*, 1981).

Several cases of Rickettsiales-like organisms (RLO) have been associated with mortality of bivalve species (Buchanan, 1978; Comps & Raimbault, 1978) as well as scallops (Gulka *et al.*, 1983), and have been implicated in the high mortality among black abalone in the last fifteen years (Gardner, 1995). This epidemic, now known as withering syndrome (WS), can be observed as far north as Pacifica, CA (Friedman *et al.*, unpublished data). RLOs are frequently observed free in the hemolymph and infected cells are generally clustered. Antigens such as these generate a phagocytic response with the generation of toxic oxygen radicals. Cytotoxicity is exerted through enzyme inhibition, lipid peroxidation and genetic damage (DiGuglio *et al.*, 1989).

Exposure to environmental pollutants could depress phagocytic activity and the associated microbicidal activity of molluscan hemocytes, thereby increasing disease susceptibility (Anderson *et al.*, 1981; Cheng, 1988a; Alvarez *et al.*, 1991, Anderson *et al.*, 1981; Sami *et al.*, 1993, Chu and Hale, 1994). Chu and Hale (1994) found that pollutant exposure enhanced infection in the oyster *Crassostrea virginica* and increased their

susceptibility to experimentally induced infection, in a dose-dependent manner. Anderson and Anderson (1997) found that PCP inhibits both the production of NADPH and superoxide in a dose-dependent manner, and Fisher (1990) found a dose-dependent suppression of luminal-dependent chemiluminescence (LDCL) in response to tributyltin treatment in *C. virginica*. The virulence of infectious agents in the field has been correlated with high salinity and temperature (Chu and Hale, 1994). As molluscs are osmoconformers, they readily conform to the salinity of the external environment and must regulate intracellular volume accordingly. Fisher and Newell (1986) found that elevated salinity reduced the rate of hemocyte locomotion towards an antigen and may therefore pose an additional stress on these animals.

This investigation utilized CL to determine the effects of a well-known biocide, pentachlorophenol (PCP), on the oxidative killing capacity of abalone hemocytes. PCP has been shown to adversely effect a variety of defensive and metabolic functions among molluscs (Roszell and Anderson, 1993, 1994, 1996; Martello, 1998). PCP is an EPA priority pollutant widely used in industrial processes and agriculture as a wood preservative and biocide. Previous studies in our laboratory have demonstrated that *in vivo* exposure of abalone to PCP effectively reduces the intracellular phosphagen pool by 50% within 3.5 h for red abalone (*Haliotis rufescens*) and 6.5 h for black abalone (*H. cracherodii*). These intervals have been defined as metabolic endpoints (MEPs). Diminished ATP production may reduce an organism's defenses against microorganisms. The objective of this investigation was to examine whether *in vivo* exposure of red and black abalone to combined natural and chemical stressors can compromise hemocyte

microbicidal activity. Furthermore, to assess the validity of the MEP defined using NMR as it relates to immune function (Martello *et al.*, 1998).

Materials and Methods

Chemicals

PCP sodium salt (p*K*a, 5.3; water solubility at 20 °C, 80 mg/L; Merck Index, 1996) were purchased from Aldrich Chemical Co. (Milwaukee, WI), and methanol was purchased from Fisher Scientific, Inc. (Pittsburgh, PA). Natural seawater was collected and filtered (0.2 μm) at the UCSC Long Marine Laboratory, stored at 14 °C, and used within 3 d. Instant Ocean (Aquarium Systems, Mentor, OH) was used to adjust water salinity to 45 $^{0}/_{00}$, while distilled water was used to dilute seawater to 25 $^{0}/_{00}$.

Animals

Red abalone (*H. rufescens*), averaging 9-14 cm in shell length, were provided by U.S. Abalone, Davenport, CA. Black abalone (*H. cracherodii*) averaging 10-16 cm, were obtained from Ano Nuevo Island, San Mateo County, CA. They were maintained in flowing seawater, fed giant kelp (*Macrocystis pyrifera*), and acclimated to 14°C for at least two weeks prior to use. Animals were measured and weighed prior to exposure to insure uniformity.

Exposure System

PCP exposures were conducted according to Martello *et al.* (1998). Briefly, abalone was placed in a Tedlar– fluoropolymer bag (Aeroenvironment, Monrovia, CA),, which was anchored into an acrylic chamber and sealed. Aerated and temperature-controlled seawater was pumped from a 20-liter carboy with a peristaltic pump through silicon tubing. PCP was combined with the seawater flow using a second pump from a 6-liter collapsible fluoropolymer gas-sampling bag.

Exposures

Based on PCP toxicity range-finding test performed on juvenile in red abalone (6-h LC_{50} 1.6 mg/l; 6-h NOEL=0.8 mg/l)animals were exposed in flowing seawater (14^{o}C) to a sublethal PCP concentration of 1.2 mg/l and a water salinity of either 25, 35, or 45 $^{0}/_{00}$. Red (n=6) and black (n=6) abalone were individually exposed to high (45 $^{0}/_{00}$), ambient (35 $^{0}/_{00}$), or low (25 $^{0}/_{00}$) salinities for 3.5 and 6.5 h, respectively, not only to serve as controls but to determine whether salinity alone would cause an adverse response. Three sets of red (n=6) and three sets of black (n=6) abalone were then separately exposed to 1.2 mg/L PCP in ambient, low, and high seawater salinities at 14^{o}C for 3.5 h. Another three sets of black abalone (n=6) were individually exposed to 1.2 mg/L PCP for 6.5 h at ambient, low and high seawater salinities. The dosing schedule was organized around the fact that abalone are only able to withstand the loss of 500 ul of hemolymph/month necessitating a staggered schedule of animal use. The 3.5 h and 6.5 h time intervals

chosen represented the lowest average metabolic endpoints (MEPs) found using surface-probe localized ^{31}P NMR spectroscopy for red and black abalone, respectively, and were therefore chosen as the exposure periods for the following experiments (Tjeerdema *et al.*, 1996, Martello *et al.*, 1998). Moreover, the exposure periods represent the time within which animals were consistently able to recover. The dosing solution was prepared by adding 5.6 mg of PCP to 700 ml of filtered seawater in a 6-LTedlar bag for the 3.5-h periods. The final exposure concentration of 1.2 mg/L was prepared by mixing a 3 ml/min flow from the dosing bag to a 17 ml/min flow from the 20-liter seawater carboy. The volumes were doubled for the 6.5-h exposures.

