Heat shock protein (HSP70) response in the eastern oyster, *Crassostrea virginica*, exposed to various contaminants (PAHs, PCBs and cadmium)

Luis A. Cruz Rodriguez  
*College of William and Mary - Virginia Institute of Marine Science*

Follow this and additional works at: https://scholarworks.wm.edu/etd

Part of the Ecology and Evolutionary Biology Commons, Environmental Sciences Commons, Freshwater Studies Commons, Oceanography Commons, and the Toxicology Commons

**Recommended Citation**

https://dx.doi.org/doi:10.25773/v5-dwj4-r408

This Dissertation is brought to you for free and open access by the Theses, Dissertations, & Master Projects at W&M ScholarWorks. It has been accepted for inclusion in Dissertations, Theses, and Masters Projects by an authorized administrator of W&M ScholarWorks. For more information, please contact scholarworks@wm.edu.
HEAT SHOCK PROTEIN (HSP70) RESPONSE IN THE EASTERN OYSTER, 
CRASSOSTREA VIRGINICA, EXPOSED TO VARIOUS CONTAMINANTS (PAHs, 
PCBs and CADMIUM)

A Dissertation
Presented to
The Faculty of the School of Marine Science
The College of William and Mary in Virginia

In Partial Fulfillment
Of the Requirements for the Degree of
Doctor of Philosophy

by

Luis A. Cruz Rodríguez
APPROVAL SHEET

This dissertation is submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

Luis A. Cruz Rodríguez

Approved, September 2001

Fu-Lin E. Chu, Ph.D.
Committee chairman / Major Advisor

Robert C. Hale, Ph.D.

Peter van Veld, Ph.D.

Robert Díaz, Ph.D.

Brian P. Bradley, Ph.D.
University of Maryland, Baltimore Campus
Baltimore, Maryland
Dedication

I dedicate this work to my dear wife Dr. Denise J. Deckert-Cruz for her love and support in pursuing this dream. Thanks for never giving up. And to my son Ricardo Luis Cruz for bringing his boundless energy and hope to my life.
Heat shock Protein (HSP70) Response in the Eastern Oyster, *Crassostrea virginica*, Exposed to Various Contaminants (PAHs, PCBs and Cadmium)

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table of Content.................................................................iv</td>
</tr>
<tr>
<td>Preface..........................................................................................vi</td>
</tr>
<tr>
<td>Acknowledgements........................................................................vii</td>
</tr>
<tr>
<td>List of Figures...............................................................................ix</td>
</tr>
<tr>
<td>Abstract..................................................................................xiv</td>
</tr>
<tr>
<td>Chapter 1: General Introduction.................................................2</td>
</tr>
<tr>
<td>Pollutant effects...........................................................................11</td>
</tr>
<tr>
<td>Objectives................................................................................19</td>
</tr>
<tr>
<td>References..................................................................................22</td>
</tr>
<tr>
<td>Chapter 2: Heat-shock protein (HSP70) response in the eastern oyster, <em>Crassostrea virginica</em> (Gmelin), exposed to PAHs sorbed to suspended artificial clay particles and suspended field contaminated sediments</td>
</tr>
<tr>
<td>Abstract..................................................................................56</td>
</tr>
<tr>
<td>Introduction................................................................................57</td>
</tr>
<tr>
<td>Methods and Materials..............................................................58</td>
</tr>
<tr>
<td>Results.....................................................................................65</td>
</tr>
<tr>
<td>Discussion................................................................................67</td>
</tr>
<tr>
<td>References................................................................................75</td>
</tr>
<tr>
<td>Chapter 3: Effects of PCBs sorbed to algal paste and sediments on the stress protein response (HSP70) in the eastern oyster, <em>Crassostrea virginica</em> (Gmelin)</td>
</tr>
<tr>
<td>Abstract..................................................................................92</td>
</tr>
<tr>
<td>Introduction................................................................................92</td>
</tr>
<tr>
<td>Methods and Materials..............................................................94</td>
</tr>
<tr>
<td>Results.....................................................................................98</td>
</tr>
<tr>
<td>Discussion................................................................................100</td>
</tr>
<tr>
<td>References................................................................................106</td>
</tr>
</tbody>
</table>
Chapter 4: HSP70 levels in oyster *Crassostrea virginica* (Gmelin) exposed to cadmium sorbed to algal food and suspended clay particles

Abstract ...........................................................................................................120
Introduction ......................................................................................................122
Methods and Materials ...................................................................................126
Results ..............................................................................................................131
Discussion .........................................................................................................133
References .........................................................................................................142

Chapter 5: Variation in the levels of stress protein (HSP70 family) under natural conditions in the eastern oyster, *Crassostrea virginica* (Gmelin) from the lower Chesapeake Bay

Abstract .............................................................................................................161
Introduction .......................................................................................................162
Methods and Materials ....................................................................................165
Results ...............................................................................................................167
Discussion ..........................................................................................................170
References ..........................................................................................................176

Chapter 6: The HSP70 response in the eastern oyster exposed to various contaminants:

Summary of findings and future research

Introduction ......................................................................................................190
Summary ..........................................................................................................191
Conclusions ....................................................................................................195
Future research ...............................................................................................198
References ........................................................................................................199
Vita ........................................................................................................205
Preface: Note To The Reader

The chapters 2 through 5 of this dissertation are written as separate manuscripts for publication. At the time of this writing, half of chapter 3 was published in Marine Environmental Research 50:341-345, and chapter 2 was accepted for publication. Due to the common theme underlying this study, readers will note some redundancy in the chapters particularly with methods and background information.

Chapter 1 presents an introduction about stress proteins function and potential for a biomarker. Chapter 2 measured the HSP70 response in laboratory exposures to suspended field contaminated sediments (SFCS) and PAH-sorbed to clay particles. Chapter 3 measured the effects of PCB sorbed to algal food on the stress protein in sexually mature and sexually immature oysters. Chapter 4 measured the stress protein response in oysters exposed to Cd-contaminated food and clay particles. Chapter 5 measured natural variability in the HSP70 in oysters collected from Point of Shoals in the James River, Virginia. Finally, chapter 6 summarizes the results of the studies, point to limitations in the use of stress proteins and the studies herein, finishing with research needs.
Acknowledgements

I would like to take this opportunity to thank all whose collaboration and support make this dissertation possible. First, my deepest and most felt gratitude to my advisor Dr. Fu-Lin E. Chu whose unending energy, efforts, economic assistance and willingness to succeed made possible the completion of this dissertation. Thanks to Dr. John Milliman, always a source of friendship and positive reinforcement during my time at VIMS. Dr. Robert C. Hale for his help in environmental chemistry, a field I had no previous knowledge, and his helpful comments in editing this dissertation. Dr. Robert Diaz always willing to listen my unending questions about statistics. Dr. Peter van Veld and Dr. Brian Bradley who generously accepted the unenviable task of been part of my graduate committee. Thanks to Dr. Michael Newman and Dr. Gary Rice for allowing me the use of their laboratories to perform metal analysis, an area of total obscurity for me before this project. Also thanks to Dr. Newman in his capacity as Dean of Graduate Studies for the economic assistance and advice provided. I would like to acknowledge Dr. Morris H. Roberts, Jr., chair of the Department of Environmental Sciences, for his assistance in securing economic aid for equipment, materials and travel during my time at VIMS. Many thanks to all the students and technicians at VIMS past and present for their help, collaboration and friendship. Dr. Philippe Soudant, Dr. Eric Lund, Dr. Aswini K. Volety, Mrs. Georgetta Constantin, Vincent G. Encomio, Anthony J. Baucum, Lee N. Steider, Shawn Stickler, Michael O. Gaylor, Elizabeth Bush, Matt Mainor, Mark LaGuardia, George Vadas, Ellen Harvey, David Powell, David Ownby, Amy Bohannon, Alanna MacIntyre, Dr. Gustavo Calvo, Lisa M. Ragone-Calvo, Rita Crockett, Jennifer Cardinal, Jan McDowell, Ken Goldman, Roy Pemberton and Reynaldo Morales Alamo.
This work was supported in part by a grant from the Exploratory Research Program of the Environmental Protection Agency (EPA Grant No: R825349-01-0), the School of Marine Science Graduate Students Association research grant and The College of William and Mary research grant.
List of Figures

Chapter 2:

1) Figure 1 ............................................................................................................84
Western Blot showing two HSP70 isoforms of 69 kDa and 71 kDa in gills of the eastern oyster, *Crassostrea virginica*. Oysters were heat-shocked for 1 hour at 37°C followed by a 48 hours accumulation period at ambient temperature. Lanes 1, 3--7 show the two isoforms detected using anti HSP70 monoclonal antibody (Affinity BioReagents MA3-006). Lane 2 shows low range molecular weight marker.

2) Figure 2 ........................................................................................................85
Representative slot blot of soluble protein extracted from the eastern oyster *Crassostrea virginica*. Rows 1 and 2 show “reference sample” serial dilution loaded from opposite directions with 0.25, 0.50, 1.0, 1.5, 2.0 and 2.5 μg total protein. The remaining wells were randomly loaded with 1.5μg protein of samples exposed to 1.0, 1.5 or 2.0 g SFCS. Slot blot technique measures total HSP70 levels (constitutive and induced) in samples.

3) Figure 3 ........................................................................................................86
Representative chromatogram of the PAH mixture (fluoranthene, pyrene, benzo(e)pyrene and benzo(a)pyrene) used to expose the oysters. PAHs were analyzed by capillary chromatography (DB-5 column, 60m) with flame ionization detection. An internal standard (p-terphenyl) was added immediately prior to GC analysis for quantitation.

4) Figure 4 .........................................................................................................87
HSP70 response in oysters *Crassostrea virginica*, after 40 days exposure to suspended unspiked clay particles (0, 1.0, 1.5, or 2.0 g daily) , or to suspended PAH-sorbed clay particles (1.0, 1.5 or 2.0 g daily, corresponding to 65.6, 159, 242 μg PAHs respectively). No significant difference between treatments for oysters exposed to suspended unspiked clay particles (Mean ± CI, N=9-11). Oysters exposed to suspended PAH-sorbed clay particles show a significant increase in HSP70 levels compared to controls. However no dose dependency is observed (Mean ± CI, N=8). CI= 95% confidence interval.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
5) Figure 5...................................................................................................................88
PAH bioaccumulation in oyster gills after 40 days exposure to suspended PAH-sorbed
clay particles. Oysters show a dose related increase in accumulated PAHs (Mean ± SD,
N=3).

6) Figure 6...................................................................................................................89
Bioaccumulation of PAH by compound in oyster gills after 40 days exposure to
suspended PAH-sorbed clay particles. Oysters were exposed to a mix of PAH
(Fluoranthene, Pyrene, B(e)P and B(a)P, 100μg/ml of each) sorbed to clay particles.
Oysters preferentially bioaccumulate B(e)P over Fluoranthene, Pyrene and B(a)P (Mean
± SD, N=3).

7) Figure 7...................................................................................................................90
HSP70 response in the eastern oyster, C. virginica exposed to (a) 0 (b) 1.0 (c) 1.5 and (d)
2.0 g suspended field contaminated sediments (SFCS) for 5 days, 10 days, 20 days, and
40 days (Mean ± CI, N=7-9). * Denotes significantly different from day 5 within each
treatment (p<0.05). CI = 95% confidence interval.

Chapter 3:

8) Figure 8...................................................................................................................115
Representative chromatogram showing the PCB mixture (Aroclor 1242, 1254, and 1260)
used to expose the oysters. PCBs were analyzed by gas chromatography with electrolytic
conductivity detection (GC-ELCD) equipped with a DB-5 fused silica capillary column
(60m x 0.32 mm x 0.25 μm film thickness) using a splitless injection system at 300°C and
helium as carrier gas. PCB30, PCB65 and PCB204 were used as surrogates to assess the
extraction yield. Pentachlorobenzene (PCBt) was used as internal standard.
Quantification was performed by comparison with response of congeners with identical
degrees of chlorination.
9) Figure 9...........................................................................................................116
Total HSP70 in reproductive active oysters exposed to 0, 0.1 or 1.0 µg PCB/oyster/day for 15 and 30 days. (A) Gills showing a decrease in total HSP70 after 15 days with a subsequent increase after 30 days. This changes are not statistically significant different from controls. (B) Mantle showing decreases in HSP70 with dose, however these changes are not statistically significant different (p=0.118).

10) Figure 10...........................................................................................................117
PCB accumulation in gill+mantle of reproductively active oysters exposed 0, 0.1 or 1.0 µg PCB daily sorbed to algae for 15 and 30 days. PCB accumulation showed increase with dose and length of exposure (Mean ± SD, N=3).

11) Figure 11.............................................................................................................118
Total HSP70 in reproductive inactive (conditioned) oysters. (A) HSP70 response in gill of oysters exposed to PCB sorbed to algal paste and artificial sediments (Mean ± 95% CI, N=9-10). (B) HSP70 response in mantle of oysters exposed to PCB sorbed to algal paste and artificial sediments (Mean ± 95% CI, N=8-9). No dose effects due to PCB exposure were observed in gill or mantle. The addition of sediments produced a significant increase in total mean HSP70 response in gills (p<0.0001) and mantle (p=0.0317).

12) Figure 12............................................................................................................119
PCB accumulation in reproductive inactive (conditioned) oysters. (A) Oysters exposed to PCB contaminated algal diet containing 0, 0.35 or 3.5 µg PCB / daily, no clay particles added. (B) Oysters exposed to PCB contaminated algal diet containing 0, 0.3 or 3.5 µg PCB / daily + 0.3 g clay particles for 56 days. PCB accumulation showed increase with dose and length of exposure (Mean ± SD, N=3). Mantle showed differences in the accumulation of PCB with and without the addition of clay particles (802 versus 1342 ng / g DW). Gills showed no difference in the accumulation of PCBs with or without the addition of clay particles (512 and 550 ng PCB / g DW respectively).
Chapter 4:

13) Figure 13........................................................................................................156
HSP70 levels in oysters exposed to: (A) Cd-sorbed to algal paste (Experiment 1) and (B) Cd-sorbed to suspended clay particles (Experiment 2). Oysters were exposed to 15 and 25ppb Cd / oyster / day) (Mean ± 95% confidence interval, n=8-10). Different letters represent significant difference (p<0.05).

14) Figure 14........................................................................................................157
HSP70 levels in oysters exposed to Cd-sorbed to algal paste and to suspended clay particles (Experiment 3). Oysters exposed to 15 and 30ppb Cd / oyster / day. (Mean ± 95% confidence interval, n=8-10).

15) Figure 15........................................................................................................158
Cadmium accumulation in gills of oysters exposed to Cd sorbed to algal paste (Experiment 1) or suspended clay particles (Experiment 2). Oysters were exposed to 15 or 25ppb Cd / oyster / day. (Mean ± SD, n=6). Different letters represent significant difference (p<0.05).

16) Figure 16........................................................................................................159
Cadmium accumulation in gills of oysters exposed to Cd sorbed to algal paste or suspended clay particles (Experiment 3). Oysters exposed to 15 or 30ppb Cd / oyster / day. (Mean ± SD, n=6). Different letters represent significant difference (p<0.05).

Chapter 5:

17) Figure 17........................................................................................................186
Temperature (°C) and disease prevalence (% infected) by the parasite *P. marinus* in oysters collected in (A) May, August and October 1999, and (B) January, March and August 2001 from Point of Shoals in the James River, VA. Infection prevalence is highly associated to the ambient water temperatures (n= 7 – 9).
Condition Index in oysters collected from Point of Shoals in the James River, Virginia, in (A) 1999 and (B) 2001. Bars with different letters denote significant difference (p < 0.001) (Mean ± SD, n = 7 - 9).

HSP70 levels in the eastern oyster, *Crassostrea virginica* under natural conditions sampled in (A) November 1996, January, March and August 1997, (B) May, August and October 1999 and (C) January, March and August 2001. Numbers above bars indicate water temperature on sampling date. Bars with different letters denote significant difference (p < 0.05). Mean ± CI (95% Confidence Interval), n = 7 - 9, NR = not recorded.
Abstract

The stress protein response has been proposed as a general indicator of exposure to stress as their expression might integrate overall biological impact and interactions among multiple stressors. Stress proteins can be found constitutively in cells acting as chaperones helping in the correct folding and transport of proteins, stabilize and/or unfold proteins, disaggregate protein aggregations, and translocate newly synthesized proteins across membranes. The HSP70 family is highly conserved across taxa and accounts for much of the translational activity in cells responding to environmental stress. As a marker of contaminant effects, a major advantage is the premise of higher sensitivity over other indices such as condition index, scope for growth and survival. However, the role of the stress proteins in environmental toxicology depends on the outcome of studies examining their responses under environmentally realistic conditions.

Field contaminated sediments were collected from the Elizabeth River, a highly contaminated estuary in Virginia. Laboratory exposure to suspended field contaminated sediments (SFCS) elicited a stress protein response in the eastern oyster, (*C. virginica*). The SFCS is a mixture of different contaminants and the stress response probably resulted from the combined effect of various contaminants including PAHs, metals and trace amounts of PCBs in the suspended sediments. Exposure to suspended clay particles spiked with a mixture of PAHs (Fluoranthene, Pyrene, Benzo(e)Pyrene and Benzo(a)Pyrene) caused an increase in the levels of HSP70. No changes in the condition index or mortality were observed. B(e)P accumulated at a higher level than any of the other compounds. It seems B(e)P is preferentially accumulated, retained or its degradation is slower than the other PAHs. This study appears to indicate that the HSP70 provides a sensitive indicator of exposure to PAHs. In principle this findings are promising in establishing the stress proteins as a tool to evaluate exposure under stressor regimes.

The effect of PCBs on the HSP70 was investigated in sexually mature and immature oysters. Sexually mature oysters were fed 0.2 g algal paste containing 0, 0.10 or 1.00 μg PCBs for 15 and 30 days. Sexually immature oysters were conditioned before gametogenesis and fed 0.7 g algal paste containing 0, 0.35 or 3.50 μg PCBs daily for 56 days. Exposure to PCBs (Aroclor 1242, 1254, and 1260) sorbed to suspended algal food did not produce statistically significant changes in the stress protein levels. The PCB concentrations used or the length of exposure might not have been sufficient to promote a significant stress response in the oyster.

Cadmium has been described as a common pollutant in estuaries and to accumulate in the biota. In the current experiments long-term exposure to 15 – 30ppb Cd for 40 days, generally did not cause changes in stress protein levels in oysters exposed to Cd sorbed to algal food and suspended clay particles. These results along the PCBs results, might suggest that not all toxic compounds would elicit a stress protein response. In bivalves the effective sequestration and removal of metals from the cell’s cytosol may prevent them from further interact with protein components or other structures, thus preventing stimulation of a stress protein response.

Oysters collected form Point of Shoals in the James River, Virginia, exhibited variation related to water temperature in the HSP70 levels. There seems to be an inverse relation between ambient water temperature and levels of stress protein (i.e. low temperature related to high levels of stress protein in gills). However, because under field conditions...
conditions is difficult to separate the effects of various environmental factors, at this time we cannot describe the variation in HSP70 levels as the result of ambient water temperature.

The stress protein response has potential as a biomarker of exposure to contaminants. However, there are limitations to its application. Different contaminants conceivably can affect different targets in the organism, not necessarily implicating a stress protein response. Organisms could have other mechanisms to more effectively cope with those contaminants precluding a stress protein response. A contaminant might not necessarily targets protein damage or the contaminant effects are more apparent when they are interacting with other contaminants. Adaptations to contaminated areas and life history traits may influence the response of individuals to stress, making these individuals more tolerant.