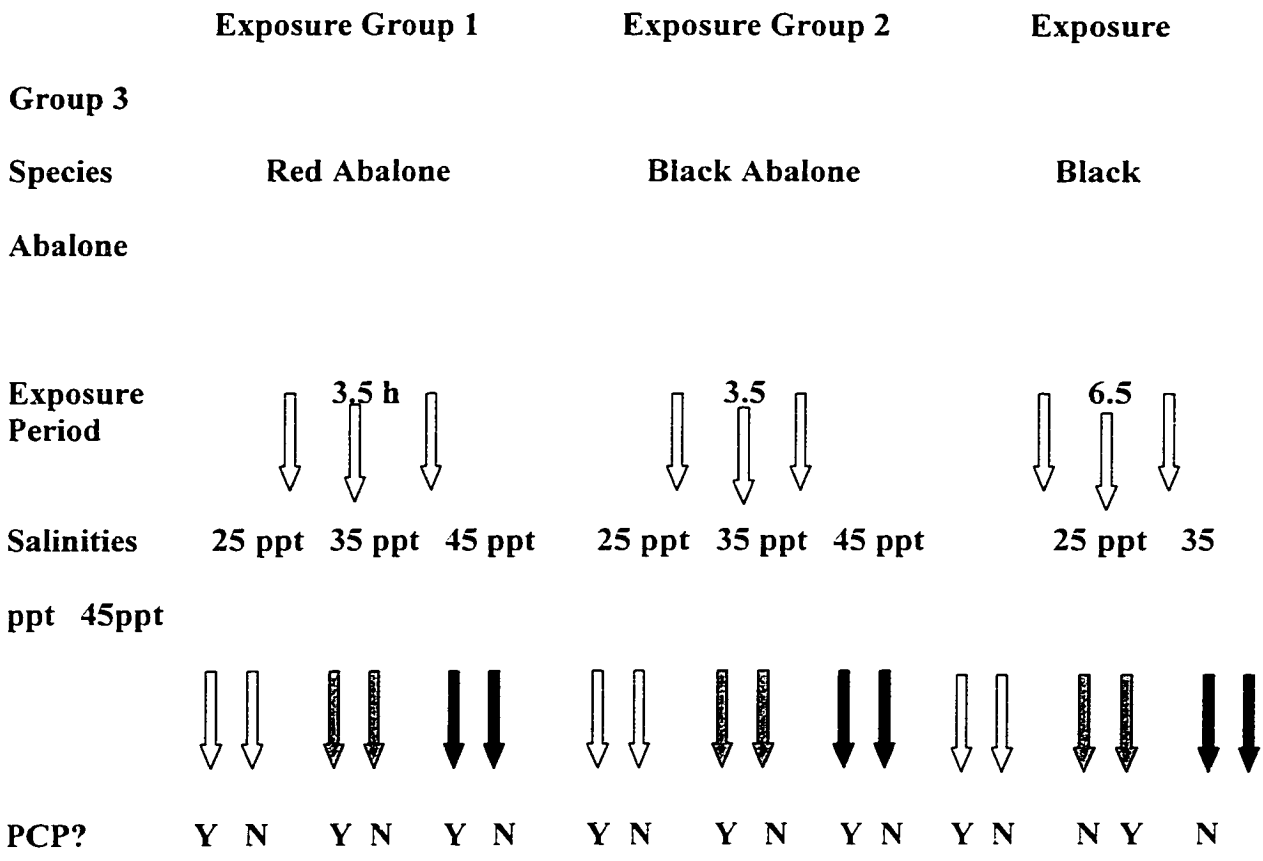
Comparisons were made on a time equivalent basis (red abalone 3.5h exposure to black abalone 3.5h exposure) as well as a metabolic equivalent basis. These were established from the results of the ^{31}P NMR spectroscopy study (Chapter 2) that indicated that PCP manifests its effects in red abalone after 3.5 h and in black abalone after 6.5 h. Therefore, red abalone after 3.5 h of exposure were also compared to black abalone after 6.5 h of exposure.

Assignments:

Exposure group 1: Red abalone exposed to PCP/salinity for 3.5 h

Exposure group 2: Black abalone exposed to PCP/salinity for 3.5 h

Exposure Group 3: Black abalone exposed to PCP/salinity for 6.5 h



Objectives:

-Determine the effect of *in vivo* exposure to PCP on the generation of reactive oxygen species (ROIs) among abalone.
-Determine the interactive effects of PCP combined with salinity stress on the animals' ability to generate ROIs.
-Determine the inherent differences in ROI generating abilities in red versus black abalone after exposure to salinity and PCP stress.
-Determine whether the "metabolic endpoint" (MEP) is a viable tool for use in exposure studies that examine physiological mechanisms of toxicity.

Differential Counts and Apoptosis

Hemolymph samples were collected from the pallial sinus after each treatment and differential hemocyte counts were made on slides stained with Giemsa stain (Sigma St. Louis, MO). In order to assess cell viability, a 25-µl cell suspension (1 X 10^6 cells/ml) was incubated with 1 µl of acridine orange (AO)/ethidium bromide (EB) solution (1 part 100 µl/ml AO in PBS; 1 part 100 µl/ml EB in PBS) to determine apoptotic cells. Cell suspensions from each abalone were placed on glass slides and at least 300 cells were examined using fluorescence microscopy with a fluorescein filter and a 60X objective. Nuclear morphology and chromatin condensation were assessed immediately after addition of AO/EB mixture.

Chemiluminescence

Cell support medium (CSM) included fetal bovine serum, antibiotic/antimycotic solution, d-glucose (Sigma, USA), and filtered seawater (FSW) and served to opsonize the zymosan. Zymosan particles (Sigma, USA) were used as the phagocytic stimulus and were suspended in fresh ambient seawater (FSW), boiled for 30 min, washed and re-

suspended in FSW, aliquoted and stored at -70°C. Number of zymosan particles was determined using a hemocytometer (Fisher Scientific); hemocyte to zymosan ratio was approximately 1:80.

Luminol stock solution (7.9 mM) was prepared according to Scott and Klesius (1981) by dissolving 0.78 g KOH, 0.618 g boric acid, and 0.014 g luminol (Sigma) in 10 ml ultrapure, deionized water. One-ml stock solution was mixed with 6.9 ml ultrapure water as the working solution.

Chemiluminescent activity of abalone hemocytes was determined as follows: 1 ml hemocyte suspension (2 x 10^6 cells/ml) was added to 1.275 ml CSM and aspirated. Luminol working solution (0.025 ml) was added and vial was counted in a model TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) in 5-min intervals for 30 min to allow background LDCL activity to stabilize. Finally, 0.2 ml zymosan suspension was added and vials were counted for at least 1.5 h at 21°C.

Lysozyme Measurement

Lysozyme was quantified based on the method described by Shugar (1952). Briefly, hemolymph is centrifuged at medium speed to remove hemocytes and was then used immediately. Four antigenic stimuli were used for detection and quantification of lysozyme. A 1-ml suspension of *Micrococcus lysodeikticus* (0.15 mg/ml, 0.66 M sodium phosphate buffer, pH 6.24) was mixed with 50 ul of serum to give a final volume of 1.050 ml according to the method described by Shugar (1952). Treatments with yeast, zymosan and *Pseudomonas stutzeri* in like suspensions were also assessed. The reaction was

carried out at 21°C at an absorbance of 450 nm for 10 min in one minute intervals on a Beckman DU650 spectrophotometer. Hen egg-white (HEW) lysozyme was used to construct a standard curve. Lysozyme activity was expressed as concentration of HEW lysozyme equivalent in micrograms per milliliter.

Statistical Analysis

Statistical analysis was conducted using the general linear model procedure of SAS version 6.12 (1989). Data were analyzed as an ANOVA with a 3x3x2 factorial arrangement of treatments. If the F-test was significant, then differences among individual means were tested using the SAS multiple comparison PDIFF procedure with a Bonferroni adjustment. Differences were considered significant if the P-value was < 0.05.

Comparisons were based on time equivalents of 3.5 h for both red and black abalone as well as metabolic equivalents of 3.5 h for red abalone and 6.5 h for black abalone which are the respective metabolic endpoints derived from the initial ^{31}P NMR spectroscopy experiments (Chapter 2).

Results

No significant differences were found in total hemocyte counts or percentage Type II hyalinocytes (Shields *et al.*, 1996,1997) between control and exposed abalone. Cellular viability was assessed immediately after *in vivo* exposure as well as just prior to analysis and was consistently over 94% in each application. Furthermore, using ^{14}C PCP it was determined that salinity has an insignificant influence on both seawater pH (8.0) and the

solubility of PCP at the concentration used ($p > 0.05$; Martello *et al.*, 1998). Regardless of the antigenic stimuli, lysozyme could not be detected during any of the treatments for either red or black abalone.

Total Chemiluminescent Response (CLtotal)

Table 1

Effects in Model	F-Test P-Values from ANOVA
Exposure group	0.0001
Salinity	0.0021
PCP	0.0485
Exposure group * Salinity	0.0032
Exposure group* PCP	0.0025
Salinity * PCP	0.8850
Exposure group * Salinity * PCP	0.0002

ANOVA Results

There was a highly significant main effect of exposure group on the total chemiluminescent response (CLtotal) where black abalone demonstrated a significantly greater ability to generate reactive oxygen species (ROIs) over a specific period of time than red abalone (ANOVA,$p=0.0001$). Furthermore, there was an overall effect of salinity whereby high salinity caused a reduction in CLtotal (ANOVA, $p=0.0021$). There was a main effect of PCP whereby exposure to PCP caused a reduction in CLtotal (ANOVA, $p=0.0485$). There was a significant interaction between salinity and exposure group where

red abalone were more sensitive to salinity changes than black abalone at either exposure period (3.5 h or 6.5 h; ANOVA, p=0.0032). There was also an interaction between PCP and exposure groups where PCP caused a stimulatory effect on red abalone CLtotal and an inhibitory effect on black abalone CLtotal (ANOVA, p=0.0025). On the other hand, there was no significant interaction between PCP and salinity. The ANOVA showed that there was a highly significant three-way interaction for CLtotal (ANOVA, p=0.0002). In order to assist in interpreting this interaction specific pair-wise comparisons were performed.

Comparison of Individual Means (CLtotal)

Relevant pair-wise comparisons of means were performed using the SAS multiple comparison PDIFF procedure with a Bonferroni adjustment. Although the analysis of variance showed significant effects most of the pair-wise comparisons did not reach significance due to the inherent conservatism of the Bonferroni as well as the number of comparisons made. Figure 1 presents the influence of salinity on PCP exposure and CL_{total} in red versus black abalone. Black abalone do not demonstrate any effect of salinity between 3.5 h of exposure and 6.5 h of exposure. Although nonsignificant, black abalone at ambient salinity demonstrate a CLtotal of nearly 1.5 times that of red abalone at ambient salinity in the absence of PCP. Black abalone at high salinity demonstrate a CLtotal of nearly 4 times that of red abalone at high salinity ($p < 0.0001$). Neither high nor low salinity caused a significant difference in CL_{total} among black abalone at either

3.5 or 6.5-h of exposure although high salinity alone caused a significant reduction in CL_{total} compared to low salinity among red abalone ($p < 0.037$).

Another noteworthy trend is the apparent stimulatory effect of PCP on red abalone at ambient and high salinities and the apparent inhibitory effect of PCP on black abalone at those same salinities. In fact, the stimulation of red abalone to PCP at ambient salinity was so great that it increased CL_{total} to twice that of black abalone under the same conditions. Furthermore, although exposure of black abalone to PCP inhibited CL_{total} at ambient salinity ($p<0.0439$) it had no significant effect at low and high salinities.

Maximum Chemiluminescent Response (CLmax)

Table 2

Effects in Model	F-Test P-Values from ANOVA
Treatment	0.0001
Salinity	0.0001
PCP	0.0003
Treatment * Salinity	0.1932
Treatment * PCP	0.0001
Salinity * PCP	0.1538
Treatment * Salinity * PCP	0.0093

ANOVA results

There was a highly significant main effect of exposure group on the maximum chemiluminescent response (CLmax) where black abalone demonstrated a significantly greater peak response regarding the generation of reactive oxygen species (ROIs) than red abalone (ANOVA,p=0.0001; Table 2). Furthermore, there was an overall effect of salinity whereby salinity variations caused an overall reduction in CLmax (ANOVA, p=0.0001).

There was a main effect of PCP as well whereby PCP caused an overall reduction in CLmax (ANOVA, p=0.0003). Interestingly, there was no significant interaction between salinity and exposure group as existed when measuring CLtotal indicating that red abalone were no more sensitive to salinity changes than black abalone at either exposure period (3.5 h or 6.5 h). There was also a highly significant interaction of PCP between exposure groups where, once again, PCP caused a stimulatory effect on red abalone CLmax and an inhibitory effect on black abalone CLmax (ANOVA, p=0.0001).

A significant three-way interaction was demonstrated for CLmax as indicated in the ANOVA table 2 (ANOVA, p=0.0093). Specific pair-wise comparisons of means were performed to assess these interactions.

Pair-wise Comparison of Means (Clmax)

To determine the specific differences that caused this interaction, pair-wise comparisons of means were performed using the SAS multiple comparison PDIFF procedure with a Bonferroni adjustment. Figure 2 presents the influence of salinity on PCP exposure and CL_{max} in red versus black abalone. Significant differences exist between red and black abalone at both ambient and high salinity in the absence of PCP ($p < 0.0003$) where black abalone illustrate a CLmax twice as great as red abalone at ambient salinity and a CLmax that is nearly four times greater at high salinity than red abalone. Varying the salinity did not have an effect on CLmax within either red or black abalone exposure groups.

While not significant, a trend observed while measuring CLtotal was repeated for CLmax where exposure of red abalone to PCP *stimulated* CLmax at ambient and high salinity while exposure of black abalone to PCP after 6.5 h *inhibited* CLmax at each salinity (significantly at ambient salinity among black abalone 6.5 h, $p < 0.0001$). Interestingly, no significant differences exist between red and black abalone exposed to 25 and 45 ppt salinity plus PCP. However, red abalone exposed to ambient salinity plus PCP was significantly greater than that of black abalone despite the fact that black abalone CLmax was nearly twice as great as red abalone CLmax in the absence of PCP at ambient salinity. No significant PCP or salinity effect on black abalone CLmax was demonstrated within the 3.5 h exposure period.

Time to Maximum Chemiluminescent Response (Tmax)

Table 3

Effects in Model	F-Test P-Values from ANOVA
Exposure Group	0.0007
Salinity	0.0764
PCP	0.4300
Exposure Group * Salinity	0.9234
Exposure Group * PCP	0.0121
Salinity * PCP	0.0026
Exposure Group * Salinity * PCP	0.0949

ANOVA Results

There was a highly significant main effect of exposure group on the time to maximum chemiluminescence (Tmax) where black abalone demonstrated a significantly

greater Tmax compared to red abalone (ANOVA,p=0.0007; Table 3). This indicates that it takes a longer period of time for black abalone to attain their peak respiratory burst than it does for red abalone. There was also a significant interaction of PCP between exposure groups (Figure 3; ANOVA, p=0.0121). This suggests that PCP increased the time to maximum chemiluminescent response differently for red and black abalone where red abalone demonstrated a greater increase in Tmax after PCP exposure than black abalone.

Furthermore, there was an effect of salinity on PCP (Figure 4) as demonstrated by the ANOVA (p=0.0026). This graph suggests that exposure of red and black abalone to high salinity caused a reduction in Tmax when compared to high salinity in the presence of PCP. However, PCP increased Tmax at high salinity counteracting the high salinity effect resulting in no effect of high salinity in the presence of PCP versus ambient salinity in the presence or absence of PCP.

A significant three-way interaction was demonstrated for CLmax as indicated in the ANOVA table 2 (ANOVA, p=0.0093). Specific pair-wise comparisons of means were performed to assess these interactions.

Pair-wise Comparison of Means (Tmax)

To determine the specific differences that caused this interaction, pair-wise comparisons of means were performed using the SAS multiple comparison PDIFF procedure with a Bonferroni adjustment. Figure 5 presents the influence of salinity on PCP exposure and T_{max} in red versus black abalone. However, there is a high probability that a type I error will occur among these comparisons because the F-test from the

ANOVA indicate that there is no significant three-way interaction. While red and black abalone demonstrate similar Tmax at ambient salinity there is a trend, though not significant, of reduced Tmax among red abalone upon exposure to low and high salinity. There are no significant differences in Tmax due to salinity among black abalone between 3.5 and 6.5h exposures. Thus, no significant salinity effects were demonstrated *within* either species.

No significant differences *between* red and black abalone were apparent after PCP exposure at any salinity tested. Although, red abalone exposed to low salinity did show a significant effect of PCP ($p < 0.0463$) this was not the case for red or black abalone for any of the other treatments. Still the trend suggests that PCP exposure may be impacting the timeliness of the respiratory burst.