The stress protein response might still be useful as part of a suite of biomarkers combining indicators at several levels of biological organization to facilitate data interpretation and identification of deleterious effects caused by stressors (Sanders, 1993; Forbes and Forbes, 1994). Future research areas might include; (1) examination of the response under realistic conditions of exposure using other known pollutants, or pollutants with known mechanism of action, (2) evaluating its relation to various other toxicity end points, (3) long-term studies of exposure to chronic stressor regimes, (4) comparison to species tolerance, habitat and prior exposure (put the response in ecological context), and (5) study the effects of endogenous factors on the stress protein response. The ultimate evaluation is the extent in which results of these researches can be used to monitor real-world environmental issues.

Luis Angel Cruz Rodríguez

School of Marine Science
The College of William and Mary in Virginia
HEAT SHOCK PROTEIN (HSP70) RESPONSE IN THE EASTERN OYSTER, 
CRASSOSTREA VIRGINICA, EXPOSED TO VARIOUS CONTAMINANTS (PAHs, 
PCBs and CADMIUM)
Chapter 1: General Introduction
Much of the body of biochemical indicator research has dealt with measurements of effects such as enzyme activity and biochemical composition of tissues (Mazeaud and Mazeaud, 1981; Neff, 1985). Biomarkers involved in protection and defense have been suggested to be sensitive indicators of contamination exposure and/or adverse biological effects (Sanders et al., 1991). Stress proteins (SPs) are one type of indicator. They have been implicated in activities designed to counter negative effects on the cell subjected to environmental insults. Therefore measuring alterations in their activities can give us an early indication of stress as expressed through these protein systems.

Heat shock proteins (HSPs), generally referred to as stress proteins, are inducible upon exposure to stress. Their discovery happened by chance in the early 1960's when Ferruccio Ritossa noticed a new chromosomal puffing pattern in the salivary glands of Drosophila subjected to a heat shock (Ritossa, 1962). This pattern was taken to indicate new synthesis of mRNA. The results held true for agents other than heat, such as ATPase uncouplers (dinitrophenol, salycilate) and anaerobiosis. The use of different tissues, different species of Drosophila, or development time (age) gave similar results with respect to the induction of this chromosomal pattern. This was the first evidence of a general HSP response to various agents and across Drosophila species (Ritossa, 1963a, 1963b).

In all eukaryotic cells there are various recognizable SPs families named according to their apparent molecular weight on SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). The families recognized include HSP90, HSP70,
HSP60 (Cpn60), HSP20-30, ubiquitin and GRPs (glucose regulated proteins). In addition there are actin-like (45 KDa) protein, metallothionein (MT), and the stress kinases Jun N-terminal Kinase (JNK) and p-38, among others. The stress 70 family includes HSP70, HSP72, and Hsc70, as well as BiP (a 78 KDa endoplasmic reticulum HSP) and GRP75, two of the glucose regulated proteins (Engel, D.W., and M. Browner, 1989; Morimoto, R.I., 1993; Lindquist, S. and E.A. Craig, 1988; Sanders, 1993; Hightower, L. E., 1991).

Marine bivalves have been used extensively as indicators of pollution (Widdows and Johnson, 1988; Smaal et al., 1991), and are among the most suitable bioaccumulators of trace contaminants (Phillips, 1980). For example the scallop, (Mizuhopecten yessoensis), accumulates heavy metals in its tissues (Gould et al., 1985) as a function of metal concentration and exposure time (Chelomin and Belcheva, 1991). In mussels factors such as scope for growth (SFG) and clearance rate may be affected by organic contaminants (Widdows et al., 1985) and PCBs (Martin, 1985). Mussels act as integrators of toxic pollutants and are often used to determine and assess levels of contamination in coastal areas (Martin 1985).

Many marine bivalve species are economically important. In the Chesapeake Bay the oyster fishery is a very important economic resource. Declines in oysters populations have been observed since the 1950's (Andrews, 1988). During the 1980's, a combination of long-term climate changes such as high winter temperatures, and low precipitation in conjunction with infectious diseases caused by, (Perkinsus marinus) and (Haplosporidium nelsoni), and pollution may all have exacerbated oyster mortalities.
(Andrews, 1988; Andrews and Ray, 1988; Chu and Hale, 1994). More recently, drastic reductions in populations since 1992 have been attributed to factors such as disease, over harvesting and pollution (Chu and Greene, 1989; Chu and LaPeyre, 1993; Ragone and Burreson, 1993; Burreson and Ragone-Calvo, 1996; Chu and Hale, 1994).

The induction of stress proteins in marine bivalves caused by stress agents such as Cd\(^+2\), Cu\(^+2\), changes in salinity and heat shock have been demonstrated (Sanders et al., 1988; Sanders et al., 1994; Werner and Hinton, 2000). Exposure to agents such as, Cd\(^+2\) and heat shock resulted in the induction of HSP70 in the mussel, *M. edulis* (Sanders, 1988). Following a heat shock of oyster, (*C. virginica*), we detected increases in total HSP70 (unpublished data). Similarly a heat shock response (HSP70) was detected in the pacific oyster, (*C. gigas*) by others (Clegg et al., 1998; Shamseldin et al., 1998). HSP70 along with 51 and 28 kDa proteins have been detected in mussels exposed to Cu or a heat shock (Steinert and Pickwell, 1988). No induction in stress proteins was detected in the oyster (*C. virginica*), and the mussel, (*M. edulis*), exposed to 0.7ppb TBT in the water (Pickwell and Steinert, 1988). However, in a later study mussels demonstrated a significant increase in HSP70 when the TBT (0.7ppb) was sorbed to their food (Steinert and Pickwell, 1993).

The HSP70 family is highly conserved across taxa. They help to stabilize and/or unfold proteins, disaggregate protein aggregations, and translocate newly synthesized proteins across membranes (Craig, 1985; Chirico et al. 1988; Rothman, 1989; Welch, 1990). The isoform HSP72 is induced exclusively under stress (Smerdon et al., 1995) and
is not found under normal unstressed cellular conditions (Stegeman et al. 1992). Under stress conditions HSP72 migrates toward the nucleolus where it is speculated to resolubilize denatured pre-ribosomal complexes. During recovery, HSP72 migrates to the cytoplasm and associates with the ribosomes and polysomes where it may help in the resolubilization of denatured proteins (Welch and Feramisco, 1985; Pelham, 1988).

Stress proteins can be found constitutively in cells not experiencing stressful conditions (Pelham, 1986). Under these conditions two members of the HSPs, Cpn60 (mainly mitochondrial) and HSP70, act as chaperones helping in the correct folding and transport of proteins. They are involved in assuring the delivery of nascent proteins through the cytosol (Gething and Sambrook, 1992). Generally, they help to maintain cellular homeostasis.

The HSP70 family has shown good induction and conservation of reaction across species including marine invertebrates such as mussels, (M. edulis) and echinoderms, (Strongylocentrotus purpuratus) (Sanders, B., 1990; Stegeman et al. 1992; Sanders, B., 1993). HSP70 from mollusc and human cells have similar antigenic and ATP-binding domains despite the taxonomic differences (Margulis et al, 1989). Stress protein 70 from different tissues of various fishes reacted with a polyclonal antibody prepared against purified catfish, (Ictalurus punctatus) liver stress70 (Abukhalaf et al., 1994). The polyclonal antibody reacted with liver, muscle, and gill tissues from fathead minnows (Pimephales promelas), red shiners (Cyprinella lutrensis), black bass (Micropterus salmoides), and bluegill (Lepomis macrochirus) (Abukhalaf et al., 1994).
A series of factors have been identified that can influence the induction of stress proteins. Heat shock proteins are known to be synthesized at higher levels when the organisms are challenged with certain environmental stimuli such as high temperature, toxic chemical exposure and xenobiotics, making them a potentially useful marker of exposure to those challenges (Morimoto, 1993; Sanders, 1993). For example, in human HT29 cells subjected to heat and cold shock, 8% ethanol, and 2.5% 1-propanol, the expression of stress proteins was substantially induced. HSP70 response was concluded to be a more sensitive to environmental insults than changes in growth rates (Delmas et al 1995). This study adds to a growing body of evidence suggesting the higher sensitivity of molecular biomarkers compared to other physiological markers.

The presence of abnormal proteins seems to be the signal inducing a stress protein response in the cell. In HeLa cells many of the agents that enhance HSP gene expression are known to affect protein conformation. This suggests that accumulation of unfolded (denatured) proteins may be an obligatory intermediate in HSP activation (Baler et al., 1996). Ananthan et al. (1986), in studies utilizing (Xenopus sp.) oocytes, provided direct evidence that denatured proteins can induce the activation of HSP genes. In their studies, only the presence of denatured proteins in the oocytes triggered the expression of HSP genes. Lack of a capacity to effect protein denaturation (proteotoxicity) has been suggested as a factor constraining a stress protein response (Salminen et al., 1996; Lewis et al., 2000). In human hepatoma cells (HepG2) agents such as CCl₄ and cocaine failed to induce a HSP70 response (Salminen et al., 1996). These agents or their metabolic intermediaries do not bind to proteins and demonstrate reduced proteotoxicity. The low
proteotoxicity of the triazine herbicide Irgarol 1051 was suggested as the reason for a lack of induction HSP70 levels in the macroalga, *(Enteromorpha intestinalis)* (Lewis et al., 2000).

Another important factor is the amount or concentration of denatured proteins in the cell. Besides the presence of denatured proteins, their quantity may affect the induction of HSP70 in the cell. At low levels of denatured protein in the cell, base amounts of HSP70 may suffice, but at higher levels increased synthesis may be required. Studies with *(Escherichia coli)* show that it is the concentration of degraded protein, not its degradation itself, that it is important in inducing a heat-like response in the cell. Mutant cells expressing an unfolded protein (lambda repressor) showed that concentration was the important element inducing a stress protein response (Parsell and Sauer, 1989). The stress response ensues once the denatured protein accumulates in the cell.

The amount of heat shock protein cognate (Hsc70) influences how much HSP70 will be needed when a stress is encountered. Manzerra et al (1997) have suggested that the amount of preexisting constitutive Hsc70 can affect induction of HSP70; i.e. endogenous levels of Hsc70 may modulate the level of induction. Locke and Tanguay (1996) also suggest that some ‘critical amount’ of HSP72 (inducible form of HSP70) may be required to provide protection, although no suggestion of an actual amount is made. Free HSP70 binds to denatured proteins releasing the heat shock factor (HSF) that now activate the HSP70 genes synthesizing, among others, the HSP72 isoform. Locke and
Tanguay (1996), in studies using rat hearts, suggest that only the free form of HSP72 would be available to restore and/or repair proteins that are damaged. Thus evidence suggests that certain amounts of HSP70 of both the constitutive as well as the inducible forms, are required to provide adequate protection against stressors.

Another factor affecting the induction of stress proteins is stress severity. In the hemocytes of the eastern oyster, (C. virginica) the magnitude of a heat shock influences the magnitude and duration of the heat shock protein synthesis process (Tirard et al.1995b). In their study when the temperature was increased up to 46°C for 1 hour, the synthesis of HSP70 continued for 4 days even after the cells were returned to the control temperature (20°C). In Drosophyla sp. the induction of HSP70 RNA is higher in individuals exposed for longer times to high temperature, this effect occurring in individuals of all ages (Niedzwiecki et al.1991). Length of exposure acts as an agent increasing the severity of heat stress.

Variability in the stress protein response due to changes in life history is important in assessing their utility as biomarkers. The expression of heat shock genes during development is documented in various species, but the mechanisms responsible and their functions are not well understood. In (D. melanogaster) HSP26, HSP23 and HSP22 mRNA were present in early embryos, although no temperature shock was applied (Zimmerman et al. 1983). In mouse embryos at the two cell stage, accumulation of HSP68 and HSP70 was observed in the absence of a stress agent (Bensaude et al. 1983).
Biens (1984) studying *Xenopus* sp.) found that the only cells to contain HSP70 mRNA without heat shock were the oocytes.

Another aspect related to HSPs induction is the variation in the levels with chronological age. Human skin cultures taken from 30 individuals ranging in age from 17 to 86 were heat shocked at 45°C for 1 hour and returned to normal temperature (37°C). The stress response was analyzed using a monoclonal antibody. Although the time course of the heat induced HSP72 expression was similar between the young and aged groups a lower level of induction was observed in the aged group (Muramatsu, et al., 1996). The evidence so far points to a decline in the binding and/or recognition between HSF and heat shock element with age in response to stress, thus producing a decrease in HSP70 mRNA and a subsequent decline in HSP70 levels.

Environmental fluctuations associated with seasonal changes are of major importance in establishing adjustments in the physiology and behavior of aquatic organisms (Dickson and Giesy, 1981). When measuring the levels of stress proteins it is important to realize the potential effect that natural variations can have. One possibility is that variability in the environment can be reflected as variations in the levels of HSPs. This variation does not necessarily mean a stress response to adverse conditions. A study by Fader et al (1994) supported the concept that variability in the stress response can be caused by seasonal variations. Using four different species of fish (*Pimephales promelas, Salmo trutta, Ictalurus natalis, Ambloplites rupestris*) the lowest levels of HSC/HSP70 measured occurred in winter followed by a high level in spring, a significant decrease in
summer and another decrease in fall. Dietz and Somero (1992) suggested that seasonal variations in temperature and acclimation regimes might affect the synthesis of HSPs in goby fishes. Winter-acclimated (18°C) individuals show induction of HSP90 at lower temperatures than summer-acclimated (28°C) individuals. The summer-acclimated individuals possessed a constitutively higher tissue level of HSP90 compared to the winter-acclimated individuals.

Adaptations to the environment or habitat and the levels of HSPs have been investigated in related (sister) species inhabiting vastly different environments. Norris et al (1995) showed that several species of tropical topminnows, (Poeciliopsis gracilis) adapted to desert environments had lower levels of inducible HSP70 polymorphism than the tropical species. The constitutive forms were identical in both groups of topminnows and in a confamilial species (Gambusia affinis). In addition northern desert topminnows synthesized a subset of inducible HSP70 isoforms identical to the tropical species.

The presence of xenobiotics also can induce the expression of HSPs. In experiments utilizing immunoblots to quantify protein induction, comparison of control and TBT-exposed mussels, (M. edulis) showed a linear increase in a 70 kDa stress-protein concentration (Steinert and Pickwell, 1993). Mussels exposed to the highest doses had levels more than ten times those of controls. At the same time a physiological indicator, filtration rate, showed a linear decrease with an increase in HSP70 following exposure to pollutants. Ryan and Hightower (1994) measured the induction of stress proteins following exposure to Cd^{2+} and Cu^{2+} and correlated it to cytotoxic effects in cell
cultures. Using fish hepatoma derived cells (PLHC-1) they showed an increase in various stress proteins of 25, 27, 32, 35, 70 and 75 KDa with an increase in the metal concentrations. Correlated with this increase they observed that at the highest sublethal Cd$^{2+}$ concentration (10 µg/ml), HSP70 and HSP75 reached their highest levels. This represents the highest concentration after which cytotoxic effects were noted by the neutral red (NR) assay. Possibly the presence of high levels of stress proteins helped to maintain cellular functions. Beyond this point the cellular damage may have overwhelmed the cell’s capacity to protect itself.

A study by Dyer et al. (1991) in the fathead minnow, (Pimephales promelas) showed tissue specificity in the heat shock proteins induced, minimum temperatures required for induction, and maximum temperatures at which each tissue synthesized heat shock proteins. Gill tissue synthesized six proteins of 60, 68, 70, 78, 90 and 100 KDa at an induction temperature of 28°C. Muscle tissue synthesized four proteins of 70, 78, 90, and 100 KDa at a temperature of 31°C. Brain tissue synthesized three of 68, 70, and 90 KDa at 33°C. Not only was there a difference in response to a heat shock as a function of tissue temperature, but the temperature at which the induction took place also differed.

Few studies have investigated the effects of PAHs on the stress protein expression in vivo. Monaghan and Bradley (1993) observed that β-naphthoflavone (BNF) and benzo(a)pyrene (BaP) elicited the induction of HSP70 and HSP90 in the catfish and medaka respectively. Induced levels of HSP70 and HSP90 compared to the controls were observed after a 5h exposure to BNF and after 15 days exposure to B(a)P. Werner and
Nagel (1997) reported reduced levels of HSP71, compared to their control, in (*Hyalella azteca*) exposed to 500 µgL\(^{-1}\) fluoranthene for 24 hours. In a preliminary report clams, (*Macoma nasuta*) exposed to fluoranthene expressed HSPs of apparent molecular weights of 51, 70 and 110 kDa, whereas those exposed to acetone expressed HSPs of 45, 51, 70, and 110 kDa (Randall, Lee and Sanders 1989).

In summary, there are a series of factors influencing the induction of stress proteins, specifically the HSP70 family: the presence and amount of denatured proteins, the amount of HSP70 present in the cell prior to a stress event, and the severity of the stress. In addition there are developmental, seasonal, habitat, tissue, and stress agent specificity in the HSP induction. Stress protein studies must account for as many of these factors as possible in order to reduce potential sources of variability. For example, individuals should have the same chronological age, be from the same population and season and habitat should be similar.

**Pollutants**

Two major classes of organic contaminants in the environment are the PAHs and PCBs. Exposure of marine invertebrates to such pollutants frequently results in a loss of ecological fitness, manifested as a reduction in scope for growth, reproductive output (Widdows, 1985) or increased mortality (Lowe and Pipe, 1987). Petroleum hydrocarbons including alkanes and polycyclic aromatics may reduce food intake and alter metabolic pathways in aquatic organisms (Capuzzo et al., 1984; Widdows et al., 1987). As a result, exposure to these agents can increase catabolism of endogenous energy reserves (Fraser,
1989) and alter lipid metabolism. A decrease in both synthesis of energy reserves (TAG) and mobilization of sterols was observed among oil-exposed lobsters (Capuzzo et al., 1984). Situations in which this pattern was observed resulted in increased catabolism of endogenous energy reserves in larval fish, bivalves, and crustaceans (Fraser, 1989).

PAHs, due to their hydrophobic nature, can be stored in body lipids or reproductive products. For example in pre-spawn mature mussels, (*M. edulis*) PAHs are more likely to be in the lipid reserves of the eggs (Lowe, 1988). Stored in these lipids, PAHs can be precluded from exerting toxic effects on the organisms. Nonetheless, upon lipid mobilization due to reproduction demands, stress or starvation these toxicants and/or their metabolites may be released, affecting the organisms, embryos and larvae. Low hatching success (dead embryos or no embryos produced) and high mortality (50% of hatchlings) were observed among the common tern, (*Sterna hirudo*) exposed to organochlorine toxicants in a contaminated riverine area (Castillo, et al., 1994). Possibly this was the result of toxics been mobilized from the yolk to the embryo during reproduction and/or feeding from contaminated sources by newly hatched birds. No evidence was given to support eggshell thinning for the low success observed.

Because of the inability of marine invertebrates to efficiently eliminate lipophilic compounds, these contaminants will accumulate in fat depots and may impact lipid metabolism. Consequently, reproductive capabilities of organisms may be affected and the survival of progeny reduced. Exposure of marine invertebrates to contaminants including PAHs can lead to the reabsorption of germinal material due to stress. Decreases in reproductive success in mussels, (*M. edulis*) and high mortality were observed after
exposure to diesel oil at different times of the year; higher mortality occurred if exposure took place just after spawning in June (Lowe and Pipe, 1987). Lower nutrient reserves in June compared to nutrient reallocation, gamete reabsorption and atresia in January was believed responsible. In addition there was also a significant decrease in the volume of ripe gametes, suggesting that energy destined for reproduction was used to compensate for the increased demand caused by the stress (Lowe and Pipe, 1986).