Discussion

Agents that decrease LDCL activity in hemocytes may reduce the antimicrobial potential of these cells, thereby increasing susceptibility to infectious disease. The data presented here suggests that *in vivo* exposure of abalone to PCP causes modulation of ROI production. Moreover, exposure to PCP in combination with salinity stress often exacerbates that modulation. While red abalone demonstrate effects of PCP after 3.5 h it was not until 6.5 h that black abalone manifested any PCP effects. These intervals reflect the respective metabolic endpoint for each species as determined by ^{31}P NMR spectroscopy (Martello *et al.*, 1998). The MEP may therefore prove to be a useful framework for physiological comparisons between species. This method can be used in

conjunction with conventional studies that use identical exposure periods in an effort to keep adverse effects from being overlooked.

As the data suggests there are strong differences between the two species inherent ability to generate ROIs. Not only do black abalone appear to have a greater ability to produce oxygen radicals but they are more resilient to salinity changes than are red abalone particularly in regards to CLtotal. Black abalone exhibit virtually no salinity stress effect as exposure to salinity variations after 6.5 h closely mimicked that of 3.5 h. Red abalone, on the other hand, demonstrated a consistent trend of decreased chemiluminescent response with increased salinity. While black abalone inhabit the intertidal zone, red abalone reside deeper in the subtidal areas and are therefore exposed to fewer natural environmental variations such as temperature and salinity changes. Furthermore, this population of black abalone reside in a location that serves as a rookery for Elephant Seals and California Sea Lions (Ano Nuevo Island , CA). The waters surrounding this island (as well as the Channel Islands and Farallon Islands, CA) undoubtedly contain high concentrations of microorganisms such as *e. coli* from animal waste. It is plausible that black abalone have adapted to this increase in microbe concentration by being able to generate higher concentrations ROIs. While this may appear to be a beneficial adaptation to fighting off microbial infections this particularly robust ROI response may, in fact, cause tissue and cellular damage to the very host that produces it.

The most common interaction demonstrated among the three measures was exposure group * PCP. One of PCPs mechanisms of action is the uncoupling of oxidative

phosphorylation, leading to a decrease in ATP available to the cell (Weinbach & Garbus, 1965). Both phosphagen concentrations and arginine kinase activity have been shown to be effected by PCP exposure (Martello *et al.*, 1998; Tjeerdema *et al.*, 1991a, b, 1993; Shofer *et al.*, 1996, 1997). Phagocytosis and the respiratory burst are energy requiring processes. Therefore, it is likely that the suppression of immune function seen in these experiments, as well as previous experiments (Anderson & Brubacher, 1992, 1993; Roszell & Anderson, 1993), result in part from a decrease in the energy available to the hemocytes. Supporting this argument are previous reports that another uncoupler of oxidative phosphorylation, 2-4 dinitrophenol, inhibits superoxide release by rat alveolar macrophages (Castranova *et al.*, 1987). Furthermore, both naphthalene and 2,4-dinitrophenol have been shown to cause a decrease in oyster (*C. virginica*) CL activity after both *in vivo* and *in vitro* exposure (Larson *et al.*, 1989).

Contact between zymosan and the hemocyte plasma membrane stimulates a membrane bound NADPH-oxidase system to generate and release oxygen radicals. An active NADPH-oxidase enzyme complex has to be assembled from putative cytosolic and membrane associated components. After internalization, this system appears to continue oxygen radical production inside the phagosome (Adema 1991). NADPH-oxidase facilitates the transfer of an electron from NADPH to molecular oxygen to produce superoxide (Anderson and Anderson, 1997). In phagocytic cells, the NADPH used to fuel the respiratory burst is generated by the pentose phosphate pathway, which uses glucose 6-phosphate (G6P) supplied by glycolysis or glycogen metabolism. ATP is required in several steps in the production of G6P by glycolysis, as well as in the activation of the

enzymes phosphorylase kinase and glycogen phosphorylase, both of which are necessary for the cleavage of G1P from glycogen. ATP may also be necessary for the assemblage of NADPH oxidase. PCP is similar to dinitrophenol in that it acts as a proton shuttle across mitochondrial membranes, depleting the proton gradient required for ATP production (Cantelmo *et al.*, 1978). Because ATP is required for NADPH production, PCP has the potential to limit the availability of NADPH for ROI production. Continuous replacement of active NADPH-oxidase enzyme complexes is necessary to sustain the respiratory burst during interaction with nonself and may be impaired by exposure to metabolic inhibitors. PCP exposure in *Fundulus heteroclitus* phagocytic blood cells was also shown to produced a dose-dependent inhibition of phagocytically induced CL, O2- generation, and bacteriocidal activity (Roszell and Anderson, 1993). Previously it was believed that contaminants such as dioxins and furans present in technical grade PCP were responsible for diminished lymphocyte-dependent immune functions but analytical grade PCP has been shown to be strongly immunotoxic to medaka phagocytes, as measured by reduced CL responsiveness (Kerkvliet *et al.*, 1985). Analytical grade PCP also produced decreased resistance to bacterial infection in the clam *M. mercenaria* as a consequence of impaired hemocyte-mediated antibacterial capacity (Anderson et al, 1988a).

Anderson and Anderson (1997) demonstrated that in vitro exposure of the oyster *C. virginica* to PCP reduces CL and inhibits NADPH production by immunostimulated hemocytes. Because NADPH is a required cofactor in the production of superoxide, it appears that the immunosuppressive action of PCP results from reduced NADPH production. The assembly of new active NADPH-oxidase complexes in the cell membrane,

essential for ongoing ROI production, has been shown to be prevented by quinones that result from a reaction between catechols and either ROIs or ROIs together with peroxidase (Adema *et al.*, 1993), and may be a mechanism of action of PCP as well. Other possible explanations for decreased superoxide production include direct interference with NADPH-oxidase assembly or altered cellular redox status (decreased NADPH:NADP+), leading to lipid peroxidation and enzyme inactivation (Anderson *et al.*, 1996; Chu and Hale, 1994). Because oxy-radical production is an important element in microbicidal defense in molluscan hemocytes, the inhibition of this pathway by exposure to environmental contaminants could have serious consequences in terms of resistance to infectious diseases.

Although both superoxide production and NADPH production by oyster hemocytes are decreased after exposure to PCP, significant decreases in NADPH appear at lower concentrations of PCP than does decreased superoxide production (Anderson and Anderson, 1997). The fact that NADPH appears to be slightly more sensitive to PCP implies that the coupling between NADPH production and superoxide production may not be tightly linked. This is understandable as NADPH is an important cofactor in numerous other cellular functions unrelated to the production of ROIs. The degree of association between the two may explain some of the species differences demonstrated.

Phenolics are redox cycling compounds and can stimulate superoxide production in the molluscan hepatopancreas, but the degree of stimulation can vary between species (Wenning and Digiulio, 1988). Stimulation of ROI production by red abalone at ambient and high salinities may reflect this. Many molluscs have been found to possess

antioxidants (SOD, catalase, and GSH) that are designed to protect against oxidative stress, including those imposed by redox cycling compounds (Wenning and Diguilio, 1988). Perhaps antioxidant defenses are stimulated among black abalone upon exposure to PCP. Varying concentrations of these ROI scavengers responsible for neutralizing or packaging potentially reactive oxygen intermediates may exist between species and may account for the increase in ROIs among red abalone after PCP exposure.

Both Dyrynda et al. (1998) and Livingston et al. (1990) demonstrated pollution-enhanced oxy-radical production in mussels. The extent to which enhanced oxy-radical production causes oxidative damage is difficult to gauge. As among red abalone, it is unknown whether the higher levels released from hemocytes at contaminated sites arose from augmented generation, or the suppression of inhibitory antioxidant enzymes that may be demonstrating some level of cellular damage.

Although no changes in total or differential hemocyte counts were detected here, Fries and Tripp (1980) found a reduction in granulocyte number in a dose-dependent manner in *M. mercenaria* upon exposure to phenol. Furthermore, no apoptotic or necrotic deterioration was detected which would reduce hemocyte efficacy. We observed inherent differences between the ROI producing ability of red and black abalone. The overall response of black abalone is greater than that of red abalone particularly in regards to CLmax and at ambient and high salinity for CL total. Exposure to high salinity has a lesser impact on black abalone than red in that inhibition as well as stimulation of phagocytosis may be due to cellular damage not detected by the apoptosis assay.