Capuzzo and Leavitt (1988) studied mussels (*M. edulis*), and crabs (*Carcinus maenas*) exposed to aromatic hydrocarbons and/or PCBs in the field. Changes in the distribution of lipid classes with the tissue concentration of pollutants were observed. Mussels from the site containing the highest doses of PAHs and polychlorinated biphenyls (PCBs) showed a decrease in phospholipid content, and increases in neutral lipid:polar lipid ratios and TAG:phospholipid ratio. In crabs collected along the contaminant gradient increases in TAG content, reductions in phopholipid and sterol and increases in neutral lipid:polar lipid and TAG:phospholipid ratio were observed. The authors suggested that stress responses to the contaminants included alterations in mobilization of TAG, sterol turnover and tissue degeneration noted by changes in the lipid ratios. The changes associated with the use of lipids, as energy reserves and/or structural components, seem to indicate altered lipid metabolism in the exposed organisms. Studies in oysters have shown that TAGs are not the only compounds affected during stress. For example, Riley and Mix (1981) did not report significant effects on the glycogen or neutral lipids in naphthalene exposed adult oyster, (*O. edulis*). Yet, there was
a decrease in protein and polar lipid concentrations suggesting that the pollutant
stimulated protein and polar lipid catabolism.

    Toxic effects of hydrophobic organic contaminants such as PAHs, involve the
impairment of certain activities and behaviors. Low molecular weight alkanes and
cycloalkanes can cause narcosis and anesthesia in various invertebrates (Blumer, et al.,
1969), and narcosis is the result of cell membrane disruption (Nelson-Smith, 1972).
Crustacean larvae have been found to be among the most sensitive organisms to oil in the
water (Wells and Sprague, 1976; Brodersen, 1987). Larvae of the king crab,
(Paralithodes camtschatica) and kelp shrimp, (Eualus suckleyi) exposed to oil water
soluble fractions (0.5-2 ppm aromatic hydrocarbons) stopped swimming within minutes,
apparently the result of narcosis (Brodersen, 1987). Morton and Wu (1977) reported
similar loss of swimming ability in nauplii of (Balanus amphitrite amphitrite) and (B.
variegatus variegatus) exposed to kerosene. In fish, Schimer et al., (1998) screened 16
PAHs using a rainbow trout, (Onchorhyncus mykiss) gill cell line (Rtgill-W1) and
concluded that direct cytotoxicity by the PAHs was caused by general perturbation of the
cell membrane. At the cellular level, contaminants such as PAHs can disrupt the
regulatory faculties of the cell membrane.

    PCBs were widely used compounds in industry and recognized as aquatic
pollutants (Boon et al., 1989). PCBs have high octanol/water partition coefficients (K_{ow}),
and low water solubilities (Zaranko, et al., 1997). They are of high concern due to their
chemical stability, high environmental persistence and hydrophobic nature. They are a
ubiquitous pollutant in the environment, in which they occur as a mixture of many congeners (Schulz et al., 1988). PCBs manufacture was banned in the United States in the 1970's but they remain in the environment and are recycled through air, water, and biota. Through leakage from electric capacitors, use as electric insulators, as additives to paints and hydraulic fluids, PCBs have been released into the environment. Close to \(330 \times 10^3\) M tonnes of PCBs have been released and is estimated that about 20\% of total production now resides in the oceans (Tanabe, 1988).

Waterborne PCBs enter estuarine systems and bind therein (DiPinto et al., 1993; Young et al., 1977; Steen et al., 1978). PCBs will tend to achieve their highest concentrations around the point of entry in the aquatic environment or at a settling area were currents slow down sufficiently for the settling of particles to occur. Organisms living in the sediments will be exposed to higher PCB levels than pelagic ones (DiPinto et al., 1993). The principal sources include leakage from capacitors, storage sites, and contaminated sediments.

In biota PCBs are sequestered in the lipid tissues (Phillips, 1986). Hamdy (1980) in a study using \(^{14}\text{C}\) labeled PCB (Arochlor 1254) found most of the PCB accumulated in cellular lipids. Once in the food web PCBs can be accumulated in successively greater concentrations in the higher trophic levels. Zaranko et al (1997) after analysis of fish, leeches, crayfish, oligochaetes, and chironomids from a polluted creek, concluded that in aquatic systems biomagnification is the mechanism governing PCB transfer.
PCBs are known for interfering with reproduction in different organisms. Because of their lipophilic character they tend to accumulate in gravid individuals, particularly in the gametes. In the copepod, (*Microarthridion littorale*) sediment-associated PCBs significantly lowered the reproductive capacity of females and reduced the LC$_{50}$ values of males. Depuration of PCB via eggs was suggested as the cause of this difference (DiPinto et al., 1993). In addition, naupliar survival significantly decreased upon exposure to Aroclor 1254. In the copepod, (*Acartia tonsa*) McManus et al (1983) found that egg deposition was the primary mechanism of PCB elimination. Feeding PCB (Clophen A50) to guinea pigs delayed the onset of puberty in the offspring (Lundkvist, 1990). In the Great Lakes, PCBs and DDT have been implicated in the reproductive impairment of fish-eating birds (Tillit et al., 1989), delaying the onset of breeding and thinning egg shells of water birds such as pelicans and cormorants (Peakall, 1970).

One mechanism of PCB toxicity in vertebrates is associated with arylhydrocarbon hydroxylase (Ah) receptor activity. This in turn affects the expression of cytochrome P$_{450}$ having consequences in the metabolism of fatty acids, hormones, and lipophilic pollutants. Exposure to polychlorinated organic compounds in fish-eating water birds resulted in the induction of cytochrome P$_{450}$ mixed function oxygenase enzymes, depletion of hepatic reserves, retinoids and vitamin A, porphyria, and wasting syndrome (Giesy et al., 1994). In white perch, (*Morone americana*), larval success (Holm et al., 1993) and maturation of adult females (Monosson et al., 1994) were reduced. The zebrafish, (*Danio rerio*) exposed to three doses (0.008, 0.08 and 0.4 µg of each congener / g food) of a mixture of 20 PCBs for 13 weeks, showed reduction in liver, ovary, and body...
weights, as well as in somatic and gonadal indexes compared to unexposed controls (Örn et al., 1998). The inhibition of cytochrome P₄₅₀ by PCB congeners has also been reported. In the rainbow trout, (O. mykiss) intraperitoneal injection of Clophen A50 caused a reduction in CYP1A1 mRNA and protein (Celander and Förlin, 1995). In scup, (Stenotomus chrysops) simultaneous induction of CYP1A1 mRNA and inhibition of CYP1A1 protein have been observed (White et al., 1997).

Despite the toxicity associated with PCBs, exceptions have been reported. In the cladoceran, (Daphnia magna) minimal effects on survival, reproduction and growth were observed at mean tissue concentrations of 2-130 ng mg⁻¹ dry weight (Dillon et al., 1990). Survival was high (88-100 %), number of neonates were unaffected or enhanced, and biomass was little or unaffected by the various congeners used after 21 days of static renewal exposure. Low binding affinity between the Ah receptor and PCBs in marine invertebrates was offered as a possible explanation for the lack of toxic effects.

The vertebrate toxicity model for PCBs seems to have little application to marine invertebrates. The induction and activity of Ah receptor seems to be very low in aquatic invertebrates (Borgmann et al., 1990). The binding affinity of PCBs for the Ah receptor in marine invertebrates could be low significantly affecting their susceptibility to halogenated aromatic compounds (Denison et al., 1986; Dillon et al., 1990).

PCBs can alter lipid mobilization and utilization in organisms (Addison, 1982; Reddy and Rao, 1989). Ferreira and Vale (1992) found that triglycerides are the main
lipid reserve of (C. angulata), and PCB accumulation followed the triglyceride variations.
In the oyster (C. angulata), Madureira et al (1993) found a decrease in triglyceride levels and adenylate energy charge (AEC) as PCB concentration increased. These studies show that TAG is extensively mobilized in PCB-stressed oysters. This is suggestive of energy demands and consumption as a consequence to PCB stress. Stress by organochlorines caused reduction of reserve lipids in the shrimp, (Metapenaeus monoceros) (Reddy and Rao, 1989). In cultures of the alga, (Scenedesmus sp.) and field samples from Lake Michigan, Swackhamer and Skoglund (1993) noticed variations in the lipid mass fraction (grams lipid per gram of algae) of exposed cultures. An increase during the first 24h was followed by a decrease in lipids spanning the length of the study (20 days). Decreases occurred in both the neutral lipids and phospholipids.

Cadmium

Metals are introduced into aquatic systems as a result of weathering of soils and rocks, volcanic eruptions and human activities involving mining, processing and use of metals (Laws, 1981). Benthic organisms are likely to be most affected by sediment metal concentrations, since the benthos is ultimately a repository of particulate material entering aquatic systems (Klein and Goldberg, 1970; Laws, 1981). Cadmium is a metal similar to zinc and mercury widely distributed in the lithosphere but usually at very low concentrations (GESAMP, 1974; OECD, 1975; O’Neill, 1985). Cadmium is used in electroplating, in plastic stabilizers, in pigments, solder and nickel-cadmium batteries (Laws, 1981; O’Neill, 1985). The toxicity of metals is in large degree based on binding to intracellular components. Metals with greater covalent characteristics will have a high
affinity for biological macromolecules with oxygen, nitrogen and sulfur (Niebohr and Richardson, 1980). The inappropriate binding to enzymes and membranes and production of free radicals that can undergo peroxidation reactions have been considered to be mechanisms for metal toxicity (Mahler, 1961; Harrison et al., 1983; Viarengo, 1989).

Cadmium, a known pollutant in the marine environment, is accumulated by bivalve filter feeders (Janssen and Scholz, 1979; Zaroogian, 1980; Farag, et al., 1998; Mouneyrac et al, 1998; Barak, et al., 1999; Tedengren, et al., 2000). Marine organisms including bivalves are known for their capacity to accumulate heavy metals (Janssen and Scholz, 1979; Fowler, 1979; Wikfors et al., 1994). Concentrations of 15ppb Cd are considered higher than concentrations encountered in chronically stressed natural environments (Zaroogian, 1980), and concentrations 40ppb – 60ppb are considered representative of heavily polluted areas (Hung, 1982). In the oyster, metals including cadmium, chromium, copper, and zinc can reduce the condition index (Shuster and Pringle, 1969; Roesijadi and Klerks, 1989), elevate respiration rates (Engel and Fowler, 1979), inhibit enzymes involved in shell formation such as carbonic anhydrase (Frazier, 1976; Hinkle, et al., 1987), affect cellular defense mechanisms (George et al., 1983), interfere with larval development (Okazaki, 1976), spat settlement and reproduction (Phelps and Mihursky, 1986; Akberali et al., 1984). Since many bivalves are sedentary and accumulate metals, they are considered good indicators of metal pollution in the environment (Zaroogian, 1980 Farrington, et al., 1983; Landrum et al. 1991).
Objectives

This study will examine the stress response to various contaminants in the eastern oyster. The studies herein, will investigate whether a stress protein response is elicited in the eastern oyster, (C. virginica) exposed to suspended field contaminated sediments (SFCS), PAHs, PCBs and a heavy metal (Cd$^{2+}$). At the molecular level, there is still need for validation of stress proteins as molecular biomarkers (Ryan and Hightower, 1994).

The effects of a mixture of PAHs (fluoranthene, pyrene, benzo[a]pyrene, benzo[e]pyrene), a heavy metal (Cd$^{2+}$) and uncontaminated suspended clay particles on the induction of HSP70 in oysters will be studied. A seasonal study will determine the natural variation in HSP70 for the eastern oyster in the Chesapeake Bay.
References


Heinonen, J., Kukkonen, J., Penttinen, O.-P. and Holopainen, I.J. 1997. Effects of hypoxia on valve closure time and bioaccumulation of 2,4,5-trichlorophenol by the


Kay, R.J., Russnak, R.H., Jones, D., Mathia, C. and Candido, E.P.M. 1987. Expression of intron-containing *C. elegans* heat shock genes in mouse cells demonstrates divergence of 3' splice recognition sequences between nematodes and vertebrates and an inhibitory


Chapter 2

Heat-shock protein (HSP70) response in the Eastern Oyster, *Crassostrea virginica*, exposed to PAHs sorbed to suspended artificial clay particles and suspended field contaminated sediments
Abstract

Sediments are a potentially significant source of pollutants, containing both organic contaminants and heavy metals. The heat shock protein response (HSP70 family) in the eastern oyster, *Crassostrea virginica* exposed to suspended clay particles spiked with polynuclear aromatic hydrocarbons (PAHs) and to suspended field contaminated sediments (SFCS) was investigated. In experiment 1, oysters were exposed to 1.0, 1.5 or 2.0 g suspended clay particles with concentrations of 65.6, 159.0 or 242.6 μg PAHs per g of wet clay particles respectively, and sampled after 40 days. Controls were exposed to 0, 1.0, 1.5, or 2.0 g suspended unspiked clay particles. In experiment 2, oysters were exposed to 0, 1.0, 1.5, or 2.0 g SCFS and the HSP70 expression determined after 5, 10, 20 and 40 days. Oysters exposed to suspended clay particles spiked with PAHs showed a significant increase in HSP70 levels, while oysters exposed to 1.0, 1.5 or 2.0 g suspended unspiked clay particles did not show changes in HSP70 levels compared to the group receiving 0 g clay particles. Exposure to the SFCS resulted in a significant increase in HSP70 as a function of exposure and treatment. The response however was not dose dependent. Compared to 0 g SFCS, groups exposed to 1.0, 1.5 or 2.0 g SFCS reached significantly higher levels in HSP70 at 40 days of exposure, with those exposed to 2.0 g SFCS expressing the highest levels. The HSP70 expression for each treatment showed fluctuations at various time intervals. No mortalities were recorded during the exposure experiments. The major contaminants in the SFCS were PAHs, heavy metals and PCBs. These results reveal that exposure to PAHs sorbed to clay particles and to SFCS induced a HSP70 response in the eastern oyster.
Introduction

Initially termed “heat-shock” (HSPs) proteins the stress proteins include a number of protein families induced during stress events. Stress proteins are synthesized at higher levels when cells are challenged with certain environmental stimuli such as high temperature and toxic chemicals, making them a potentially useful marker of exposure (Morimoto, 1993; Sanders, 1993). The HSP70 accounts for much of the translational activity in cells responding to environmental stress. HSP70 is inducible and highly conserved among various phyla including marine invertebrates such as mussels *Mytilus edulis* exposed to Cu$^{2+}$ (Sanders et al., 1993), Pacific oysters *Crassostrea gigas* exposed to elevated temperature (Clegg et al., 1998), in hemocytes of the eastern oyster, *C. virginica* exposed to 41°C for 1 hour (Tirard et al., 1995) and echinoderms *Strongylocentrotus purpuratus* exposed to a heat shock (25°C, 1 hour) (Stegeman et al. 1992; Sanders, B., 1994). We have also detected the presence of two HSP70 isoforms in the eastern oyster of 69kDa and 71kDa (Fig 1), 48 hours after a heat shock for 1 hour at 37°C.

Sediments are a source of toxicity for aquatic organisms containing not only organic contaminants but heavy metals as well. As such, sediments are a potentially significant source of pollutants in aquatic ecosystems. Sediments usually contain a higher concentration of hydrophobic contaminants in comparison with the water phase (Eertman *at al.*, 1995). The potential adverse effects of sediment-associated contaminants is generally assessed by determining their concentration directly from sediments, organisms on sites, or determined from bioassays of exposed animals (ASTM, 1993). However,
these determinations are not always a good indicator of effects in all cases (Long et al. 1995).

The majority of previous studies have focused on the stress protein response to contaminants dissolved in the water phase (e.g., Monaghan and Bradley, 1993; Sanders et al. 1994a) or settled sediments (e.g., Werner et al. 1998; Werner and Hinton, 1999). In all of these studies, a stress protein response was observed upon exposure to heavy metals, PAHs, or complex mixtures of contaminants associated with the water phase or in deposited sediments.

To my knowledge no study has been conducted, to test pollutants sorbed to either suspended particulates or suspended field contaminanted sediments (SFCS) on the HSP70 response in benthic filter feeders such as oysters. Bivalves are sessile filter feeders incapable of moving to avoid unfavorable conditions in their immediate environment and are known to bioaccumulate contaminants (Obana, et al. 1981; Farrington, et al. 1983; Landrum et al. 1991). The present study was conducted to test whether exposure of oysters to various doses of PAH-sorbed to suspended clay particles and SFCS induce a HSP70 response.

Methods and Materials

Experiment 1: HSP70 response in oysters exposed to PAHs sorbed to suspended clay particles.

The effects of suspended clay particles spiked with PAHs or suspended clay particles alone on the stress protein response were tested. Oysters collected from the
Damariscotta River, Maine, an area rarely infected by the parasite, (*Perkinsus marinus*) were used in this experiment. Acclimation to local conditions was initiated by bringing the oysters to 18\% (York River salinity) and temperatures of 23° - 24°C over nine days in two 600 L tanks. After acclimation, a subsample of oysters (n=10) were examined for *P. marinus* infection and results were negative.

Clay particles for the experiments were prepared by pulverizing green shale (Illite 46E0315, Wards/Cenco, Rochester, New York) to an average size of 50 μm. The clay particles were then hydrated in York River water (YRW, 1 μm filtered water), and stored at 4°C until use.

A solution containing a mixture of PAHs (fluoranthene, pyrene, benzo(a)pyrene and benzo(e)pyrene; 100 μg PAH / ml each) was prepared in acetone. The PAH solution was added to hydrated clay particles to obtain a nominal concentration of 400 μg PAHs / g hydrated clay particles (HCP). The mixture was stirred continuously and small amounts of distilled water were added occasionally until the carrier evaporated and the mixture became slurry. The PAH spiked clay particle slurry was mixed with unspiked clay particle slurry to obtain three nominal concentrations: 40.0, 133, and 200 μg PAHs / g of hydrated clay particles. These nominal concentrations represented exposure treatments of 40.0, 200 μg and 400 μg PAH / day in 1.0, 1.5 and 2.0 g hydrated clay particles respectively. Subsequent extraction and GC analysis of the spiked clay particles revealed the actual concentrations to be 65.6, 159 and 242 μg PAHs / g of hydrated clay particles. Clay particle suspension were prepared by stirring the desired amounts of spiked clay particles.
for treatment groups and unspiked clay particles for control groups into filtered YRW for two hours to ensure the adequate suspension of the clay particles prior use.

Acclimated oysters were divided into seven groups. Treatment groups (3) were exposed daily to 1.0, 1.5 or 2.0 g suspended spiked clay particles (corresponding to 65.6, 238 and 485 µg PAHs / oyster daily, n=12) for 40 days and sampled for measurement of HSP70 responses. Control groups (4) were exposed to 0 g, 1.0, 1.5 or 2.0 g suspended unspiked clay particles to test the effects of clay particle dosage on HSP70 response. Oysters were maintained in 2L containers with aeration and fed 0.2 g algal paste daily. York River water (1µm filtered) was changed every other day. At the end of the experiment (40 days post-exposure), gills from individual oysters were excised and used for HSP analysis. Gills were selected because they are directly exposed to waterborne contaminants and particulates. Aliquots of rectal, mantle + gills, and adductor muscle tissues were also removed to analyze for possible infection by the oyster parasite, (*P. marinus*).

Experiment 2: HSP70 response in oysters exposed to suspended field contaminated sediments (SFCS).

The HSP70 response was part of a wider study examining cellular responses in oysters exposed to SFCS (Chu et al., in press). These sediments were collected from the Elizabeth River, a highly contaminated estuary in Virginia. Chemical analysis of these sediments revealed the presence of PAHs (70.2±5.95 mg/kg dry sediments), PCBs (0.41±0.09 mg/kg dry sediments) and a variety of heavy metals (Chu et al., in press).
Oysters were collected from the Damariscotta River, Maine. After acclimation, 50 oysters were examined for (P. marinus) infection and the rest divided into four groups, maintained in individual 2L chambers with aeration, fed 0.2 g algal paste/oyster daily, and exposed daily to 0, 1.0, 1.5, or 2.0 g SFCS. Infection by (P. marinus) on the 50 acclimated oysters was negative. York River water was changed every other day. Oysters were sampled for HSP70 analysis 5, 10, 20, and 40 days post exposure. After collecting hemolymph for measuring cellular responses, tissues from individual oysters were excised for HSP analysis (gills) and examined for possible (P. marinus) infection (rectal, mantle+gills, and adductor muscle).

Heat-shock protein analysis

HSP70 in gill tissues was assessed by slot blot. Gills were homogenized using a hand held blender (Ultraturrax T-25 Homogenizer) at 24,000 rpm for 30 seconds, on ice in 2 ml of buffer (66 mM Tris pH 7.2, 3% Nonidet, 0.1 mM PMSF). The homogenate was centrifuged at 19,800xg on a fixed angle rotor for 30 minutes at 4°C, and the supernatant collected. Total protein concentration was determined using Biorad DC Protein Assay (Lowry et al. 1951).

Routinely Western blot and immunoassays have been used to analyze stress proteins in target tissues and organisms. While the use of western blot is effective to evaluate stress protein response, the assay is time consuming and laborious. In comparison, the slot blot technique for estimating total HSP70 is relatively quick. This technique has been used previously in HSP determinations comparing fishes from
contaminated and relatively clean sites in southern California (Brown and Bay, 1999), in oysters exposed to algae contaminated with PCBs (Cruz Rodríguez, et al., 2000) and in a macroalga exposed to environmental stressors (Lewis, et al., 2001). However, because the binding of a secondary antibody to the first antibody is not always 1:1 and the ratio between antigen and antibody is not necessarily constant, the application of the technique used is semi-quantitative. Notwithstanding, the blots serve to obtain an estimate of HSP accumulation when comparing treatments.

Prior to using slot blot for evaluation of the HSP70 in oyster gill tissues, the specificity of the primary antibody and the efficacy of the approach were tested. First, the specificity of the primary monoclonal antibody, raised against human HSP70 protein (Affinity BioReagents MA3-006), was assessed. After electrophoretic separation in 12.5\% SDS-polyacrylamide gel (Laemmli, 1970) and Western blotting, this primary monoclonal antibody was found to recognize two HSP70 isoforms, 69kDa and 71kDa, in eastern oyster soluble protein extract (Fig. 1). The antibody did not cross react with any other antigen in the oyster samples. The secondary antibody (Goat anti-mouse alkaline phosphatase conjugated) likewise did not cross react with any antigen in the oyster samples, but the primary antibody.

Second, the efficacy of the slot blot (Bio-Dot SF Microfiltration Apparatus) was tested. The linearity of the response was confirmed by serial dilutions of a “reference sample” (soluble protein obtained by heat shocking four oysters for 1 hour at 37\(^{0}\)C followed by a 48 hour accumulation period at ambient temperature). The linear range of
the assay was determined to be between 0.25 μg and 2.5 μg protein. The gradient showed a strong positive correlation ($r^2 > 0.96$) between the amount of protein loaded and the intensity of the signal. Non-specific cross reactivity of the antibodies was not visible in the slot blot. In addition, samples were run by both western blot and slot blot. Correlation between the two methods was good showing a similar pattern in HSP70 response. Slot blot measures total HSP70 (inducible and constitutive forms).

The blotting procedures consisted in directly applying and immobilizing 1.5 μg total protein per tested sample in triplicates onto the nitrocellulose (0.45 μm). The “reference sample” gradient (0.25, 0.50, 1.0, 1.5, 2.0 and 2.5 μg protein) was loaded in every blot to adjust for interblot variability. The 1.5 μg dilution in each series was used for data normalization. The blot was blocked with 5% non-fat dry milk in TTBS (0.05% Tween, 500 mM NaCl, 15 mM Tris pH 7.5) for 30 minutes, followed by two washes in TBS (500 mM NaCl, 15 mM Tris pH 7.5) for 10 minutes. Antibody dilutions (1:5000 primary antibody and 1:1000 secondary antibody) used were such that the quantity of antigen, not antibody, was limiting. Primary monoclonal antibody against HSP70 (Affinity Bioreagents, MA3-006) was applied for 90 minutes, followed by one wash with TTBS and two washes with TBS for 10 minutes each. The secondary antibody (Goat anti-mouse AP conjugated) was applied for 90 minutes. Subsequently the blot was washed twice with TBS for 10 minutes, then placed in a developing solution containing NBT ($p$-nitroblue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolyl phosphate). Bands started to develop after half an hour and development was completed after three hours. The blot was then stored in deionized water until densitometric analysis (Fig. 2).
Densitometric analysis was performed by scanning the blots using SepraScan software (ISS Enprotech, MA, USA). The areas of the samples were recorded and each sample area normalized against the area of the 1.5 μg dilution from the dilution series loaded in each blot. Arbitrary units of HSP, expressed as Units HSP70, were defined as the normalized values divided by 1.5.

\[
\text{Normalized area} = \frac{\text{Sample area}}{\text{Reference area}} \quad \text{Units Hsp70} = \frac{\text{Normalized area}}{1.5}
\]

Statistical analysis

When necessary, logarithmic transformation of the data was carried out to comply with normality and equality of variances requirements. One-way ANOVA was used to test for differences as a function of treatment in stress protein expression in experiment #1. Two-way ANOVA was used to test for differences in HSP70 expression between treatments and over length of exposure in experiment #2 (SAS Institute, Cary, NC). The Tukey test was used to compare means when ANOVA was significant (p<0.05). Results are expressed as mean ± CI (95% confidence interval).

Condition Index

The condition index (CI) is a measure of the total physiological condition of the organism. Condition index was calculated as tissue dry weight divided by shell dry weight multiplied by 100 (Lucas, A. and P.G. Beninger, 1985). Results are expressed as mean and S.D.
Bioaccumulation

Organic pollutant burdens in the oysters were determined by freeze drying the samples prior to analysis. Surrogate standards containing d$_8$-naphthalene, d$_{10}$-fluorene and 1-1' binaphthyl were added. Samples were extracted with dichloromethane at 100°C and 1500 psi for 10 minutes in an accelerated solvent extractor (Dionix ASE 200). Extract purification and gas chromatographic analysis procedures have been described previously (Chu and Hale, 1994). Interfering biogenic material was removed by fractionation using solid phase extraction (SPE) column chromatography (2g silica 100-200 mesh) sequentially eluted with hexane, hexane:dichloromethane (40:60) and dichloromethane:acetone (25:75). The PAHs were recovered in the hexane:dichloromethane fraction. Purified extracts were analyzed by capillary chromatography (DB-5 column, 60m) with flame ionization detection for PAHs. An internal standard (p-terphenyl) was added to the extracts immediately prior to GC analysis for quantitation. GC/MS was used for authentication of analyzed peaks. Recoveries of the surrogate standards were typically greater than 77%. Results are presented as mean and SD. Figure 3 is a representative chromatogram showing the PAH mixture used to expose the oysters.

Results

Experiment 1: HSP70 response in oysters exposed to PAHs sorbed to suspended clay particles.

Oysters exposed for 40 days to 1.0, 1.5 or 2.0 g suspended clay particles containing PAH (fluoranthene, pyrene, benzo(a)pyrene and benzo(e)pyrene) showed a
statistically significant increase in the HSP70 levels compared to controls, however no
dose dependency was noted (Fig. 4). Exposing oysters to 1.0, 1.5 or 2.0 g suspended clay
particles alone (controls) did not cause significant increases in HSP70 levels compared to
oysters not exposed to suspended clay particles (0 g clay particle, Fig. 4). Oysters exposed
to the highest dose of suspended PAH-contaminated particles showed a four-fold increase
in the accumulated PAH compared to the lowest dose (17.2 µg PAH/g tissue DW and
69.0 µg PAH/g tissue DW respectively) (Fig. 5). PAHs were not detected in control
oysters (Fig. 5). The bioaccumulation of PAHs did not appear to directly correlate to the
stress response in a dose-dependent nature. Higher levels of B(e)P were present in oyster
gill compared to B(a)P, Pyrene and Fluoranthene (Fig. 6). There was no mortality in the
exposed oysters. No statistically significant difference was observed in the condition
index (CI) between oysters exposed to different amounts of suspended clay particles
compared to the controls. No significant difference in CI was observed between controls
and suspended clay particles spiked with PAH. Infection by the oyster parasite, (P.
marinus) was not detected in any experimental oysters.

Experiment 2: HSP70 response in oysters exposed to SFCS

Exposure to SFCS resulted in a general increase of total HSP70 (p<0.001) in
oysters in all treatments. Oysters exposed to 0 g SFCS did not show statistically
significant changes in HSP70 levels (Fig. 7a). Treatment receiving SFCS showed
increases in HSP70 levels overtime, but these were not strictly linear in each case.
Oysters exposed to 1 g SFCS showed a minor increase after 20 days, with a subsequent
significant increase after 40 days (Fig. 7b). Oysters exposed to 1.5 g SFCS showed a
sustained increase after 20 days (Fig. 7c). Oysters exposed to 2 g SFCS showed fluctuations in the levels with a series of increases and decreases, and an eventual significant increase after 40 days (Fig. 7d). The oysters in this group reached highest levels of HSP70 expression of any treatment (Fig. 7b,c, d).

HSP70 levels remained high in all the exposed oysters after 40 days. Compared to the 0 g SFCS, the HSP70 levels in oysters exposed to 1.0, 1.5 and 2.0 g SFCS were statistically significantly higher. Even when a treatment effect was observed (p=0.006) no dose dependency was present. There was no mortality in the exposed oysters. Oysters exposed to SFCS showed no statistical significant difference in CI between treatments and controls. Infection by the oyster parasite, (*P. marinus*) was not detected in any experimental oysters. Analysis of PAHs revealed concentrations of 0, 1.45, 1.49, 2.46 mg PAHs/ kg wet tissues in 0, 1.0, 1.5, and 2.0 g SFCS after 40 days in exposed oysters (Chu et al., in press).

**Discussion**

Bioavailability of sediment or particle-associated contaminants plays a decisive role in toxicity. Contaminants bound to suspended particles have been shown to be bioavailable (Pollet and Bendell-Young, 1999; Björk and Gilek, 1996) and to pose potential adverse effects to filter feeders (Weltens et al. 2000, Hermsem et al., 1994) and other aquatic organisms (Brummelen and Stuijfzand 1993). Pollet and Bendell-Young (1999) showed Cd$^{2+}$ accumulation in the mussel, *M. trossulus*, exposed for 4 hours to spiked suspended particulate matter. Björk and Gilek (1996) showed phenanthrene uptake
in the blue mussel, *M. edulis*, from ingested suspended particulate organic matter concurrent with decreases in body condition index.

Few studies have tested the effects of contaminants associated with suspended particles on organisms (Weltens, et al., 2000; Peeters, et al., 2000; van den Belt, et al., 2000; Hermsen et al., 1994; Van Brummelen and Stuijfzand, 1993). The effects of contaminants sorbed to suspended particles on organisms have been reported using indicators at the organismal level such as condition index, feeding rate, survival and growth. Van Brummelen and Stuijfzand (1993) showed reductions in available energy for growth in terrestrial isopods, *Onniscus asellus* and *Porcellio scaber*, exposed to benzo(a)pyrene. Peeters et al. (2000) showed decreased growth rate in *Asellus aquaticus* exposed to suspended sediments spiked with B(a)P. Hermsen et al. (1994) reported that sediments spiked with lindane reduced feeding rate in the mussel *M. edulis*.

Stress proteins have been proposed as a potential indicator of sublethal effects to contaminants in the environment (Sanders, et al., 1993). The stress protein response in aquatic organisms such as fish, echinoderms, and bivalves has been described following exposure to contaminants in the water phase (Randall, Lee and Sanders, 1989; Monaghan and Bradley, 1993; Sanders et al.1994; Sanders and Martin, 1994; Werner and Nagel 1997) or associated with settled sediments (Werner et al. 1998; Werner and Hinton, 1999). Monaghan and Bradley (1993) observed that β-naphthoflavone and benzo(a)pyrene elicited the induction of HSP70 and HSP90 in catfish and medaka, respectively. Sanders et al (1994) demonstrated increases in chaperonin 60 (Cpn60) and
HSP70 in mussels exposed to Cu\textsuperscript{2+}. Induction in the levels of HSP70 and HSP60 were reported in *Hyalella azteca*, *Ampelisca abdita* and *Rheoxynius abronius* exposed to solutions containing cadmium, diazinon, dieldrin or fluoranthene (Werner and Nagel, 1997). Differential induction and inhibition of HSP64 and HSP70 were observed in amphipods, *A. abdita*, exposed to field collected sediments (Werner et al., 1998).

Transplant studies placing Asian clams, *Potamocorbula amurensis*, at two field sites showed increases in HSP70 and HSP76 after a mild heat shock (37\textdegree C for 15 minutes followed by 6.5 hour recovery period at control conditions) (Werner and Hinton, 1999). The sediments from both field sites were shown toxic to the amphipod *Eohaustorius estuarius*. Studies reporting stress protein response in organisms exposed to contaminants sorbed to suspended particles, however, are lacking.

The effects of contaminants bound to suspended particulate matter may be particularly acute to filter feeders such as oysters. Filter feeding has been a successful strategy, opening vast food resources to bivalves and many other aquatic organisms. However, this also has exposed these organisms to contaminants via suspended particles in addition to direct uptake from water through body surfaces. The present study demonstrated that exposing oysters to either suspended clay particles spiked in the laboratory with PAHs or to SFCS elicited a stress protein response (HSP70). The bioavailability of PAHs associated with suspended clay particles and SFCS is supported by their accumulation in the oysters' tissues. However, the potential adverse effects of sediment-associated contaminants could not be assessed from alterations in the condition index as no difference in this indicator was observed between exposed and control
Exposure of PAHs sorbed to suspended clay particles in the laboratory showed differential accumulation in oyster gills. The PAH mixture used contained equal amounts of each PAH, however a preferential uptake or retention of benzo(e)pyrene was observed. Bender et al. (1988) showed that benzo(e)pyrene had a slower depuration rate than benzo(a)pyrene, fluoranthene and pyrene in the eastern oyster, (C. virginica) exposed to contaminated sediments from the Elizabeth River, Virginia. The net result is a higher bioconcentration factor for benzo(e)pyrene. Baumard, et al., (1998) also observed the preferential accumulation of benzo(e)pyrene relative to its isomer benzo(a)pyrene in the mussel, Mytilus galloprovincialis. Since both compounds are characterized by similar $K_{ow}$ values (Karcher, 1988) and bioavailability, the difference in concentration found in tissues was hypothesized to be caused by the preferential biotransformation of benzo(a)pyrene while benzo(e)pyrene was preferentially accumulated in the lipids. Similarly, the results from this study show higher accumulation of benzo(e)pyrene compared to benzo(a)pyrene in the oyster. However, this study cannot elucidate which mechanism, preferential retention of benzo(e)pyrene or preferential biotransformation of benzo(a)pyrene, is causing the differential accumulation observed in the oyster.

Although the bioaccumulation of PAHs shows a dose-related increase in the oysters' tissues, no dose dependency in the stress protein (HSP70) response was noted. Stress proteins have been observed to exhibit a fluctuating response with time, dose or both (Köhler et al. 1999; Lewis, et al. 2001; Theodorakis et al. 1992). In a study using the
terrestrial isopod, *Onniscus asellus*, exposed to γ-Hexachlorocyclohexane (γ-HCH), pentachlorophenol (PCP), benzo[a]pyrene (B[a]P) and 2,2',5,5'-tetrachlorobiphenyl (PCB52) in soil samples did not produce a dose dependency in the heat-shock protein response (Köhler et al. 1999). These authors showed HSP70 levels fluctuating between induction and suppression in exposed isopods. They speculated that rapid metabolism/excretion of the contaminants via cytochrome P₄₅₀-like enzymes, or similar bioavailable doses in all treatments were responsible for the lack of a significant change in stress protein response. Lewis et al. (2001) reported a significant increase in HSP70 levels in the macroalga *Enteromorpha intestinalis* exposed to up 200 μg/l Cu⁺², but no dose dependency. In studies with the bluegill sunfish, *Lepomis macrochirus*, Theodorakis et al. (1992) reported increases in HSP70 levels for the first two weeks followed by decreases below control levels after 16 weeks. Possible explanations proposed included: acclimation (genetic), compensatory mechanisms or adaptation (physiological) to the stress. The apparent upregulation and downregulation of the HSP70 response observed in the SFCS is difficult to explain. The SFCS is a complex mixture of contaminants, whose synergism or antagonism may influence the nature of the stress protein response. Werner et al. (1998) exposed the amphipod, *A. abdita*, to sediments collected from the San Francisco Bay. They reported that HSP64 levels were positively correlated to total PAHs, but HSP71 levels were negatively correlated to benzo(b,k)fluoranthene and benzo(g,h,i)perylene. Either of these responses, upregulation or downregulation, could be considered as a potential indicator of adverse effects in field situations (Werner and Hinton, 1999). Continuing studies investigating complex mixtures of relevant contaminants are necessary to fully understand the stress protein response and its utility as...
a general biomarker of contamination. In the present study, as suggested by Köhler et al. (1999), the lack of a dose dependency in the HSP70 response could be due to similar bioavailable doses in all treatments especially with the narrow range of doses used.

Suspended solids in the water column may exert direct mechanical effects on filter feeders by increasing abrasion, clogging the respiratory surfaces of gills, and/or interfering with feeding mechanisms (Hughes 1976; Hellawel 1986). Negative response (closing of valves) has been noted in oysters exposed to silt concentrations above 1 g/l for periods longer than 48 hours, opening at time intervals apparently to test the water conditions (Loosanoff and Tommers, 1948; Loosanoff, 1962). However, habitat and species feeding ecology may play a role in the responses of organisms to high suspended sediment inputs (Peeters et al., 2000). Weltens et al. (2000) reported that exposure to uncontaminated sediments up to 500 mg/l did not cause mortality in filter feeder Daphnia magna. Van den Belt et al. (1999) showed no mortality in the zebra fish Danio rerio, exposed to clay particles up to 2000 mg/l. In the eastern oyster, no harmful effects caused by suspended silt have been reported. Engle (1952), and McKinney and Case (1973) did not observe apparent detrimental effects in oysters suspended in baskets next to dredging operations. Mackin and Hopkins (1961) showed that turbidities up to 0.7 g/l did not cause apparent harmful effects on oysters. Similarly, the amounts of suspended clay particles used in the present study did not appear to produce any negative impact on the exposed oysters since no changes were noted in either HSP70 levels or condition index in oysters exposed up to 2 g suspended unspiked clay particles. In addition, no mortalities in the oysters exposed to suspended unspiked clay particles were recorded.
However, enhanced HSP70 expression was noted in oysters exposed to suspended clay particles sorbed with PAHs and SFCS. Exposure to SFCS was also found to elevate *P. marinus* disease expression and modulation of cellular responses in oysters (Chu, et al., in press). Thus, it is believed that the increased levels in HSP70 response is due to the PAHs sorbed to the suspended clay particles and contaminants present in the SFCS. However, while the enhanced HSP70 in oysters exposed to PAH-sorbed particles was apparently a response to the presence of PAHs, the increased HSP70 response in SFCS exposed oysters was probably a response to the combined effect of various contaminants including PAHs, metals and trace amounts of PCBs contained in the SFCS rather than PAHs alone.