Generally, the permeability of aquatic organisms is lower in low salinity than in high salinity environments (Blust *et al.*, 1992). Red abalone, in particular, seem to be more sensitive to high salinity in the absence of and in combination with PCP (Martello, unpublished data). Salmon smolt were twice as sensitive to Prudhoe Bay crude oil and benzene in high salinity possibly due to interference of their osmo- and ion regulating capabilities (Stickle and Sabourin, 1982). Transfer of smolt to high salinities also caused a transient increase in O_2 consumption rate. Their overall tolerance to toluene and naphthalene after placement in high salinity was considerably lower than at ambient conditions possibly due to ion regulation failures. Finally, *Fundulus heteroclitis* was more sensitive to naphthalene when exposed in hyper-osmotic than in hypo-osmotic salinities (Levitan and Taylor, 1979). It seems plausible that exposure of red abalone to high salinity in the presence of PCP may demonstrate an inhibition of regulatory mechanisms required for the controlled release of ROIs.

Protein induction may be affected by exposure to salinity variations as well. Copepods exposed to low and high salinities showed differences in the up and down regulation of specific proteins (Gonzalez and Bradley, 1994) The proteins induced may be involved in cellular transport machinery regulating solute efflux and influx. The rate of increase of free amino acids during hyper-salinity stress has been shown to vary dramatically between species of molluscs (Amende and Pierce, 1980a). The subtidal red abalone may have a diminished capacity for volume regulation in response to hyper-osmotic stress in that they occupy a tidal zone that undergoes less salinity variation than the intertidal black abalone.

Anderson (1995) demonstrated that oysters at a high salinity site were 100% infected with *Pecten maximus* and those at a lower salinity site were only 31% infected suggesting that bacterial virulence may be augmented at high salinity. Interestingly, the high salinity oysters that were completely infected at the end of year one were still highly infected (96%) when examined six months later. Ingestion of certain parasites by bivalve hemocytes fails to trigger CL, even though phagocytosis of zymosan by the same cells produces a strong CL response. For example, *P. marinus* infection of *C. Virginica* and Rickettsiales-like organisms of Pecten maximus (LeGall *et al.*, 1991) fail to elicit production of ROIs during phagocytosis (Anderson *et al.*, 1995) which may explain the ability of these parasites to survive within hemocytes of black abalone. Resistance and susceptibility to parasites may depend not only on genetically determined levels of defense capacity but also on modulation of existing defense mechanisms by parasites (Anderson, 1995).

Although lysozyme is considered to be well conserved among eukaryotes there are many examples of species that utilize other types of bacteriolysins. It is thought that among molluscs the primary cell releasing lysozyme into the serum is the granulocyte. As abalone have only few granulocytes and are dominated by Type I and II hyalinocytes it may not be surprising that abalone secrete little or no lysozyme and may therefore be employing different types of antimicrobial enzymes. Cushing et al. (1971) demonstrated a rise in bacteriocidal activity in pre-immunized red and black abalone demonstrating that, in fact, abalone do possess bacteriolytic activity of some kind. Lopez *et al.* (1997) characterized enzymes of the carpet sheli clam (*Ruditapes decussatus*) and found that

while this species of clam lacked NADPH oxidase and therefore lacks oxidative metabolism coupled to phagocytosis, they do possess significant concentrations of lysozyme. These defense mechanisms are essentially the reverse of those mechanisms employed by abalone. These clams also possess significantly high numbers of circulating granulocytes as well as hyalinocytes, again indicating that lysozyme may be dependent on granulocyte activity.

While Penaeid shrimp serum proved to be bactericidal there was no evidence of lysozyme activity whatsoever (Noga *et al.*, 1996). In fact both gram-positive and gram negative bacteria proved to be susceptible to shrimp serum indicating that Penaeid shrimp are able to produce inducible antibacterial factors after immunostimulation. Adams (1991) detected little or no bactericidin in shrimp before they were exposed to antigen. This may reflect differences in immune mechanisms even in closely related species as is demonstrated in red versus black abalone. Interestingly, it has recently been determined that the oyster, *C. Gigas*, may also lack lysozyme while its close relative *C. Virginica* has demonstrated very high concentrations of the enzyme (Dr. Michael Offret, pers. comm., 1999). Furthermore, lysozyme secretion from black abalone suffering from withering syndrome has yet to be demonstrated (Dr. Carolyn Friedman, per. comm., 1999).

Although pollutants have not been found to be directly responsible for the decline in the abalone population, the only sizable populations that remain are around islands off the coast of California (channel Is., Farrallon Is.) and coastlines north of San Francisco. It is plausible that the cumulative stress of over-harvesting, sea otter predation, loss of habitat, and human inputs have contributed to these declines. The research performed

herein attempts to address a mechanistic approach to studies of toxicology but is restricted by certain limitations inherent to the design of any acute approach to toxicology. Although estuarine seawater salinities may range as much as 20-25 ppt it is unusual for subtidal animals to experience salinity deviations of more than 5-7 ppt from ambient although runoff events feeding tide pools could decrease the salinity to 25 ppt (John Pearse, person. Comm). Furthermore, PCP concentrations in the mg/L amount are typically found near industrial outfalls and wood and paper mills only.

Extensive variability in immune function exists in natural invertebrate populations and such changes are not solely induced experimentally. The multi-stressed nature of the environment, in which both anthropogenic and natural factors may exert effects (Fisher *et al.*, 1996), makes it difficult to attribute immunomodulation to any one causative agent. In addition, immune parameters may be either enhanced or suppressed, making the elucidation of a mechanism behind the success of certain epizootics difficult to establish.

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Fig 1. Comparison of total chemiluminescent response (CL{total} in red (3.5h) vs black abalone (3.5h and 6.5h) after exposure to 25, 35, and 45 $^{0}/_{00}$ salinity plus 1.2mg/L of pentachlorophenol.

Data were analyzed as an ANOVA. Error bars represent the standard error of the means.