In the Chesapeake Bay region, declines in populations of the eastern oyster have been observed since the 1950’s (Andrews, 1954) with drastic reductions since 1992 (Ragone and Burreson, 1993). These reductions have been attributed to factors such as disease, over harvesting and a decrease in water quality due to pollution (Chu and LaPeyre, 1993; Ragone and Burresson, 1993; Burreson and Ragone-Calvo, 1996; Chu and Hale, 1994; Anderson et al., 1996; Fisher et al., 1999). The results from the present study indicate that contaminants associated to suspended sediments are stressful to filter feeders such as the eastern oyster at sublethal concentrations. Increases in HSP70 levels in response to the presence of contaminants associated to suspended sediments were clearly demonstrated. The resuspension of sediments could continually expose filter feeders to contaminants long after these compounds are clear from the water column. Thus, water quality must be a concern particularly to filter feeders as shown by the stress response exhibited in the oyster (i.e. increases in HSP70 levels).
The HSP70 response seems to be a sensitive indicator of toxicity to contaminants sorbed to suspended sediments to benthic filter feeders such as oysters. In the present studies, while oysters did not show changes in condition index or mortalities, a stress response was detected by the stress protein analysis. The stress protein response appears to be a potentially useful marker, in combination with a suite of other biomarkers, in aquatic toxicological studies.

In summary, results of the present studies exposing oysters to artificial suspended clay particles, contaminants sorbed to suspended clay particles or SFCS give a clear indication of a possible sublethal toxic effect caused by the presence of suspended contaminated sediments in the water column. To my knowledge, this is the first report on HSP70 response as a measure of a stressful condition in filter feeders exposed to contaminants associated to suspended sediments and clay particles. Because of the bioaccumulation potential of bivalve filter feeders, contaminants could be magnified up trophic levels posing serious consequences for top consumers and human health if consumed. The stress protein response appears to be a potentially useful marker in combination with a suite of other biomarkers in environmental monitoring. The contribution of suspended contaminated particles must be taken into consideration when evaluating polluted waters. Baseline studies are also needed to assess the natural variations in the HSP70 response, as well as the mechanisms involved in the stress protein response to various contaminants.
References


Pollet, I. and Bendell-Young, L.I. 1999. Uptake of $^{109}$Cd from natural sediments by the


Stegeman, J.J., Brouwer, M., DiGuilio, R.T., Forlin, L., Fowler, B.A., Sanders, B.M. and


Figure 1. Western Blot showing two HSP70 isoforms of 69 kDa and 71 kDa in gills of the eastern oyster, *Crassostrea virginica*. Oysters were heat-shocked for 1 hour at 37°C followed by a 48 hours accumulation period at ambient temperature. Lanes 1, 3--7 show the two isoforms detected using anti HSP70 monoclonal antibody (Affinity BioReagents MA3-006). Lane 2 shows low range molecular weight marker (Bio Rad Prestained SDS-PAGE Standards, Low Range).
Figure 2. Representative slot blot of soluble protein extracted from the eastern oyster, *Crassostrea virginica*. Row 1 show “reference sample” serial dilution loaded from left to right with 0.25, 0.50, 1.00, 1.50, 2.00 and 2.50 μg total protein. Row 2 show the “reference sample” loaded in opposite direction from row 1. The remaining wells were randomly loaded with 1.50μg protein of samples exposed to 1.00, 1.50 or 2.00 g SFCS. Slot blot technique measures total HSP70 levels (constitutive and induced) in samples.
Figure 3. Representative chromatogram of the PAH mixture (fluoranthene, pyrene, benzo(e)pyrene and benzo(a)pyrene) used to expose the oysters. PAHs were analyzed by capillary chromatography (DB-5 column, 60m) with flame ionization detection. An internal standard (p-terphenyl) was added immediately prior to GC analysis for quantitation.
Figure 4. HSP70 response in oysters (*Crassostrea virginica*), after 40 days exposure to suspended unspiked clay particles (0, 1.0, 1.5, or 2.0 g daily), or to suspended PAH-sorbed clay particles (1.0, 1.5 or 2.0 g daily, corresponding to 65.6, 159.0, 242.0 µg PAHs / g sediments respectively). No significant difference between treatments for oysters exposed to suspended unspiked clay particles (Mean ± CI, N=9-11). Oysters exposed to suspended PAH-sorbed clay particles show a significant increase in HSP70 levels compared to controls. However no dose dependency is observed (Mean ± CI, N=8, CI= 95% confidence interval). (*) Asterisk denotes significantly different (p<0.05).
Figure 5. PAH accumulation in oyster gills after 40 days exposure to suspended PAH-sorbed clay particles. Oysters show a dose related increase in accumulated PAHs (Mean ± SD, N=3). Different letters represent significant difference (p<0.05). Control oysters had levels below detection (0.1μg / mL).
Figure 6. Accumulation of PAHs by compound in oyster gills after 40 days exposure to suspended PAH-sorbed clay particles. Oysters were exposed to a mix of PAH (fluoranthene, pyrene, benzo(e)pyrene and benzo(a)pyrene, 100 μg / ml of each) sorbed to clay particles. Oysters accumulated higher levels of benzo(e)pyrene over fluoranthene, pyrene and benzo(a)pyrene (Mean ± SD, N=3).
Focusing on polyaromatic hydrocarbons such as Fluoranthene, Pyrene, B(e)P, and B(a)P, the chart illustrates the concentration of PAHs in tissue dry weight for different sample sizes (1g, 1.5g, 2g). The data suggests varying degrees of accumulation, with B(a)P showing the highest concentration among the substances tested.
Figure 7. HSP70 response in the eastern oyster, *Crassostrea virginica* exposed to (a) 0 (b) 1.0 (c) 1.5 and (d) 2.0 g suspended field contaminated sediments (SFCS) for 5 days, 10 days, 20 days, and 40 days (Mean ± CI, N=7-9). (*) Asterisks denotes significantly different from day 5 within each treatment (p<0.05). CI = 95% confidence interval.
c)

Exposure (days)

b)

Exposure (days)

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Chapter 3

Effects of PCBs sorbed to algal paste and sediments on the stress protein response (HSP70) in the Eastern oyster, *Crassostrea virginica* (Gmelin)
Abstract

This study investigated whether there is a stress protein response (HSP70 family) in sexually mature and immature oysters fed algal paste laced with PCB (Aroclor 1242, 1254, and 1260). Sexually mature oysters were fed 0.2 g algal paste / oyster / day containing 0, 0.10 or 1.00 μg PCBs for 15 and 30 days. Sexually immature oysters were conditioned before gametogenesis and fed 0.7 g algal paste / oyster / day containing 0, 0.35 or 3.50 μg PCBs for 56 days. To test the combined effects of PCB and sediments, 0.3 g of clay particles were added to the containers of a second group of PCB-exposed immature oysters (i.e. 0, 0.35 or 3.50 μg PCB daily). After PCB exposure, oyster tissues (mantle and gill) were sampled and analyzed for HSP70 and PCB. In general for both sexually mature and immature oysters, there was no significant effect in mean HSP70 response after feeding PCBs sorbed to algal paste. PCB concentration was up to 931 ng/g DW and 1795 ng/g DW in gill and mantle of sexually mature oysters after 15 and 30 days respectively. In sexually immature oysters, PCB concentration was 1342 ng/g DW in the mantle, and 180 ng/g DW in gill after 56 days. The addition of clay particles caused a significant increase in mean HSP70 levels of gills and mantle although the mantle was less sensitive. It did not appear that PCB concentrations used elicit a significant stress protein response in the oyster.

Introduction

In the Chesapeake Bay region, declines in populations of the eastern oyster *Crassostrea virginica* have been attributed to disease and a decrease in water quality due to pollution. Bivalves are sessile filter feeders incapable of moving to avoid unfavorable
conditions in their environment and are known to bioaccumulate contaminants (Obana, et al., 1981; Farrington, et al., 1983; Landrum et al. 1991). Polychlorinated biphenyls are synthetic aromatic compounds widely used in industry and recognized as aquatic pollutants (Boon et al., 1989). In the environment, PCBs occur as a mixture of many congeners (Schulz et al., 1989). PCBs have been banned in the U.S. since the 1970s. Sources of PCBs in the environment include leakage from electric capacitors, storage sites, release from paints and hydraulic fluids and association to sediments or organic matter (Borlakoglu and Haegele, 1991). PCBs have the characteristics of high octanol/water partition coefficient (K\text{ow}) and low water solubility (Zaranko, et al., 1997). PCBs pose a threat to marine organisms due to their chemical stability and persistence in the environment. These compounds tend to accumulate in lipid-rich tissues and subsequently transfer up trophic levels (Hamdy, 1980; Phillips, 1986; Borlakoglu and Haegele, 1991; Chu et al., 2000).

Stress proteins (SPs) are known to be synthesized at elevated levels when organisms are challenged with environmental stimuli such as high temperature or toxic chemicals making them a potentially useful marker of exposure (Morimoto, 1993). Members of the HSP70 comprise one of the most highly conserved proteins known (Sanders et al, 1994) and have shown good induction and conservation of reaction across species, including marine invertebrates such as mussels (Mytilus edulis), Pacific oyster (Crassostrea gigas), and echinoderms (Strongylocentrotus purpuratus) (Sanders, 1990; Sanders et al, 1994; Clegg et al., 1998).
The induction of HSP70 after exposure to elevated temperatures has been reported in the eastern oyster *C. virginica*, and the pacific oyster, *C. gigas* (Tirard et al., 1995; Clegg et al., 1998). In our laboratory, we have detected the presence of two HSP70 isoforms in the eastern oyster of 69kDa and 71kDa, 48 hours after a heat shock for 1 hour at 37°C (unpublished data).

The objective of this study was to determine whether PCB exposure elicits a stress protein response (HSP70 family) in oysters exposed to a PCB-contaminated diet. This study was part of a larger study investigating the effects of PCB on eastern oyster reproduction.

**Methods and materials**

1. **Algae preparation**

   PCB-sorbed algal paste was prepared by mixing PCBs (1:1:1 mixture of Aroclor 1242, 1254, and 1260) dissolved in acetone with algal paste (*Tetraselmis maculata, Thalassiosira weissflogii, Chaetoceros calcitrans* and *Isochrysis galbana*) acquired from the oyster hatchery of the Virginia Institute of Marine Sciences. PCBs were extracted from the filtered paste and 99.8% was found sorbed to the algae (Chu et al., 2000).

2. **Experimental oysters**

   Oysters collected from the Damariscotta River, Maine, were used for experimental exposure. A period of acclimation to the local conditions was initiated gradually bringing the oysters to 18‰ (York River salinity) and temperatures of 23°C -
24°C over 9 days in two 600 L tanks. After acclimation a sub-sample of oysters (n=15) were analyzed for *Perkinsus marinus* infection with negative results. For the exposure experiments, oysters were placed in individual 2L containers with aeration. Water in the containers (1 μm filtered York River water) was changed every other day.

3. Exposure experiments
   
   A. Sexually mature oysters
   
   Oysters were received in August 1997. A subsample (n=25) was analyzed and found sexually mature (presence of gametes). Oysters were divided into 3 groups fed 0.20g algal paste containing 0, 0.10 or 1.00 μg PCBs / oyster daily. After 15 and 30 days exposure gills and mantle were collected and analyzed (n=15/treatment) for HSP70 determination and PCB accumulation.

   B. Sexually immature oysters
   
   Oysters were received in April 1998. Twenty-five oysters were examined for the presence of gametes and none were found. The remaining oysters were divided into 3 groups conditioned by feeding 0.70g algal paste daily containing 0, 0.35, or 3.5μg PCBs / day. To study the combined effects of sediments and PCBs, 0.30g clay particles daily / oyster (Illite 46E0315, Wards/Cenco, Rochester, New York) were added to a second set of oysters in containers exposed to 0, 0.35 or 3.50 μg PCBs / day. Clay particles were prepared by pulverizing green shale to an average size of 50μm. After 8 weeks exposure,
oyster tissues (mantle and gill, n=15/treatment) were sampled and analyzed for HSP70 and PCB accumulation.

4. Heat shock protein analysis

Gills and mantle tissues were homogenized using a hand held blender (Ultraturrax T-25 Homogenizer) at 24000 rpm for 30 seconds, on ice in 2 mL of buffer (66 mM Tris pH 7.2, 3% Nonidet, 0.1 mM PMSF). The homogenate was centrifuged at 19800 x g on a fixed angle rotor for 30 minutes at 4°C, and the supernatant collected. Soluble protein concentration in the collected supernatant was determined using Biorad DC Protein Assay (Lowry et al., 1951).

Slot blot technique has been previously used in studies comparing fishes from a contaminated and a clean site in southern California (Brown and Bay, 1999), in oysters exposed to PCBs-sorbed to algal paste (Cruz Rodríguez et al., 2000), and in the macroalga Enteromorpha intestinalis exposed to contaminants (Lewis et al., 2001). Briefly, the blotting procedures consisted in directly applying and immobilizing 1.5μg protein per tested sample in triplicates onto the nitrocellulose paper (0.45μm). A “reference sample” gradient (0.25, 0.50, 1.00, 1.50, 2.00 and 2.50μg protein) was loaded in each blot as a measure to adjust for interblot variability. The 1.50μg dilution in each series was used for data normalization. The blot was blocked with 5% non-fat dry milk in TTBS (0.05% Tween, 30 mM NaCl, 24 mM Tris pH 7.5) for 30 minutes and washed two times with TBS (30 mM NaCl, 24 mM Tris pH 7.5) for 10 minutes. Primary monoclonal antibody against HSP70 (Affinity Bioreagents MA3-006) was applied for 90 minutes, followed by a wash with TTBS and two washes with TBS 10 minutes each.
The secondary antibody (Goat anti-mouse AP conjugated) was applied for 90 minutes, the blot was washed twice with TBS, and placed in a developing solution containing NBT (p-nitroblue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolyl phosphate). The blot is stored in deionized water until densitometric analysis. Densitometric analysis was performed by scanning the blots using SepraScan software (ISS Enprotech, MA, USA). The areas of the samples were recorded and each sample area normalized against the area of the 1.5μg dilution from the dilution series loaded in each blot. Arbitrary units of heat-shock protein, expressed as Units HSP70, were defined as the normalized values divided by 1.5.

5. PCB extraction

Total PCBs in oyster were extracted from tissues by supercritical fluid extraction (SFE) and analyzed by gas chromatography with electrolytic conductivity detection (GC-ELCD) according to Hale and Gaylor (1995). Briefly, samples were freeze dry and transferred to stainless steel extraction vessels (3ml). Neutral alumina oxide was added on the top of the sample until the vessel was filled, to trap lipids during the PCB extraction. Samples were spiked at the entrance of the vessel with three PCB congeners (PCB30, PCB65 and PCB204) to assess the extraction yield. Super critical extraction of PCBs consisted of: 10min static extraction at 350 atm and 150°C, 30 min dynamic extraction at 350 atm and 150°C with a compressed CO$_2$ flow rate of 3 ml/min. The PCBs were then collected on a trap at 0°C. When the extraction was completed, PCBs were desorbed at 90°C into 2 ml GC autosampler vials and internal standard (pentachlorobenzene) added. Samples were concentrated into 50 μl of hexane for
injection onto a GC-ELCD (Electrolytic Conductivity Detector) equipped with a DB 5 fused silica capillary column (60m x 0.32 mm x 0.25 µm film thickness) using a splitless injection system at 300°C and helium as carrier gas. The column was temperature programmed: 90°C for 1 min, then 320°C at 4°C/min and held at 320°C for 10 minutes. Quantification was performed by comparison with response of congeners with identical degrees of chlorination. Figure 8 shows a representative chromatogram of the PCB mixture used during exposure.

6. Statistical analysis

Data for HSP70 were analyzed using ANCOVA (soluble protein concentration as the covariate) for effects between PCB dose and length of exposure in sexually mature oysters. Data were log transformed and analyzed using ANOVA for effects between PCB dose and sediment in sexually immature oysters (SAS Institute, Cary, NC).

Results

A) Sexually mature oysters

In general for sexually mature oysters, there was no statistical significant difference in the HSP70 response in gill after 15 or 30 days exposure compared to controls. In gills of oysters exposed to the highest dose (1.00 µg PCB/day), I observed a decrease in the mean HSP70 below the control levels after 15 days and also in the soluble protein concentration. A subsequent increase in the mean HSP70 values after 30 days brings the stress protein levels back to control levels (Fig. 9A), although these fluctuations were statistically insignificant. In mantle as in gills there is no statistically
significant difference in the mean HSP70 between control and oysters exposed to PCBs. We observed a decrease in mean HSP70 levels in oysters exposed to 1.0 μg PCB daily (Fig. 9B). However, adjusting for the decrease observed in soluble protein concentration, the decrease in mean HSP70 is not significant (ANCOVA, p=0.12).

Sexually mature oysters show a dose dependent increase in the accumulation of PCB at both 15 and 30 days in all tissues analyzed including gill and mantle (Chu et al., 2000). There was no difference in the accumulation of PCBs between 15 and 30 days in oysters exposed to the low dose. However, there was a significant increase in PCB accumulation at the high dose with a 1.9 fold increase after 30 days (1795 ng/g DW) compared to 15 days (931 ng/g DW) (Fig. 10).

B) Sexually immature oysters

There was no statistically significant difference in mean HSP70 levels in mantle and gill with dose compared to the controls (Fig. 11A, B), although a slight increase in mean HSP70 levels was observed. The addition of clay particles caused a significant increase in gill and mantle mean HSP70 levels compared to oysters not exposed to clay particles (Fig. 11A, B). Mantle appeared to be less sensitive than gills to the addition of clay particles.

Accumulation of PCB was found in both gill and mantle (Soudant et al., 1999). Mantle accumulated significantly higher levels of PCBs (1342 ng / g DW) than gill (550 ng /g DW) (Fig. 12A). The accumulated PCB levels are lower in the mantle of oysters
exposed to clay particles (802 ng / g DW) than those not exposed to clay particles (1342 ng / g DW) (Fig. 12A, B). The gills showed no significant difference in the accumulated PCB with or without the addition of 0.30 g of clay particles for 56 days (512 and 550 ng PCB / g DW respectively).

**Discussion**

The accumulation of PCBs has been linked to effects in many organisms including marine bivalves (Kamohara et al., 1984; Veldhuizen-Tsoerkan et al., 1991; DiPinto et al., 1993; Madureira et al., 1993; Ferreira and Vale, 1998). PCBs have been implicated as agents of lipid peroxidation in rats (Kamohara et al., 1984), have caused reductions in triacylglycerol (TAG) and adenylate energy charge (AEC) in oyster, *(Crassostrea angulata)* (Madureira et al., 1993), and depletion of TAG in the flat oyster, *(Ostrea edulis)* (Ferreira and Vale, 1998). In mussels, *(Mytilus edulis)*, decreases in glycogen content and anoxic survival capabilities were reportedly attributed to PCB exposure (Veldhuizen-Tsoerkan et al., 1991). Exposure to sediment-associated Aroclor 1254 caused significant reductions in the reproductive capacity (i.e. decreasing number of eggs and nauplii production) of the meiobenthic copepod, *(Microarthridion litorale)* (DiPinto et al., 1993). Similarly, in the present study a dose dependent accumulation of PCB was related to changes such as reduction in the number of females and amounts of phospholipids (PL), TAG and fatty acids (FA) in sexually mature oysters (Soudant et al., 1999; Soudant, et al., 2000; Chu et al., 2000) compared to controls.
In the present study, Chu et al. (2000) showed that PCB congener accumulation profiles differed in the organs examined. Highly chlorinated congeners were preferentially accumulated in the visceral mass, gills and mantle. Lower chlorinated congeners were found preferentially in the adductor muscle. This difference suggests that low chlorinated PCBs may partition into more polar lipids, which predominate in adductor muscle lipids (Soudant et al, 1999), whereas high chlorinated PCBs partition into the non-polar lipids of the visceral mass. Relative PCB polarity is generally inversely proportional to the degree of chlorination. It was also observed that PCB congener 180 bioaccumulated to a limited extent in all tissues.

Stress proteins have been proposed as indicators of contaminant effects (Sanders, 1993). Changes in the stress protein levels in response to contaminants have been previously reported in aquatic species (Monaghan and Bradley, 1993; Ryan and Hightower, 1994; Werner and Nagel, 1997). The aromatic hydrocarbons, β-naphthoflavone (BNF) and benzo(a)pyrene (B(a)P), elicited the induction of HSP70 and HSP90 in the catfish and medaka respectively (Monaghan and Bradley, 1993). Using fish hepatoma derived cells (PLHC-1), Ryan and Hightower (1994) showed increases in various stress proteins (25, 27, 32, 35, 70 and 75 kDa) with increases in heavy metal (Cd$^{2+}$ and Cu$^{2+}$) concentrations. Werner and Nagel (1997) exposed three species of amphipods (Hyalella azteca, Ampelisca abdita and Rhepoxynius abronius) to fluoranthene, cadmium or diazinon in solution and reported increases in HSP60 and/or HSP70. These studies are indicative of stress protein induction under various exposure conditions and agents. However, feeding PCBs-sorbed to algal paste for 30 days to
sexually mature oyster and for 56 days to sexually immature oysters did not cause significant changes in mean HSP70 levels.

The concurrent decrease observed in mean HSP70 levels and soluble protein concentration in mantle at 15 and 30 days, and in gill at 15 days of sexually mature oysters was unexpected. Studies have reported that stress proteins are preferentially expressed during stress events even when general protein synthesis is inhibited (Yost and Lindquist, 1986; Kay et al., 1987; Bond, 1988). In the present study, this phenomenon was not observed in oysters. After adjusting for the decrease in soluble protein concentration, the decrease observed in mean HSP70 levels in oysters exposed to 1.00 μg PCBs-sorbed to algal paste daily was not significant (ANCOVA p=0.12). This appears to indicate that exposure to PCBs did not cause effects on the stress protein response.

Lack of a capacity to effect protein denaturation (proteotoxicity) has been suggested as a factor constraining a stress protein response (Salminen et al., 1996; Lewis et al., 2000). In human hepatoma cells (HepG2) agents such as CCl₄ and cocaine failed to induce a HSP70 response (Salminen et al., 1996). These agents or their metabolic intermediaries do not bind to proteins and demonstrate reduced proteotoxicity. The low proteotoxicity of the triazine herbicide Irgarol 1051 was suggested as a reason for not promoting alterations in HSP70 levels in the macroalga, (Enteromorpha intestinalis) (Lewis et al., 2000). The inability of PCBs to promote a sustained stress protein response has been previously documented. Exposure to PCB52 elicited a rapid but transient HSP70 response in the terrestrial isopod, (Oniscus asellus), decreasing immediately after
24 hours and eventually dropping below control levels by the third day of continuous exposure (Köhler et al., 1999). The authors suggested that lipids in the cell membrane of epithelial tissue in the hepatopancreas entrap highly lipophylic compounds precluding further interactions. The initial transient response was suggested to be due to interactions with membrane-bound proteins. Similarly, in the present study the eastern oyster showed no change in HSP70 levels after exposure to PCBs. It is tempting to speculate that the PCB doses used did not caused sufficient protein denaturation to elicit a stress response.

However, an enhanced HSP70 expression was observed in gills of oysters exposed to 0.3g suspended clay particles, including the controls, in addition to PCB (Fig. 9A). In Chapter 2, oysters exposed to 1.0, 1.5 or 2.0g clay particles suspended in the water did not show changes in the stress protein (HSP70) levels compared to controls (Cruz Rodríguez and Chu, submitted). Studies by Engle (1952), McKinney and Case (1973), and Mackin and Hopkins (1961) showed that oysters have the capacity to cope with high levels of particulate matter without any apparent damage. The increase in mean HSP70 observed in this study, seemingly caused by the presence of suspended particles, and our previous results are difficult to explain at this time. However, since the mean HSP70 increase was significant in the gills of all oysters exposed to suspended clay particles with or without added PCB, one possibility is that the increase observed resulted from a stimulatory effect at low suspended sediment concentrations (i.e. hormesis). Hormesis has been described as a stimulatory effect of any kind to low concentrations of an agent (Stebbing, 1982). This type of phenomenon and its implications in the HSP70 response requires further study.
The arylhydrocarbon hydroxylase receptor (AhR) mediates many of the toxic responses induced by polyhalogenated and polycyclic hydrocarbons, which are ubiquitous environmental contaminants (Tian et al., 1999). PAHs show toxicity through cell membrane disruption as reported on crustacean larvae exposed to water soluble fractions of oil and low molecular weight alkanes and cycloalkanes (Nelson-Smith, 1972; Morton and Wu, 1977; Brodersen, 1987). Schirmer et al., (1998) screened sixteen PAHs using rainbow trout gill cell line (Rtgill-W1) and concluded direct cytotoxicity by the PAHs was caused by general perturbation of the cell membrane. In the eastern oyster, exposure to PAHs sorbed to clay particles for 40 days resulted in enhanced HSP70 response, potentially the result of the PAHs (Cruz Rodríguez and Chu, submitted). In many organisms including the white perch, (Morone americana) (Holm et al., 1993; Monosson et al., 1994), the zebrafish, (Danio rerio) (Örn et al., 1998), and fish eating birds (Giesy et al., 1994) the toxicity of PCBs is associated with the Ah receptor activity. In marine invertebrates, Borgmann et al. (1990) has suggested low induction and activity of the Ah receptor. Denison et al. (1986) and Dillon et al. (1990) proposed that in marine invertebrates, the binding affinity of PCBs for this receptor could be low significantly affecting their susceptibility to halogenated aromatic compounds. Possibly, the low induction and activity of the Ah receptor and/or its low affinity for PCBs may account for its low effects observed in marine invertebrates. Similarly in the present study, no significant stress protein response was observed in oysters exposed for 30 days or 56 days to PCBs sorbed to the diet.
In summary, neither the PCB concentrations used in the present study or the length of exposure were sufficient to elicit a stress response in the eastern oyster. It is not known whether PCBs affect the stress protein response. Future studies are needed to answer if increases in PCB concentration and exposure time would ultimately elicit a stress protein response.

Acknowledgments

This work was supported by a grant from the Exploratory Research Program of the Environmental Protection Agency (EPA Grant No: R825349-01-0). The views expressed herein are those of the authors and do not necessarily reflect the views of EPA or any of its sub-agencies. Contribution No. 2243 from the Virginia Institute of Marine Science, College of William and Mary.
References


Soudant, P., Chu, F-L., Steider, L. and Hale, R. 2000. Does PCB accumulation affect the lipid metabolism of the reproductive active oyster *Crassostrea virginica*? Mar Environ Res. 50(1-5):244.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Soudant, P; Van Ryckeghem, K; Marty, Y; Moal, J; Samain, JF; Sorgeloos, P 1999. 
Comparison of the lipid class and fatty acid composition between a reproductive cycle in 
nature and a standard hatchery conditioning of the Pacific Oyster, *Crassostrea gigas*. 


Tian, Y., Ke, S., Deninson, M.S., Rabson, A.B. and Gallo, M.A. 1999. Ah receptor and 
NF-Kappa B interactions, a potential mechanisms for dioxin toxicity. J. Biol. Chem. 

hyperthermia in vitro on protein synthesis and accumulation in oyster hemocytes. Fish 
Shellfish Immunol. 5:9-25.

Theodorakis, C.W., D'Surney, S.J., Bickham, J.W., Lyne, T.B., Bradley, B.P., Hawkins, 
W.E., Farkas, W.L., McCarthy, J.F. and Shugart, L.R. 1992. Sequential expression of 
biomarkers in bluegill sunfish exposed to contaminated sediments. Ecotox. 1:45-73.

and metabolic parameters as stress indices in sea mussels exposed to cadmium or 


Figure 8. Representative chromatogram showing PCB congeners in the mixture (Aroclor 1242, 1254, and 1260) used to expose the oysters. PCBs were analyzed by gas chromatography with electrolytic conductivity detection (GC-ELCD) equipped with a DB-5 fused silica capillary column (60m x 0.32 mm x 0.25 μm film thickness) using a splitless injection system at 300°C and helium as carrier gas. PCB30, PCB65 and PCB204 were used as surrogates to assess the extraction yield. Pentachlorobenzene (PCBt) was used as internal standard. Quantification was performed by comparison with response of congeners with identical degrees of chlorination.
Figure 9. Total HSP70 in reproductive active oysters exposed to 0, 0.1 or 1.0 μg PCB/oyster/day for 15 and 30 days. (A) Gills showing a decrease in total HSP70 after 15 days with a subsequent increase after 30 days. (B) Mantle showing decreases in HSP70 with dose.
Figure 10. PCB accumulation in gill and mantle of reproductively active oysters exposed 0, 0.1 or 1.0 µg PCB daily sorbed to algae for 15 and 30 days. PCB accumulation showed increase with dose and length of exposure (Mean ± SD, N=3).
Graph showing the ng PCB/g tissue dry weight for different treatments (µg PCB/oyster/day) over 15 and 30 Days.
Figure 11. Total HSP70 in reproductive inactive (conditioned) oysters. (A) HSP70 response in gill of oysters exposed to PCB sorbed to algal paste and 0.3g suspended clay particles (Mean ± 95% CI, N=9-10). (B) HSP70 response in mantle of oysters exposed to PCB sorbed to algal paste and 0.3g suspended clay particles (Mean ± 95% CI, N=8-9). No dose effects due to PCB exposure were observed in gill or mantle. The addition of suspended clay particles produced a significant increase in mean HSP70 levels in gills (p<0.001) and mantle (p=0.032).
A  GILLS

B  Mantle

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Figure 12. PCB accumulation in reproductive inactive (conditioned) oysters. (A) Oysters exposed to PCB contaminated algal diet containing 0, 0.35 or 3.5 μg PCB / daily, no clay particles added. (B) Oysters exposed to PCB contaminated algal diet containing 0, 0.3 or 3.5 μg PCB / daily + 0.3 g clay particles for 56 days. PCB accumulation showed increase with dose and length of exposure (Mean ± SD, N=3). Mantle showed differences in the accumulation of PCB with and without the addition of clay particles (802 versus 1342 ng / g DW). Gills showed no difference in the accumulation of PCBs with or without the addition of clay particles (512 and 550 ng PCB / g DW respectively).
w/o clay particles

□ Gills  ■ Mantle

with clay particles

□ Gills  ■ Mantle

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Chapter 4

HSP70 levels in oyster *Crassostrea virginica* exposed to cadmium sorbed to algal food and suspended clay particles
Abstract
Bivalve filtration, filtering suspended particles, has been used as an indicator to determine whether elements such as cadmium (Cd) sorbed to particles exposed to Cd-sorbed algae-sorbed to suspended clay particles were 15 and 25 ppb respectively exposed to Cd and to normal in each group. As expected, no 
particles showed a significa (0.96 Units) and exposed to 15 and 25 ppb respectively. Algal paste (0.48 ppb) different from control. No increase in mean Cd concentration (mg/d g DW)
Abstract

Bivalve filter feeders are important components of marine environments, filtering suspended matter and in the process accumulating contaminants. This makes them useful indicators of exposure to contaminants. Three studies were conducted to determine whether or not a stress protein response is elicited by the presence of cadmium (Cd) sorbed to algal food or suspended clay particles. In experiment 1, oysters were exposed to Cd-sorbed to algal paste. In experiment 2, oysters were exposed to Cd-sorbed to suspended clay particles. The exposure Cd concentrations in both experiments were 15 and 25 ppb (nominal). In experiment 3, two groups of oysters were concurrently exposed to Cd sorbed to algal paste or suspended clay particles (15 and 30 ppb nominal in each group). In experiment 1, a significant increase in mean HSP70 (P<0.001) was found in oysters exposed to Cd-sorbed to algal paste (0.48 ± 0.12 and 0.50 ± 0.10 Units in 15 ppb and 25 ppb respectively) compared to controls (0.31 ± 0.06 Units), but no dose dependency. In experiment 2, oysters exposed to Cd-sorbed to suspended clay particles showed no significant differences in mean HSP70 between control (0.43 ± 0.09 Units) and exposed groups (0.49 ± 0.14 and 0.55 ± 0.26 Units in 15 ppb and 25 ppb respectively). In experiment 3, oysters exposed to 15 and 30 ppb Cd sorbed to algal paste (0.96 ± 0.46 and 1.04 ± 0.54 Units respectively) were not statistically different from controls (0.78 ± 0.12 Units). Likewise, oysters exposed to 15 and 30 ppb Cd sorbed to suspended clay particles (0.84 ± 0.27 and 0.93 ± 0.24 Units respectively) showed no significant difference in mean HSP70 compared to controls. However, an increase in mean HSP70 levels with exposure was noted. In experiment 1, a significant difference in Cd concentration was found in oysters exposed to 25 ppb (6.18 ± 1.07 μg Cd/g DW).
compared to controls (3.18 ± 0.93 μg Cd / g DW, P<0.05). There was no significant
difference between oysters exposed to 15ppb (3.33 ± 0.99 μg Cd / g DW, P<0.05) and
controls. In experiment 2, significantly higher levels of Cd were present in gills of oysters
exposed to 15 ppb (26.43 ± 10.10 μg Cd / g DW, P<0.05) and 30 ppb (17.67 ± 6.15 μg
Cd / g DW, P=0.02) compared to the controls (6.25 ± 1.47 μg Cd / g DW). In experiment
3, oysters exposed to 30ppb Cd-sorbed to algal paste show a significant increase in Cd
concentration (10.37 ± 1.03 μg Cd / g DW, P<0.05) compared to the controls (5.69 ±
1.15 μg Cd / g DW), but not those exposed to 15ppb (7.37 ± 0.99 μg Cd / g DW). Oysters
exposed to Cd-sorbed to suspended clay particles show a significant increase in gills Cd
at 15ppb (14.30 ± 3.95 μg Cd / g DW, P<0.001) and 30ppb (7.79 ± 2.20) compared to the
controls (5.68 ± 1.15 μg Cd / g DW). The Cd concentration in the gills of control oysters
was considerably high (3.18 – 6.25ppm). Oysters seem to have a high tolerance for Cd
toxicity without eliciting a stress protein response.

Introduction

In the Chesapeake Bay, marked declines in oyster harvests have been experienced
since the 1950’s (Andrews, 1954) with particularly severe reductions in the 1990’s
(Burreson and Ragone, 1995). Factors that may contribute include disease,
overharvesting and degradation in water quality (Chu and LaPeyre, 1993; Ragone and
Burreson, 1993; Burreson and Ragone-Calvo, 1996; Chu and Hale, 1994). Oysters are
sedentary filter feeders and play an important role in the ecosystem. They filter water
trapping suspended matter and in the process accumulating contaminants present in the
suspended matter. Resuspension of particles and sediments back into the water column
might expose oysters and other filter feeding bivalves to contaminants long after they are not present in the water. Thus filter feeders such as oysters are exposed to contaminants not only through the water but also via filtrated and/or ingested matter, affecting bioaccumulation up the trophic levels and posing a health concern if consumed by humans.

Marine organisms, including bivalves, are known for their capacity to accumulate heavy metals (Janssen and Scholz, 1979; Fowler, 1979; Wikfors et al., 1994) and the accumulated metals can affect the organisms (Shuster & Pringle, 1969; Zaroogian and Morrison, 1981). Cadmium, a known pollutant of estuaries, is highly toxic and accumulates to high levels in bivalves (Carmichael and Fowler, 1980; Roesijadi and Unger, 1993). Since many bivalves are sedentary and accumulate metals, they are considered good indicators of metal pollution in the environment (Zaroogian, 1980; Farrington, et al., 1983; Landrum et al. 1991). Cadmium concentrations greater than 15ppb have been considered higher than concentrations encountered in chronically stressed natural waters (Zaroogian, 1980) and exposure to 40 – 60ppb Cd is considered to represent areas heavily contaminated (Hung, 1982).

The presence of cadmium in the water can affect oysters. For example, Zaroogian and Morrison (1981) found that oysters subjected to 5ppb Cd in the water had tissue concentrations of 91ppm after 37 weeks and some of the larvae produced by these oysters were abnormal. When oysters were exposed to 15ppb Cd, up to 24% of the larvae were abnormal. Oysters, (C. virginica) exposed to 100ppb Cd in solution accumulated up to
72ppm wet weight and died after 18 weeks (Shuster & Pringle, 1969). Low concentrations of cadmium in solution have been shown to have a direct effect on exposed organisms. LC50 values ranging from 2.1ppb to 47ppb have been reported in marine species including water fleas, (*Daphnia magna*), mysids, (*Neomysis integer*), rainbow trout, (*Onchorhyncus mykiss*), and copepods, (*Tigriopus brevicornis*) exposed to cadmium in solution (Forget, et al., 1998; Hollis et al., 2000; Knops et al., 2001; Roast, et al., 2001). These studies analyzed physiological responses such as growth (Knops et al., 2001), mortality (Forget et al., 1998), stamina and O₂ consumption (Hollis et al., 2000; Roast, 2001).

Heavy metals associated to suspended matter can enter the food web accumulating in the organisms’ tissues and having negative effects on bivalve filter feeders such as the oyster. Accumulation of Cd was noted in mussels, (*M. edulis*) fed algae, (*Dunaliella marina*) contaminated with 100ppb Cd (Janssen and Scholz, 1979). Algae reportedly can accumulate up to 70% of the Cd present in solution (Janssen and Scholz, 1979) and oysters, (*C. virginica*) can assimilate > 80% of the metal associated to the cytosolic fraction and 36% of the metal associated to membranes of ingested algae (Reinfelder, et al., 1997). Larvae of the oyster, (*C. virginica*) fed algae, (*Isochrysis galbana*) adapted to grow in media containing 15.3ppm Cd, had higher mortality than larvae exposed to 27ppb Cd dissolved directly into the water (Wikfors and Ukeles, 1982). Cadmium can also adsorb onto inorganic matter becoming available and possibly toxic to filter feeders. Pollet and Bendell-Young (1999) reported that mussels, (*M. trossulus*) exposed to 12.5 ppb Cd-sorbed to suspend sediments accumulated more Cd from
suspended sediments than from solution in water. Exposing *D. magna* to 90ppb or 60ppb Cd-sorbed to clay or sand respectively, caused greater than 50% mortality in 48 hours (Weltens, 2000). In the zebrafish, (*Danio rerio*) LT50 was reduced from 144 hr in fish exposed to cadmium in solution to 22 hr in fish exposed to cadmium sorbed to suspended particles (Van Den Belt et al., 2000).

The stress protein response has been proposed as a general indicator of exposure to stress (Sanders et al., 1993). Stress proteins are synthesized at higher levels when cells are challenged with certain environmental stimuli such as high temperature, heavy metals or toxic chemicals, making them a potentially useful marker of exposure (Morimoto, 1993; Kothary and Candido, 1982; Sanders, 1993). HSP70 accounts for much of the translational activity in cells responding to environmental stress and members of this family of proteins are inducible and highly conserved among various phyla (Margulis et al, 1989; Welch, 1990; Abukhalaf et al., 1994). The effects of metal exposure on the HSP70 have been tested in aquatic organisms exposed to contaminated water (Veldhuizen-Tsoerkan et al., 1990, 1991; Sanders et al., 1994; Ryan and Hightower, 1994; Werner and Nagel, 1997; Lawrence and Nichols, 1998; Tedengren, et al., 2000) or to settled sediments (Werner et al., 1998; Werner and Hinton, 1999). However, these studies have produced mixed results showing increases in the stress protein response (Ryan and Hightower, 1994; Lawrence and Nichols, 1998), minimum changes (Tedengren et al., 2000), no change (Veldhuizen-Tsoerkan et al., 1991) or upregulation and downregulation of the stress protein response (Werner and Nagel, 1997; Werner and Hinton, 1999).
The use of stress proteins is predicated on being more sensitive to sublethal exposure to contaminants than other end points such as scope for growth, feeding and respiration rates (Sanders, 1993; Steinert and Pickwell, 1993). The present study was conducted to determine whether a HSP70 response is elicited in the eastern oyster, \( C.\ virginica \) exposed to sublethal concentrations (15 – 30 ppb) of Cd sorbed to algal food or suspended clay particles. To my knowledge, no study has investigated the stress protein response in filter feeders exposed to sublethal levels of metals sorbed to suspended matter.