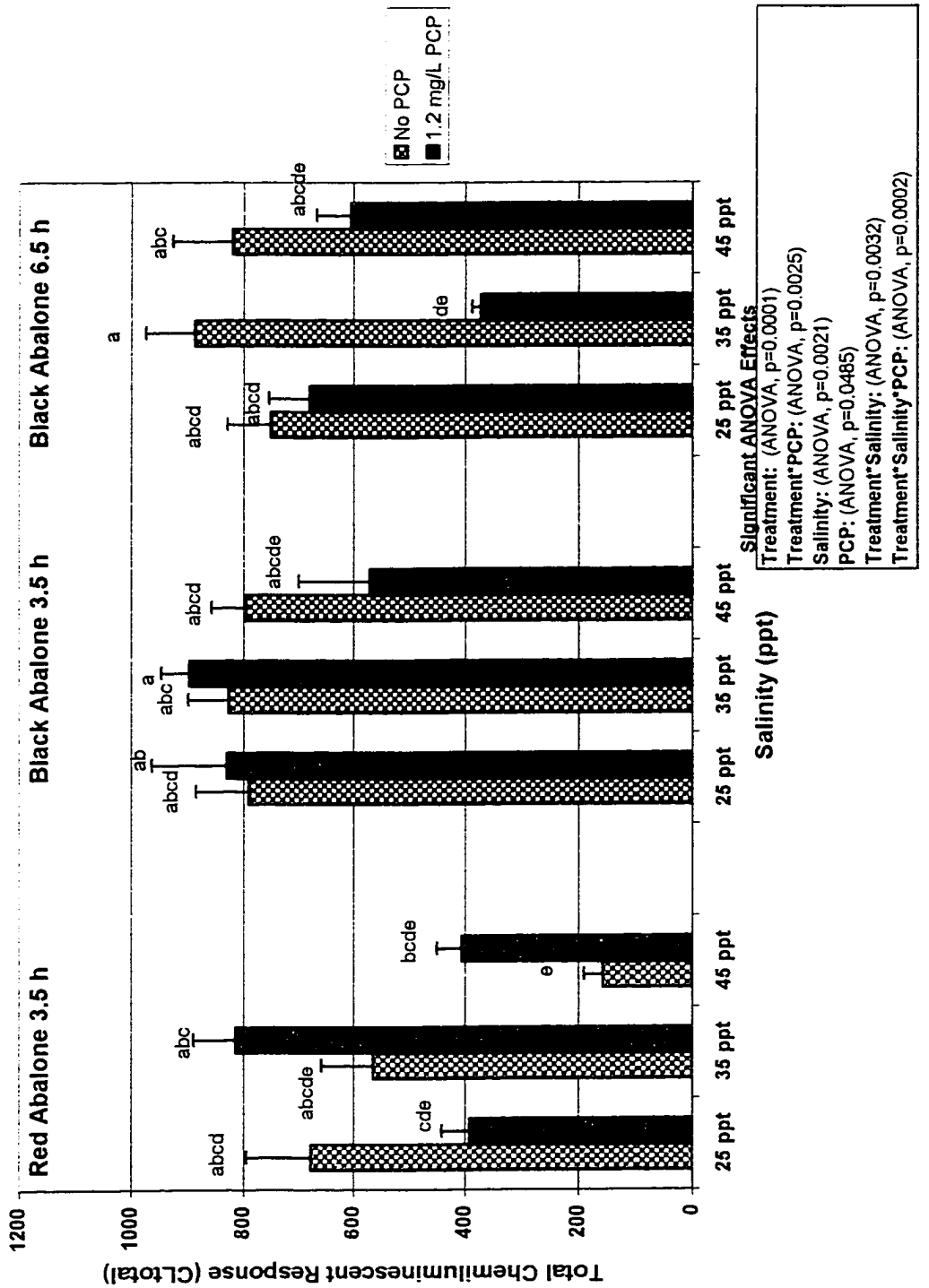


Fig 2. Comparison of maximum chemiluminescent response (CL{max} in red (3.5h) vs black abalone (3.5h and 6.5h) after exposure to 25, 35, and 45 ^{0}/00 salinity plus 1.2mg/L of pentachlorophenol.

Data were analyzed as an ANOVA. Error bars represent the standard error of the means.

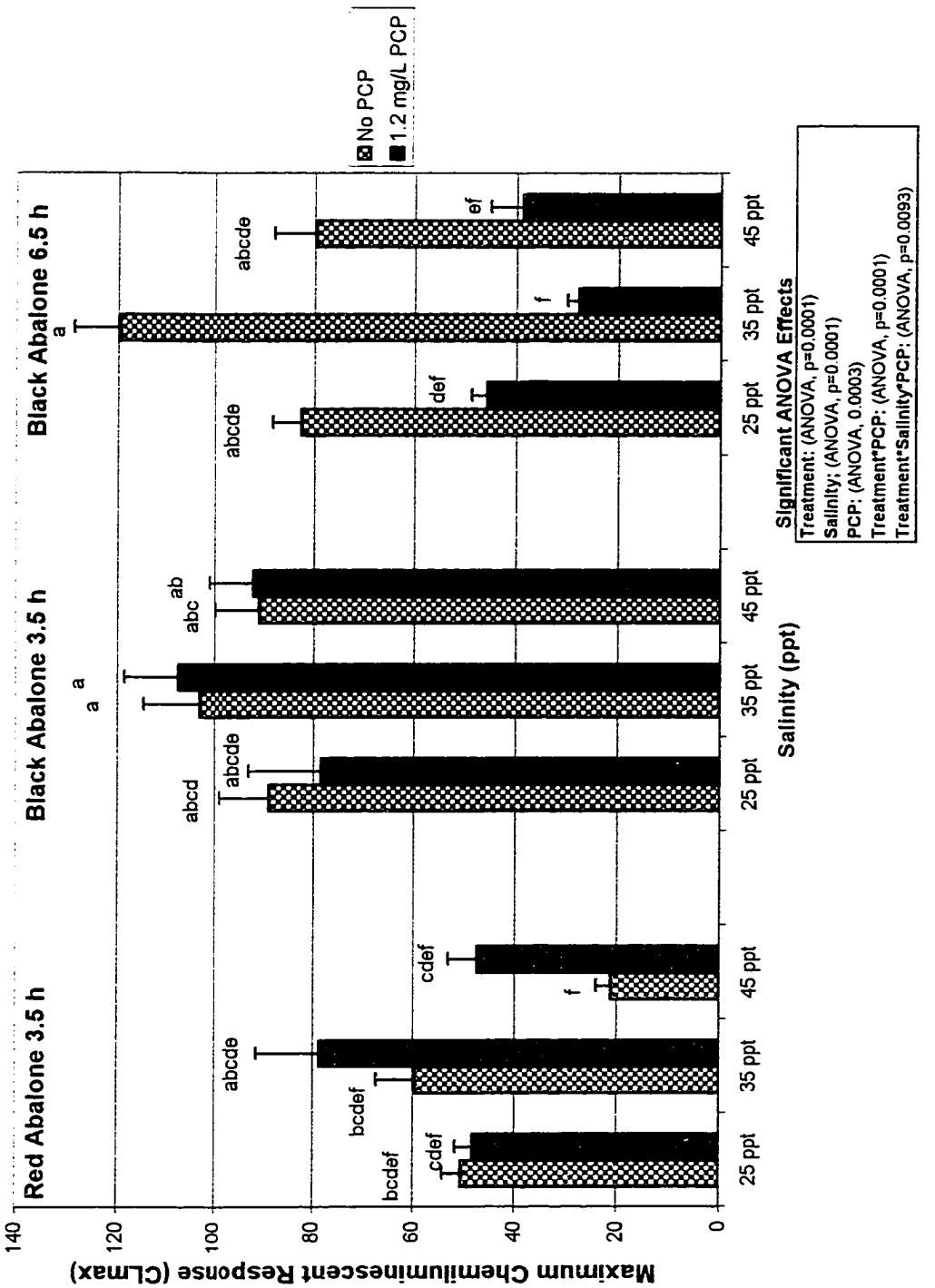


Fig 3 Comparison of time to maximum chemiluminescent response (Tmax) in red (3.5h) vs black abalone (3.5h and 6.5h) after exposure to 25, 35, and 45 $^{0}/00$ salinity plus 1.2mg/L of pentachlorophenol.

Data were analyzed as an ANOVA. Error bars represent the standard error of the means.

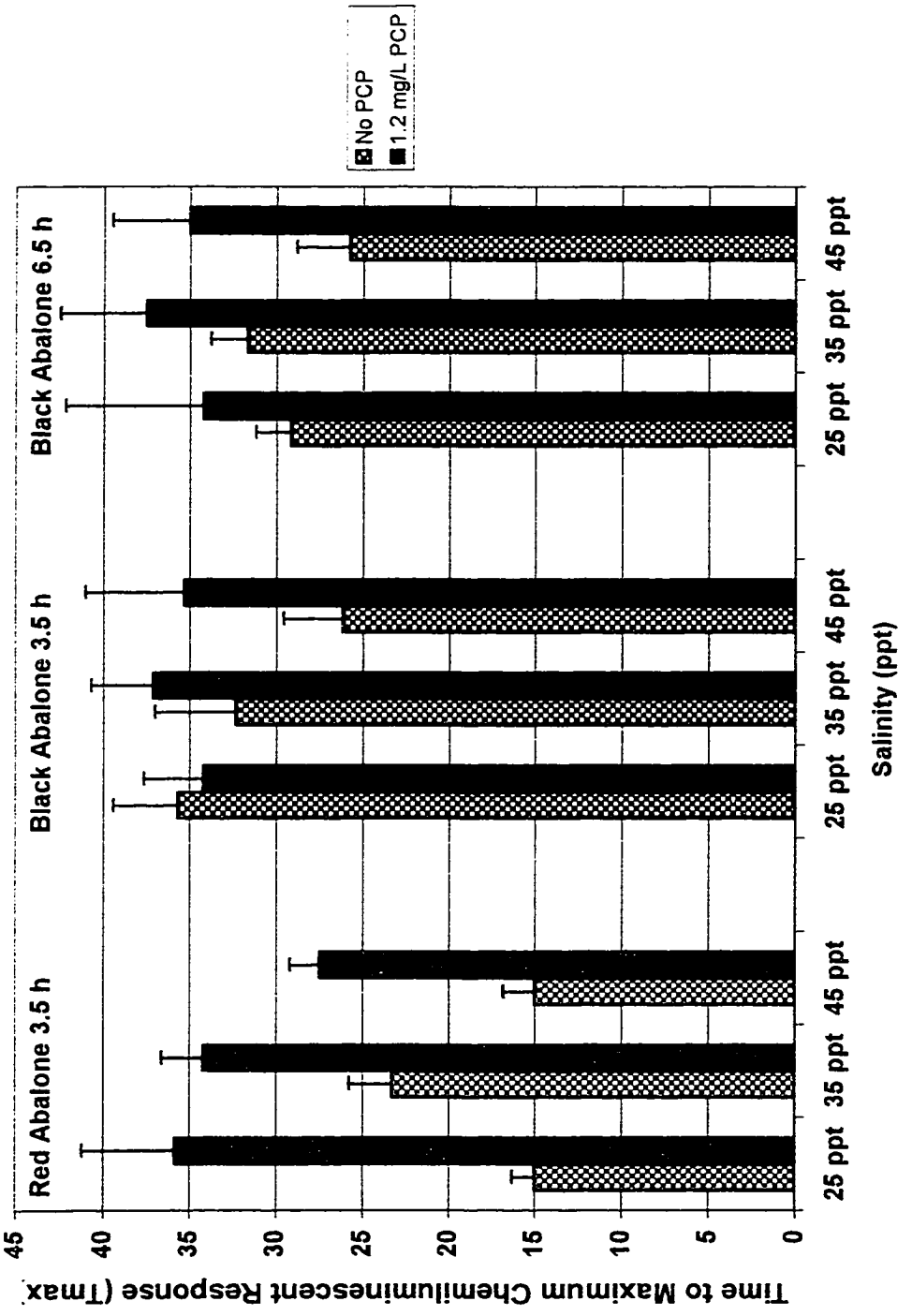


Fig 4 Interaction of PCP by exposure group in red (3.5h) vs black abalone (3.5h and 6.5h) after exposure to 25, 35, and 45 ^{0}/00 salinity plus 1.2mg/L of pentachlorophenol.

Data were analyzed as an ANOVA. Error bars represent the standard error of the means.

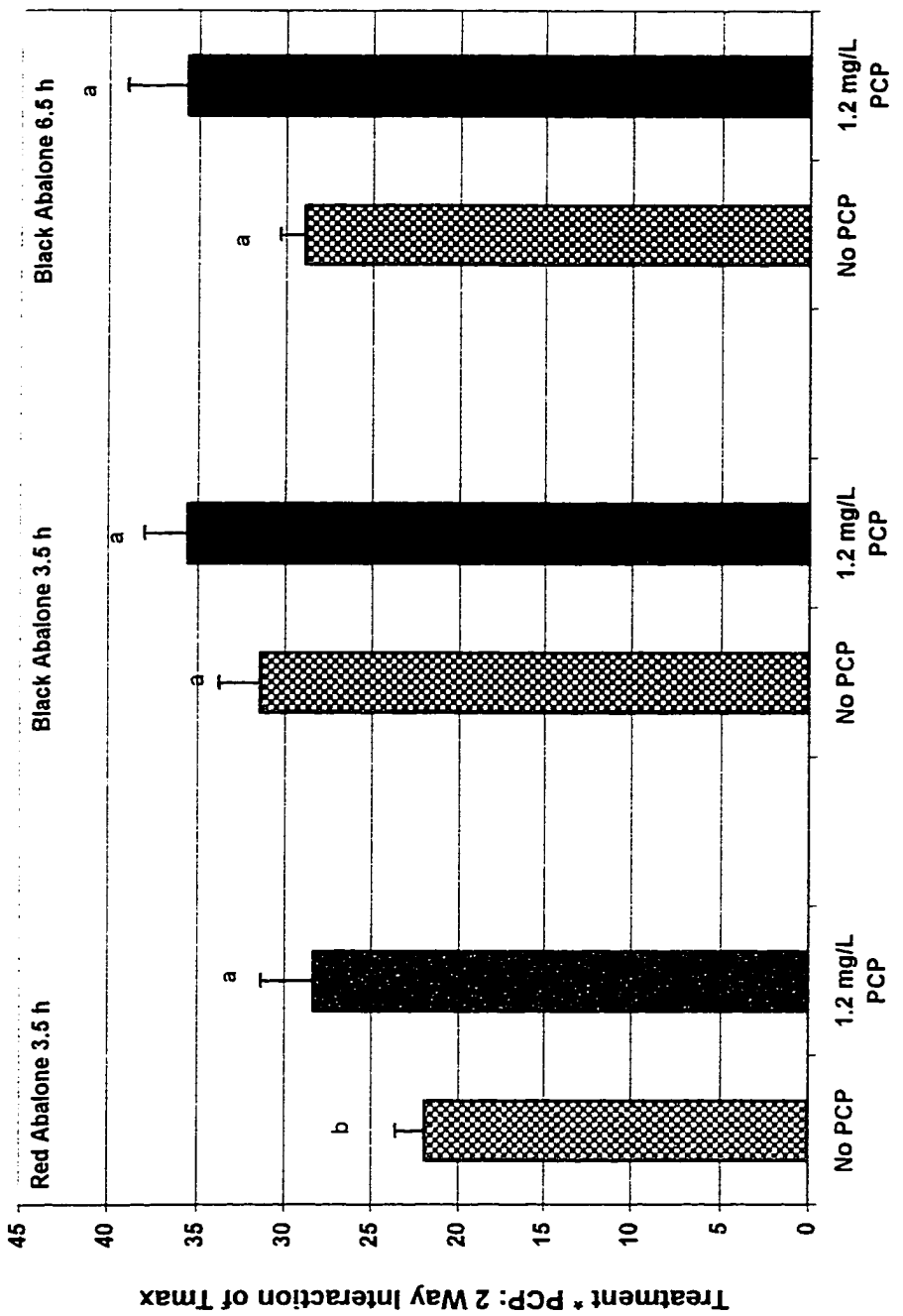
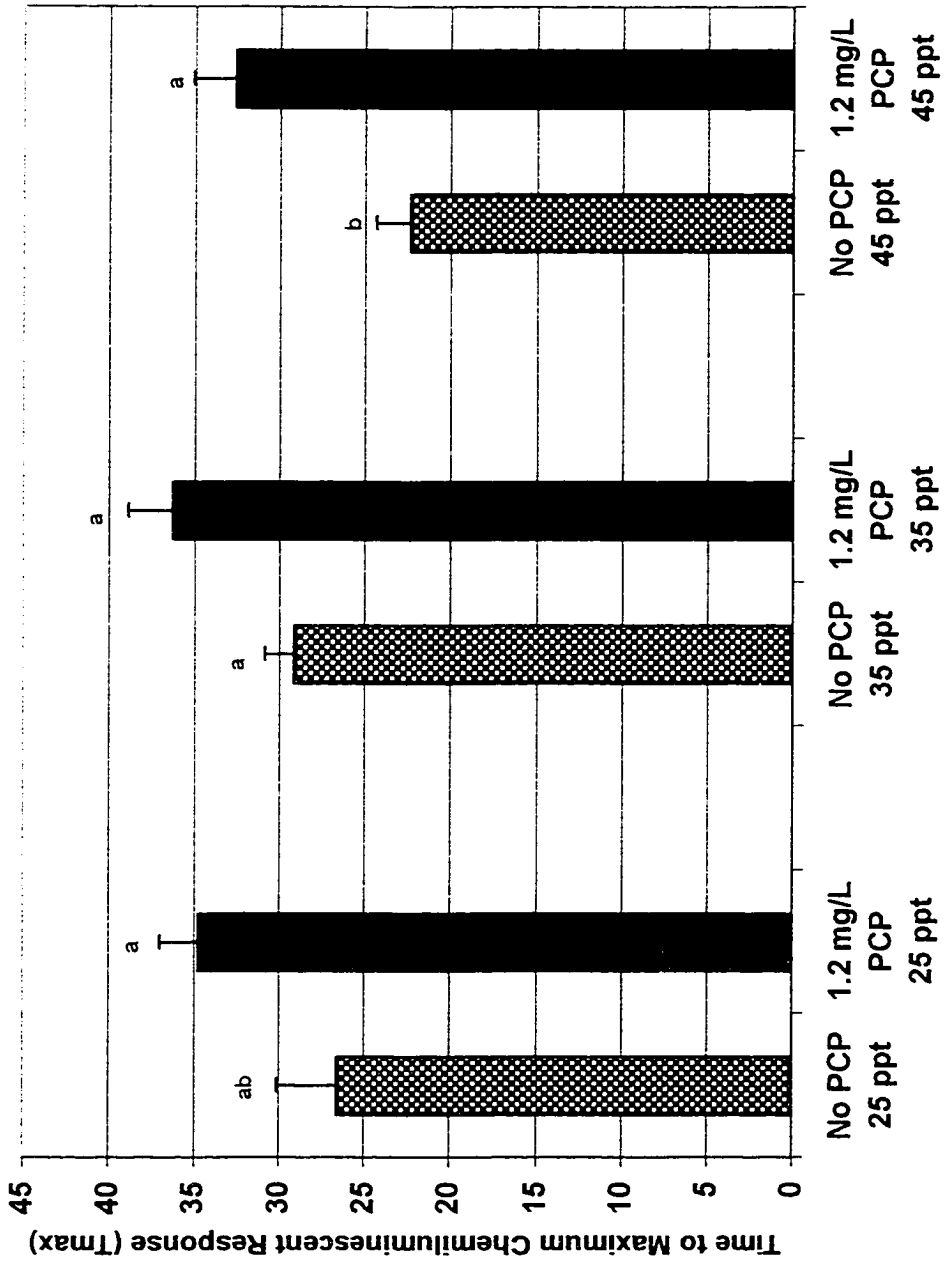