**Methods and Materials**

A) Clay preparation

Clay particles for the experiments were prepared by pulverizing green shale (Illite 46E0315, Wards/Cenco, Rochester, New York) to an average size of 50 µm. The clay particles were then hydrated in 1 µm filtered York River water (YRW) and stored at 4°C until use.

B) Algal paste

Algal paste, mixed in a 50:50 ratio of \textit{Tretaselmis} sp. and \textit{Isochrysis} sp., was purchased from Reed Mariculture Inc. (San José, CA). This mix was used as a diet and as a Cd exposure vehicle for the experimental oysters.

C) Preparation of Cd-contaminated algal paste and Cd-contaminated suspended clay particles.
The effects of algal paste or suspended clay particles spiked with Cd on the stress protein response were tested. A Cd (CdCl₂) stock solution was prepared to a final nominal concentration of 1ppm (actual concentration 0.94ppm) in water. Cd sorbed to algae was prepared each day by adding stock solution to algal paste to obtain nominal concentrations of 15 and 25ppb Cd. Cd-sorbed to clay particles was prepared by adding stock solution to hydrated clay particles to obtain nominal concentrations of 15ppb Cd (1g clay particles), and 25 or 30ppb Cd (2g clay particles). All oysters including controls were fed 0.20g algal paste / oyster daily. All treatments whether algal mix or clay particle suspensions were prepared by stirring separately the material for treatment groups and control groups into YRW prior to use.

Oysters collected from the Damariscotta River, Maine, an area rarely infected by the protozoan parasite, (Perkinsus marinus) were used in all the following described experiments. The effects of parasitic infection on the HSP70 response on invertebrate hosts are not known. There is no documentation on the alteration of HSP70 due to (P. marinus) infection on oysters. However, to avoid potential confounding effects due to the presence of the parasite, oysters were selected from a region without a reported incidence of (P. marinus). Oysters for the experiments 1, 2 and 3 were collected in summer (August) 1999, winter (February) 2000 and spring (March) 2001 respectively.

In all experiments, oysters were acclimated to local conditions, to a salinity of 12 - 18 ppt (York River salinity) and temperatures of 19°C - 21°C, over fourteen days in two 600 L tanks. After acclimation, subsamples of oysters (n=10) were examined for (P.
marinus) infection and the results were negative. At the end of every experiment all oysters were examined for (P. marinus).

**Experiment 1: Oysters exposed to Cd-sorbed to algal paste**

Acclimated oysters were separated into 3 groups each for the first two exposures experiments (i.e. Cd-sorbed to algal paste and Cd-sorbed to suspended clay particles). In Experiment 1, treatment groups were exposed daily to 15 and 25ppb Cd-sorbed to algal paste (actual concentrations 10.90 and 23.83ppb Cd / oyster / day) for 40 days. The control group was fed unspiked algal paste daily. Oysters were maintained in 2L containers with aeration. YRW was changed every other day. At the end of the experiment (40 days post-exposure), gills from individual oysters were excised and used for HSP70 analysis. Gills were selected because they are directly exposed to waterborne contaminants and particulates.

**Experiment 2: Oysters exposed to Cd-sorbed to suspended clay particles**

The experimental protocol is similar to the one described above except the treatment groups were exposed daily to 15 and 25ppb Cd / oyster (actual concentrations 15.69 and 33.58ppb Cd / oyster / day) sorbed to clay particles. After 40 days exposure oysters were analyzed for HSP70. In Chapter 2, data indicated that exposure up to 2g suspended clay particles alone did not affect the HSP70 response. Oysters were kept in individual 2L containers with aeration and fed 0.2 g algal paste daily. The aeration provided helped to keep the clay particles in suspension. YRW was changed every other
day. At the end of the experiment (40 days post-exposure), gills from individual oysters were excised and used for HSP70 analysis.

**Experiment 3: Concurrent exposures of oysters to Cd-contaminated algal food and Cd-contaminated suspended clay particles**

Subsequent to the experiments just described, another one was carried out exposing parallel groups of oysters to Cd sorbed to algal paste and Cd sorbed to suspended clay particles. Acclimated oysters were separated into 5 groups. Treatments groups (4) were exposed daily to 15 or 30 ppb Cd / oyster (actual concentrations 17.27 and 35.65 ppb Cd / oyster daily) sorbed to algal paste, and to 15 or 30 ppb Cd / oyster (actual concentrations 14.57 and 29.34 ppb Cd / oyster daily) sorbed to suspended clay particles. All treatments were continued for 40 days after which gills were sampled for determination of HSP70 levels.

D) Heat-shock protein analysis

HSP70 in gill tissues was assessed by slot blot. Western blot and immunoassays have been used routinely to analyze stress proteins in target tissues and organisms. This technique has been used previously in HSP70 determinations comparing fishes from contaminated and relatively clean sites in southern California (Brown and Bay, 1999), oysters exposed to algae contaminated with PCBs (Cruz Rodríguez, et al., 2000) and a macroalga exposed to environmental stressors (Lewis, et al., 2001).
Oyster gill tissues were homogenized using a hand held blender (Ultraturrax T-25 Homogenizer) at 24,000 rpm for 30 seconds on ice in 2 ml of buffer (66 mM Tris pH 7.2, 3% Nonidet and 0.1 mM PMSF). The homogenate was centrifuged at 19,800xg on a fixed angle rotor for 30 minutes at 4°C and the supernatant collected. Total protein concentration was determined using Biorad DC Protein Assay (Lowry et al. 1951).

The blotting procedures consisted of directly applying and immobilizing 1.5 μg total protein per tested sample in triplicates onto nitrocellulose (0.45 μm). The “reference sample” gradient (0.25, 0.50, 1.00, 1.50, 2.00 and 2.50 μg protein) was loaded in every blot to adjust for interblot variability. The 1.50 μg dilution in each series was used for data normalization. The blot was blocked with 5% non-fat dry milk in TTBS (0.05% Tween-20, 500 mM NaCl, 15 mM Tris, pH 7.5) for 30 minutes, followed by two washes in TBS (500 mM NaCl, 15 mM Tris, pH 7.5) for 10 minutes. Antibody dilutions (1:5000 primary antibody and 1:1000 secondary antibody) used were such that the quantity of antigen, not antibody, was limiting. Primary monoclonal antibody against HSP70 (Affinity Bioreagents, MA3-006) was applied for 90 minutes, followed by one wash with TTBS and two washes with TBS for 10 minutes each. The secondary antibody (Goat anti-mouse AP-conjugated) was applied for 90 minutes. Subsequently, the blot was washed twice with TBS for 10 minutes, then placed in a developing solution containing NBT (p-nitroblue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolyl phosphate). Bands started to develop after 30 minutes and development was completed after three hours. The blot was then stored in deionized water until densitometric analysis.
Densitometric analysis was performed by scanning the blots using SepraScan software (ISS Enprotech, MA, USA). The areas of the samples were recorded and each sample area normalized against the area of the 1.5 μg dilution from the dilution series loaded in each blot. Arbitrary units of HSP70, expressed as Units HSP70, were defined as the normalized values divided by 1.5.

\[
\text{Normalized}_{\text{area}} = \frac{\text{Sample}_{\text{area}}}{\text{Reference}_{\text{area}}} \quad \text{Units Hsp70} = \frac{\text{Normalized}_{\text{area}}}{1.5}
\]

E) Cadmium analysis

Samples were freeze-dried and weighted. Cadmium extractions were performed in a microwave accelerated reaction system (MARS 5, CEM corporation, NC). Samples were placed into reaction vessels with 10mL ultra pure nitric acid. Digestion took place at 200°C and 100psi for 1 hour in a closed vessel digestion system followed by a cool down period over two hours.

After extraction samples were brought to 25mL final volume in water and analyzed using a PerkinElmer atomic absorption spectrophotometer model AA800 with Zeeman correction in a graphite furnace (GFAA). The wavelength was 228.8nm, 0.7 slit width. The injector temperature was 20°C, initial furnace temperature of 110°C with maximum of 2450°C and internal gas flow of 250mL/min. Data were collected and quantified using PerkinElmer AAWinLab software.
Results

In general, increases in mean HSP70 with treatment were observed (Fig. 13A and B), but the increases were not always significant. In Experiment 1 oysters exposed to cadmium sorbed to algal food showed a significant increase in mean HSP70 of exposed groups (0.48 ± 0.12 and 0.50 ± 0.10 Units in 15 and 25 ppb respectively) compared to the controls (0.31 ± 0.06 Units) (Fig. 13A, ANOVA F=11.54, p<0.001). However the increase was not dose dependent. In Experiment 2 there was an increase in mean HSP70 levels in oysters exposed to cadmium sorbed to suspended clay particles (0.49 ± 0.14 and 0.55 ± 0.26 Units in 15 and 25 ppb respectively), but the same was statistically insignificant compared to the controls (0.43 ± 0.09 Units) (Fig. 13B). In both experiments, cadmium sorbed to algae or suspended clay particles, the mean HSP70 of exposed groups reached similar levels (Fig. 13A and B). The only difference between the two experiments is that the HSP70 of the control oysters in Experiment 2 was higher than the control oysters for Experiment 1(0.43 ± 0.09 and 0.31 ± 0.06 Units respectively). In Experiment 3, the mean HSP70 levels in the algae exposed groups were slightly higher (0.96 ± 0.46 and 1.04 ± 0.54 Units, 15ppb and 30ppb respectively) than the oysters exposed to suspended clay particles (0.84 ± 0.264 and 0.93 ± 0.24 Units, 15ppb and 30ppb respectively). Although there was an increase in the mean HSP70 levels with treatments, the increase was statistically insignificant compared to control (0.78 ± 0.12 Units) (Fig. 14).

In Experiment 1, a significant increase (P<0.05) in accumulated Cd in gills of oysters exposed to 25ppb (6.18ppm ± 1.07) compared to the controls (3.184ppm ± 0.926)
was noted. Oysters exposed to 15ppb (3.33ppm ± 0.99) were not different from controls (Fig. 15). In Experiment 2 oysters exposed to 15 and 25ppb cadmium sorbed to suspended clay particles had a significant increase of Cd in gills (26.43ppm ± 10.10 and 17.67 ppm ± 6.15) compared to the controls (6.25ppm ± 1.47) (Fig. 15). The concentration of Cd in the oysters exposed to 25ppb sorbed to the clay particles showed lower values compared to oysters exposed to 15ppb, although this is statistically insignificant (Fig. 13). In Experiment 3 oysters exposed to 30ppb Cd-sorbed to algal paste showed a significant increase in Cd (10.37ppm ± 1.03, P<0.05) compared to controls (5.68ppm ± 1.15). There was no difference between oysters exposed to 15ppb Cd (7.37ppm ± 0.99) and controls (Fig. 16). Oysters exposed to 15ppb Cd-sorbed to suspended clay particles showed a significant increase in accumulated Cd (14.30ppm ± 3.95, P<0.05) in the gills compared to the controls (Fig. 16). Oysters exposed to 30ppb (7.79ppm ± 2.20) did not show a significant difference to the controls (Fig. 16). The comparatively lower accumulation of Cd in oysters exposed to the highest dose of suspended contaminated clay particle was observed in both, experiments 2 and 3. In all of the experiments, control oysters showed cadmium present in their tissues with values between 3.18ppm ± 0.93 and 6.25ppm ± 1.47 (Figs. 14 and 16).

Discussion

The stress protein response in aquatic organisms such as fish, echinoderms, and bivalves has been described following exposure to contaminants in solution (Randall, Lee and Sanders, 1989; Veldhuizen-Tsoerkan, et al., 1990, 1991; Sanders et al.1994; Sanders and Martin, 1994; Werner and Nagel 1997; Lawrence and Nichols, 1998) or associated to
settled sediments (Werner et al. 1998; Werner and Hinton, 1999). Sanders et al. (1994) demonstrated increases in chaperonin 60 (Cpn60) and HSP70 in mussels exposed to Cu$^{2+}$. Induction in the levels of HSP70 and HSP60 was reported in amphipods, (Hyalella azteca, Ampelisca abdita and Rhepoxynius abronius) exposed to solutions containing cadmium, diazinon, dieldrin or fluoranthene (Wemer and Nagel, 1997). Lawrence and Nichols (1998) observed induction in the synthesis of 68 – 72 kDa stress proteins in mussels, (M. edulis) exposed to power plant effluents containing 10 – 70ppb chlorine. In chapter 2 of this dissertation, exposing eastern oysters to suspended clay particles spiked in the laboratory with PAHs or to suspended field contaminated sediments (SFCS) elicited a HSP70 response. In the current studies however, clear changes in HSP70 levels in oysters exposed to Cd sorbed to suspended matter (i.e. algae or clay particles) were not observed compared to the controls in experiments 2 and 3. It is possible the concentrations used in these studies might not have been high enough to cause changes in stress protein levels. In the Asian clam, (Potamocorbula amurensis) exposure to Cd concentrations up to 40ppb in the water did not elicit changes in HSP70 levels (I. Werner, University of California, Davis, personal communication). In the present study a pattern of increase in mean HSP70 with exposure and a significant increase in oysters exposed to Cd sorbed to algae in experiment 1 suggest possible and potential effects.

Variations in the mean HSP70 levels observed in oysters between experiments may be due to the oysters' been collected in different seasons and different years. For experiments 1 and 2, control oysters may be reflecting seasonal variability (increase or decrease) in mean HSP70 levels, while the exposed groups were showing a relative
constant level of stress protein due to the exposure, thus affecting the significance of the analysis. Differences in the HSP70 levels between years might also reflect seasonal influences affecting the stress protein response. For example, Weber and Janz (2001) showed differences in mean HSP70 levels in the ovaries of channel catfish, \textit{(Ictalurus punctatus)} in two different seasons. Fishes collected in summer had significantly lower levels than spring individuals. In oysters, the effects of factors such as hormonal changes and reproductive status, energy status and metabolic rates on the stress protein levels are not known and these factors show seasonal variability (Giesy and Pearse, 1974; Bayne, 1976; Giesy and Kanatani, 1987; Deslous-Paoli and Héral, 1988). Since the oysters in the different experiments were not similar in these parameters, the results of the analysis could have been confounded by any of these factors. Seasonal and year-to-year variations in stress proteins might add another variable to consider when assessing the impact of chronic metal pollution in aquatic environments.

Metals associated with particles have been shown to affect survival, growth, and weight in aquatic organisms. Wikfors et al. (1994) demonstrated significant reductions in weight of juvenile oysters \textit{(C. virginica)} exposed to a diet consisting of \textit{(Dunaliella tertiolecta)}, or a 50:50 mix of \textit{(D. tertiolecta)} and \textit{(Phaeodactylum tricornutum)} raised in media containing 60ppm Cd$^{2+}$. The zebrafish, \textit{(Danio rerio)}, showed increased mortality when exposed to Cd-contaminated clay particles (1.28 mg Cd/g clay). An LT50 of 92 hours was calculated for fish exposed to 1,000 mg/L Cd-contaminated sediments, whereas the LT50 was 22 hours when exposed to 2,000 mg/L Cd-contaminated sediments (Peeters, et al., 2000). Similarly, Erickson et al. (1996) showed that the
presence of Cu-contaminated clay particles reduced the 96hr-LC50 of exposed fathead minnows (*Pimephales promelas*), from 33.0 nM to 12.6 nM. The use of stress proteins as biomarkers has been proposed on the premise that they are more sensitive than other biomarkers (Sanders, 1993; Steinert and Pickwell, 1993; Ryan and Hightower, 1994). In the present studies however, it was not possible to observe a consisted change in mean HSP70 levels at the contaminant concentrations used, although some low level effect is suggested by a pattern of HSP70 increase with exposure.

Despite the effects demonstrated by heavy metals reported in the literature, bivalves seem capable of coping with exposure and accumulation of trace metals. Shuster and Pringle (1969) and Engle (1999), presented data indicating death occurring at elevated concentrations (>100ppm wet weight) in the tissues of oysters exposed to Cd in the water. Jana and Das (1997) upon exposure to Cd concentrations of 10, 20, and 30ppm in solution for 6 weeks showed accumulation of 165, 220 and 445ppm respectively with only reductions in body weight as implication of physiological stress in the freshwater bivalve, (*Lamellidens marginalis*). Long-term exposure (11 months) to 16.5ppb Cd dissolved in the water did not cause changes in HSP70 levels, condition index and adenylate energy charge in mussels, (*M. edulis*) (Veldhuizen-Tsoerkan et al., 1990; 1991). After a heat shock to 29.5°C for 4 hours, mussels exposed to Cd showed an enhanced HSP70 expression compared to heat-shocked control animals. However, in the present study no change in mean HSP70 levels of oysters exposed to Cd-sorbed suspend matter was observed.
The presence of the metal binding proteins, metallothionein (Mt) or metallothionein-like proteins and specialized cells (brown cells, amebocytes) might help explain the tolerance of oysters and other bivalve filter feeders to metal toxicity. A series of studies by Ruddell and Rains (1975), Carmichael and Fowler (1981), Zaroogian and Yevich (1994), and Engel (1999) have provided evidence on a multiple system to help coping with metal inputs in bivalves. One mechanism for the control of Cu and Zn in oysters, \textit{(C. gigas and C. virginica)} is the formation of intracellular inclusions in amebocytes and subsequent excretion of the metals by diapedesis (Ruddell and Rains, 1975). Zaroogian and Yevich (1994) described the presence of brown cells with the capacity to sequester metals (Cd and Ni) in the oyster, \textit{(C. virginica)}. The accumulation of metals in these cells appears to be a passive transport process. Carmichael and Fowler (1981) suggested that extrusion of calcium-phosphate concretions from epithelial cells of the kidney could be involved in metal depuration and thus advantageous in tolerating marine pollution from metals in the bay scallop, \textit{(Argopecten irradians)}. The extruded calcium-phosphate concretions are enriched in metals, and the authors suggested these concretions act as a vehicle to dispose of the excess metals. Casterline and Yip (1975), Engle and Brower (1982), and Engle (1999) have presented evidence on the role of cytosolic low molecular weight proteins (Mt) in metal binding (Cu, Cd, Hg and Zn) in bivalves. In the oyster, Mt's are proteins between 7 – 24 kDa rich in cysteine residues (~30%) induced by exposure to metals (Engle and Brower, 1982; Engle, 1999). In bivalves the effective sequestration and removal of metals by the different mechanisms thus described might prevent further interactions of the metals with protein components in the cytosol or other structures. This might prevent stimulation of a stress protein.
response mediated through protein denaturation at least at the concentrations used in this study.