Fig 5 Interaction of salinity by PCP in red (3.5h) vs black abalone (3.5h and 6.5h) after exposure to 25, 35, and 45 $^{0}/_{00}$ salinity plus 1.2mg/L of pentachlorophenol.

Data were analyzed as an ANOVA. Error bars represent the standard error of the means.



Conclusion

Stress Effects and Their Impact on Energetics and Nonspecific Immunity

There is little debate that the abalone population is in flux and that the abalone fisheries and mariculture operations are threatened. The magnitude of this threat has been demonstrated by the 1997 closure of the commercial fishery from south of the Farallon Islands to the Mexican border. According to the most recent survey, the widespread mass mortality of black abalone is no longer confined to southern California, nor is withering syndrome confined to black abalone, as it has now been observed in red, pink, and green abalone as well (C. Friedman, pers. comm.). WS seems to be progressing approximately 12 km per year, which is evidently consistent with the advancement of an infectious agent (Lafferty & Kuris, 1993). Because abalone of all sizes seem to be declining, harvesting does not explain the changes observed. Furthermore, although sea otter predation is a possibility, other large, motile invertebrates that are fed on by otters are still abundant in areas where abalone too were once abundant. This suggests that general disturbances such as burial, thermal stress or storms are also not to blame. At what point the populations have reached some critical mass is unknown but successful spawning may not occur at low population densities because fertilization may only be successful when male and female abalone are within a few meters of each other (Prince *et al.*, 1988). Furthermore, the ecological ramifications of the decline in abalone are not yet known but the decline in a dominant space holder, such as abalone, might be expected to alter the structure of the surrounding intertidal community (Altstatt *et al.*, 1996).

This study has attempted to address a cause behind the ability of microorganisms, such as the WS pathogen, to act opportunistically. The experiments performed herein have demonstrated that both energy charge and immune function are altered by exposure to PCP in combination with salinity stress. The use of NMR spectroscopy proved to be a valuable tool in the measurement of biochemical responses in vivo in its ability to

monitor changes in endogenous compounds such as phosphagens and nucleoside phosphates in real time. The experiment performed demonstrate the effects of a natural environmental stress combined with a pollutant stress on oxidative phosphorylation and mitochondrial electron transport. The exposure parameters that caused declines in phosphagens were shown to correlate with alterations in immune status among abalone. These alterations may provide infectious agents with the foothold they require to act opportunistically. Because a properly functioning immune system is energy dependent it is likely that even sublethal exposure to an energy reducing toxicant such as PCP will have a marked effect on the immune response. If a host is not able to respond quickly to parasitic invasion the explosive population growth that microorganisms are capable of will surely result in the host succumbing to the parasite.

It is not surprising that black abalone and red abalone respond differently to the application of various stresses. The differences in inherent immune capabilities between species was surprising, however. Overall, black abalone exhibited more robust locomotory and microbicidal ability, were less sensitive to changes in salinity, and were generally more resilient to PCP exposure than red abalone. Furthermore, red abalone were shown to bioconcentrate PCP more readily than black abalone and clearly exhibited the biochemical effects of PCP exposure nearly twice as quickly as black abalone. Ironically, it is black abalone that are the primary victims of withering syndrome.

The comparative defense responses between two species of abalone after stress exposure has been addressed. Black abalone have proven to be very susceptible to WS whereas red abalone have been more successful at keeping the pathogen at bay despite the data that characterizes black abalone as being more robust than red. It is noteworthy, therefore, to describe functional differences in hemocytes between the two species after stress exposure in the hopes of elucidating the bias. Finally, the study of

immunosuppression by environmental contaminants may provide insights into using immune parameters as biomarkers at industrial and treatment outfalls.

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Addendum to Chapter 2

Metabolic Endpoint

Effect	F-Test P-Value
Species	0.0001
Salinity	0.0559
Species*Salinity	0.2087

MEP

According to the ANOVA there is a main effect of species whereby black abalone demonstrate a significantly greater time to their metabolic endpoint than red abalone ($p=0.0001$). Whereas the student T-test used in Chapter 2 demonstrated a significant effect of salinity among red abalone (high salinity was significantly greater than low and ambient salinities) this was not the case demonstrated in the ANOVA where there was no significant salinity effect (ANOVA, $p=0.0559$). Finally, the lack of a significant species * salinity interaction indicates that the effect of salinity was not different across species. This is in contrast to the findings of the second chapter where the researchers found that high salinity impacted red abalone by increasing their time to metabolic endpoint whereas changes in salinity did not effect black abalone.

Total Concentration Factor

Effect	F-Test P-Value
Treatment	0.0001
Salinity	0.0001
Treatment *Salinity	0.0036

Total Concentration Factor

The ANOVA suggests that there is a significant treatment by salinity interaction. This is suggesting that salinity has a differing effect across the three different treatment groups. The reason for this is two fold. First, Black abalone do not accumulate PCP at low salinity creating an inherent difference not only within species (black abalone) but there is also a large difference in the amount of PCP accumulated at low salinity *between* the two species. The accumulation of PCP by red abalone at all salinities is comparable to that for black abalone after 6.5h at ambient and high salinities. Interestingly, the amount of PCP accumulated after 6.5 h at low salinity among black abalone is not significantly different than after only 3.5 h for black abalone. This is in contrast to the fact that black abalone accumulation of PCP after 6.5 h at ambient and high salinity is significantly greater than that at 3.5 h at ambient and high salinity (corrected PDIFF, $p < 0.0004$).

Red abalone accumulate a significantly larger amount of PCP at high salinity than low but niether are significantly different than ambient (Bonferroni corrected PDIFF, $p < 0.026$).