Cadmium, a known pollutant in the marine environment, is accumulated by bivalve filter feeders (Janssen and Scholz, 1979; Zaroogian, 1980; Farag, et al., 1998; Mouneyrac et al, 1998; Barak, et al., 1999; Tedengren, et al., 2000). Zaroogian (1980) reported accumulation up to 292ppm Cd in adult oysters (C. virginica) exposed to 5, 10 and 15ppb Cd for 40 weeks in flowing seawater. Barak et al (1999) described accumulation of trace metals including Cd in three bivalve species, (Mactra corallina, Donax sp and M. edulis), and two gastropod species, (Patella sp and Cellana rota), along the Mediterranean, Red and North Seas. The higher concentrations observed in (Patella sp) and (Donax sp) from Haifa Bay (Israel) were attributed to Cd enrichment in the suspended particulate matter, although no concentration value was reported for the particulate matter. Farag et al (1998) recorded accumulation of trace metals including Arsenic, Cd, Cu, Pb, Hg, and Zn in benthic invertebrates and fish collected from a river downstream from mining operations in Coeur d’Alene, Idaho. Tedengren et al. (2000) have shown uptake up to approximately 24ppm Cd in mussels, (M. edulis) exposed to 20ppb Cd. Janssen and Scholz (1979) showed that algae, (D. marina) sorbed about 70% of the Cd spiked into the media and metal accumulated in mussels (M. edulis) fed the algae. Similarly, in the current study the presence of Cd in oyster gills exposed to the contaminated alga and suspended clay particles was observed. However, control oysters showed Cd concentrations between 3.184 – 6.247ppm in all the studies. These values are similar to those reported by Tedengren, et al. (2000) of 4ppm – 5ppm in mussels kept in seawater tanks. In the eastern oyster values ranging from 0.39 – 13 μg / g tissue dry
weight of Cd have been reported from relatively uncontaminated sites to contaminated sites (NOAA, 1987).

While oysters exposed to suspended clay particles in Experiment 2 showed higher levels of Cd than oysters exposed to the contaminated algae did in Experiment 1, this trend of differential accumulation with exposure treatments was not observed in Experiment 3. These experiments were performed in different times of the year and in different years. It is not certain whether the differences in Cd concentration in gill tissues were because the studied oysters were collected in different times of the year and different years. Seasonal differences in tissue Cd concentrations have been reported in the oyster (Frazier, 1975; Zaroogian, 1980; Páez-Osuna et al., 1995). Frazier (1975) and Zaroogian (1980) observed decreases in Cd tissue concentration in August subsequent to a period of increased accumulation in oysters (*C. virginica*). Páez-Osuna et al (1995) reported the lowest concentrations of various trace metals including Cd when the gonad of oysters (*C. irridecens*), exhibited maximum seasonal development. These authors speculate that perhaps the differences in Cd tissue concentrations were the result of seasonal changes in body weight.

In the current studies, decreases in Cd accumulation at the highest exposure concentrations (i.e. 25ppb and 30ppb) were observed in oysters exposed to Cd-sorbed to suspended clay particles in experiments 2 and 3, but not in oysters exposed to contaminated algal food in experiments 1 and 3. The lower Cd concentration observed at the highest treatments was unexpected. However depuration is not believed to be
involved due to the slow loss and long half-life of Cd in bivalves' tissues (Engle, 1999; Okazaki and Panietz, 1981; Greig and Wenzlo, 1978; George and Coombs, 1977). Data from Engel (1999), George and Coombs (1977), Okazaki and Panietz (1981) and Greig and Wenzlo (1978) suggest that bivalves do not have an effective depuration capacity for cadmium, copper or zinc. Oysters with high concentrations of accumulated metals lost virtually none of the accumulated metal after 22 weeks of depuration in uncontaminated water (Greig and Wenzlo, 1978). Okazaki and Panietz (1981) estimated that (*C. virginica*) depurated Cd from digestive gland and kidney with a half-life of approximately 76 days. Engel (1999) showed that the eastern oyster did not lose significant amounts of Cd after 28 days of depuration following 28 days exposure to 100ppb Cd. George and Coombs (1977) reported that in exposed mussels the loss rate for Cd was 18 times slower than the rate of accumulation. In filter feeding bivalves, increases in suspended particles can cause a decrease in pumping and clearance rates, and increases in rejection through the production of pseudofeces (Loosanoff, 1962; Loosanoff and Tomers, 1948; Sobral and Widdows, 2000; Hawkins et al., 2001). In the scallop, (*Chlamys farreri*), as the volume of suspended material increases, the digestive cavity fills with the material increasing rejection as pseudofeces and or reducing the clearance rate (Hawkins, 2001). Sobral and Widdows (2000) showed that high suspended particulate matter (silt and alga) causes declining clearance rates in the infaunal clam, (*Ruditapes decussatus*) by overloading of the gill feeding mechanism. In the current study, a decrease in Cd accumulation in gill tissues was observed in oysters exposed to suspended clay particles and food, not in oysters exposed to food particles alone. One possible explanation for the decrease observed at 25 and 30ppb Cd in 2g suspended clay
particles could be that a higher input of suspended material (suspended clay particles and algal food) caused reductions in pumping rate or increased rejection of material, thus reducing the accumulation of Cd by the exposed oysters. Since no calculations were taken on clearance rate or pseudofeces production, the studies reported here cannot conclusively clarify the causes of the observed decrease.

In summary exposure to cadmium sorbed to algal paste or suspended clay particles generally did not cause a significant increase in the HSP70 levels in oysters at the exposure concentrations presently used. Although a pattern of increase in mean HSP70 with exposure and a significant increase in oysters exposed to Cd sorbed to algae in experiment 1 suggest possible and potential effects. In bivalves mechanisms other than the stress proteins may have more relevance in metal toxicity. The effective sequestration and removal of metals from the cell’s cytosol might prevent further interactions with protein components or other structures, thus preventing stimulation of a stress protein response in chronically stressed environments.
References


Chu, F.-L.E. and Hale, R.C. 1994. Relationship between pollution and susceptibility to


Figure 13: HSP70 levels in gill tissues of oyster exposed to: (A) Cd-sorbed to algal paste (Experiment 1) and (B) Cd-sorbed to suspended clay particles (Experiment 2). Oysters were exposed to 15 and 25ppb Cd / oyster / day (Mean ± 95% confidence interval, n=8-10). Different letters represent significant difference (p<0.05).
**Figure 14:** HSP70 levels in gill tissues of oysters exposed to Cd-sorbed to algal paste and to suspended clay particles (Experiment 3). Oysters exposed to 15 and 30 ppb Cd/oyster/day. (Mean ± 95% confidence interval, n=8-10).
Figure 15: Cadmium accumulation in gill tissues of oysters exposed to Cd sorbed to algal paste (Experiment 1) or suspended clay particles (Experiment 2). Oysters were exposed to 15 or 25 ppb Cd/oyster/day (Mean ± SD, n=6). Different letters represent significant difference (p<0.05).
The diagram shows the concentration of cadmium uptake in tissues (µg of cadmium per gram of dry weight) for different treatments:

- **Control**:
  - Clay particles: A
  - Algae: C

- **15 ppb Treatment**:
  - Clay particles: B
  - Algae: C

- **25 ppb Treatment**:
  - Clay particles: B
  - Algae: D

The data indicates higher cadmium uptake in Algae compared to Clay particles across all treatments.
Figure 16: Cadmium accumulation in gill tissues of oysters exposed to Cd sorbed to algal paste or suspended clay particles (Experiment 3). Oysters exposed to 15 or 30 ppb Cd/oyster/day. (Mean ± SD, n=6). Different letters represent significant difference (p<0.05).
Chapter 5

Variation in the levels of stress protein (HSP70 family) under natural conditions in the eastern oyster, *Crassostrea virginica* (Gmelin) from the lower Chesapeake Bay
Abstract

Environmental fluctuations associated with seasonal changes are of major importance in establishing adjustments in the physiology and behavior of aquatic organisms. This study examined the HSP70 levels in a winter, summer, and fall months in the eastern oyster, *Crassostrea virginica*. Oysters were collected from Point of Shoals in the James River, VA, in November of 1996, January, March, and August of 1997, in May, August and October of 1999, and in January, March, and August of 2001. Gills were excised and analyzed for HSP70. Condition index (CI) and prevalence of infection by the oyster parasite, *Perkinsus marinus* were assayed for the 1999 and 2001 samples. Regression analysis indicated that temperature exerted the largest influence on HSP70 levels in 1996-97 and 1999 samples (p<0.001, \( r^2 = 0.56 \) and p=0.004, \( r^2 = 0.30 \) respectively). HSP70 levels were higher in lower temperature months compared to higher temperature months. For 2001 a pattern of reduced HSP70 with increase in temperature was observed but was not statistically significant. Salinity seemingly had no effects on HSP70 levels. Infection prevalence by the oyster parasite, *P. marinus* closely followed changes in water temperature in 1999 and 2001. The condition index showed a pattern that is inverse to the prevalence of infection and temperature, with the highest value in May, decreasing in August and October (lowest) of 1999. In the eastern oyster, variation in the stress protein (HSP70) levels appears to be affected by seasonal cycles, as is the case in other physiological parameters.
Introduction

Environmental fluctuations associated with seasonal changes are of major importance in establishing adjustments in the physiology and behavior of aquatic organisms (Dickson and Giesy, 1981). Seasonal changes in physical and chemical parameters (e.g. temperature, salinity, and photoperiod) can have important effects in bivalve physiology. Temperature and photoperiod are described as predominant cyclical factors in the natural environment and equally important as an influence on the organisms (Whiteley et al., 1997). The reproductive cycle of the eastern oyster shows a strong coupling with season and temperature (Giesy and Pearse, 1974; Sastry, 1975). Oysters typically start to accumulate glycogen in winter and spring and use it to fuel gamete development during the summer (Chipman, 1948; Engle, 1951). Lipids increase at the expense of glycogen during the reproductive cycle (Chu et al., 1990) and decrease during spawning. In the Chesapeake Bay spawning can extends from June to late September (Andrews, 1979). Depending on energy availability and temperature, the species can spawn multiple times during a season (Hayes and Menzel, 1981).

Stress proteins comprise a group of protein families that help to stabilize and/or unfold proteins, disaggregate protein aggregations, and translocate newly synthesized proteins across membranes and through the cytosol (Nover, 1990; Gething and Sambrook, 1992; Creighton, 1993; Morimoto, et al., 1994). Generally these proteins help to maintain cellular homeostasis. HSP70 is inducible and highly conserved among various phyla including marine invertebrates such as mussels, (Mytilus edulis) (Sanders et al., 1993), oysters, (Crassostrea gigas and C. virginica) (Clegg et al., 1998; Tirard et al.,
1995) and echinoderms, (*Strongylocentrotus purpuratus*) (Stegeman et al. 1992; Sanders, B., 1994). Heat shock proteins have been proposed as indicators of exposure to contaminants in the environment (Sanders, 1990; Morimoto, 1993). The stress proteins are synthesized at higher levels when cells are challenged with certain environmental stimuli such as high temperature, PAHs, heavy metals, mixtures of contaminants and other toxic chemicals, making them a potentially useful marker of exposure (Morimoto, 1993; Sanders, 1993 Monaghan and Bradley, 1993; Ryan and Hightower, 1994; Sanders et al., 1994; Werner et al., 1998; Werner and Hinton, 1999).

Bivalve molluscs have been used as bioindicators of chemical contamination and biological effect (Farrington, et al., 1983; Sheehan and Power, 1999). For stress proteins to be useful as a biomarker, one must be able to distinguish between natural variations and responses to other stressors. In bivalves levels of important biomarkers may fluctuate throughout a year depending on nutrient availability and reproductive status (Sheehan and Powers, 1999). Studies have shown variations in the levels of biomarkers associated to seasonal events not necessarily to the presence of contaminants. In the winter flounder, (*Pseudopleuronectes americanus*) high levels in the antifreeze mRNA (AF mRNA) was expressed in fall and winter with low levels in summer. Declines in mixed function oxygenase (MFO) system enzymes in the mussel, (*M. edulis*) (Kirchin et al., 1992) and the eastern oyster, *C. virginica* (Weinstein, 1995), and increases in metallothionein in the Asian clam, (*Corbicula fluminea*) (Baudrimont et al., 1997) coinciding with late seasonal reproduction events unrelated to any exposure to contaminants have been published.
Thus, changes in a biomarker level might simply represent cyclic physiological changes unrelated to contamination (Sheehan and Powers, 1999).

Studies in fishes (Dietz and Somero, 1992; Fader et al., 1994) and in mussels, (*M. trossulus, M. californianus* and *M. galloprovincialis*) have given support to the concept that variation in the stress protein levels can be caused by seasonal events (Hoffman and Somero, 1995; Roberts et al., 1997; Minier et al., 2000). Generally, individuals collected during summer months showed higher levels of stress proteins compared to those collected during winter months. In contrast, Weber and Janz (2001) reported significantly lower levels in mean HSP70 levels in ovaries of juvenile channel catfish, (*Ictalurus punctatus*) in summer than in spring. In a study by Fader et al. (1994) with freshwater fishes (*Pimephales promelas, Salmo trutta, Ictalurus natalis* and *Ambloplites rupestris*) mean HSP70 levels were higher in January with subsequent decreases in May, August and October.

The present study examined possible variations in HSP70 levels in the eastern oyster, (*C. virginica*) collected from Point of Shoals in the James River, Virginia. Disease prevalence by the apicomplexan parasite, (*Perkinsus marinus*) and condition index (CI) was also assayed in the 1999 and 2001 samples. This parasite causes periodic epizootics in the Chesapeake Bay producing mass mortalities in the eastern oyster. Reductions in the CI in association with infection in oysters have been reported (Volety, 1995). This chapter reports the results of (HSP70) analysis in oysters sampled from the James River under natural environmental conditions.
Methods and Materials

A) Collection

Oysters were collected from Point of Shoals in the James River, Virginia. Collections were made in November (11/96), January (14/97), March (14/97) and August (12/97), in May 18/99, August 17/99, October (19/99), and in January (13/01), March (12/01) and August (15/01). Temperature and salinity were recorded \textit{in situ}. Oysters were kept in water with aeration until processed for stress protein analysis. Gill tissues from sampled oysters were analyzed for HSP70.

B) Heat shock protein analysis

Gills were dissected and levels of HSP70 determined. Oyster gills are involved in gas exchange and food collection and is the first tissue exposed to water borne particles and contaminants. Gills were homogenized using a hand held blender (Ultraturrax T-25 Homogenizer) at 24000 rpm for 30 seconds, on ice in 2 mL of buffer (66 mM Tris pH 7.2, 3% Nonidet, 0.1 mM PMSF). The homogenate was centrifuged at 19800 x g on a fixed angle rotor for 30 minutes at 4°C, and the supernatant collected. Soluble protein concentration in the collected supernatant was determined using Biorad DC Protein Assay (Lowry et al., 1951).

HSP70 in gill tissues was assessed by slot blot. Slot blot technique has been previously used in studies comparing fishes from a contaminated and a clean site in southern California (Brown and Bay, 1999), in oysters exposed to PCBs-sorbed to algal paste (Cruz Rodríguez et al., 2000), and in the macroalga \textit{Enteromorpha intestinalis}
exposed to contaminants (Lewis et al., 2001). Briefly, the blotting procedures consisted in directly applying and immobilizing 1.5μg protein per tested sample in triplicates onto the nitrocellulose paper (0.45μm). A “reference sample” gradient (0.25, 0.50, 1.00, 1.50, 2.00, 2.50 μg protein) was loaded in each blot as a measure to adjust for interblot variability. The 1.50 μg dilution was used for data normalization. The blot was blocked with 5% non-fat dry milk in TTBS (0.05% Tween-20, 30 mM NaCl, 24 mM Tris pH 7.5) for 30 minutes and washed two times with TBS (30 mM NaCl, 24 mM Tris pH 7.5) for 10 minutes. Primary monoclonal antibody against HSP70 (Affinity Bioreagents MA3-006) was applied for 90 minutes, followed by a wash with TTBS and two washes with TBS 10 minutes each.

The secondary antibody (Goat anti-mouse AP conjugated) was applied for 90 minutes, the blot was washed twice with TBS, and placed in a developing solution containing NBT (p-nitroblue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolyl phosphate). The blot is stored in deionized water until densitometric analysis. Densitometric analysis was performed by scanning the blots using SepraScan software (ISS Enprotech, MA, U.S.A.). The areas of the samples were recorded and each sample area normalized against the area of the 1.5μg dilution from the dilution series loaded in each blot. Arbitrary Units of heat-shock protein, expressed as Units HSP70, were defined as the normalized values divided by 1.5. Results are expressed as mean ± CI (95% confidence interval).

C) Prevalence
Prevalence of the oyster parasite, \(P.\ marinus\) was diagnosed using the assay described by Ray (1952). Pieces of rectal and mantle tissue were dissected and incubated in Fluid Thioglycollate Medium (FTM) for 7 days. After the incubation period the pieces of tissue were stained with Lugol's Iodine solution and the presence of the parasite was ascertained. Results are expressed as the percent of the oysters found infected in each sampling.

D) Condition Index

The condition index (CI) is a measure of the growth condition of the organism. Condition index was calculated as tissue dry weight divided by shell dry weight multiplied by 100 (Lucas and Beninger, 1985). Results are expressed as mean ± SD.

E) Statistical analysis

Regression analysis was used to test which variable(s) affect the stress protein levels and the CI. Tukey HSD was used to test for difference among the means in stress protein expression and in CI (SAS Institute, Cary, NC).

Results

HSP70 in oysters sampled in November (2.76 ± 0.46 Units) 1996, January (2.79 ± 0.34 Units) and March (2.02 ± 0.48 Units) 1997 showed no significant difference, however, oysters sampled in March had the lowest mean levels of the three sampled months. August HSP70 levels (1.42 ± 0.24 Units) were significantly lower (\(F=4.00, P=0.01\)) than the HSP70 recorded in November and January, but not March (Fig. 17A).
HSP70 levels in oysters collected during the months of May (0.70 ± 0.19 Units), August (0.72 ± 0.19 Units) and October (0.89 ± 0.20 Units) 1999 showed no statistically significant differences (Fig. 17B). However an increase in mean HSP70 was noted in October (Fig. 17B). The HSP70 in samples collected in January (1.19 ± 0.17 Units), March (1.16 ± 0.24 Units) and August (1.00 ± 0.18 Units) 2001 showed no statistically significant difference (Fig. 17C). However, the oysters collected in August showed a pattern the lowest mean HSP70 (Fig. 17C). A pattern of increasing values in mean HSP70 during cold temperature months compared to hot temperature months was noted in the different years sampled. However, differences were noticed in the mean HSP70 between different years. Samples collected in 1996-1997 had significantly higher mean HSP70 values compared to those from samples collected in 1999 and 2001 (F=53.17, P<0.05).

Generally, temperature values recorded in the sampled months showed the natural variations expected of this environmental factor in a year. Of all the sampled months, the lowest temperature recorded was in the month of January 1997 and January 2001 (5°C and 5.5°C respectively). The highest temperatures occurred in August (28°C, 1997; 30°C, 1999; 28°C; 2001). The temperature in the month of March ranged from 8°C (2001) to 13°C (1997). During warm months temperature recorded were 19°C (October 1999) and 21°C (May, 1999). In November 1996 the temperature was not recorded, but the ten years average is around 13°C (Ragone-Calvo, L and Burreson, E. 1997; Ragone-Calvo, L and Burreson, E. 1998; Ragone-Calvo, L. and Burreson, E. 2000) The recorded salinity values of the sampled dates ranged from 0 to 14 ppt. Salinity values were 5, 5, 0, 11, 12,
14, 6, 12, 8, and 11 ppt in November 1996, January, March and August 1997, May, August and October 1999, January, March and August 2001, respectively. Prevalence (%) of oysters infected by the oyster parasite, \( P. marinus \), showed low levels in May (30%), peaked at its highest levels in August (100%) and then declined in October (88%) 1999 (Fig. 18). Prevalence of infection in 2001 shows an unusually high percentage of oysters infected in January (44%), a decrease in March (8%) followed by an increase in August (80%). The condition index (mean ± SD) in the oysters decreased from May (3.24 ± 1.03) through August (2.02 ± 0.73) and was significantly low in October (0.81 ± 0.21, p<0.05) in 1999 (Fig. 19A). In 2001, the condition index recorded shows significant increases \( (F=53.14, p<0.05) \) from January (2.29 ± 0.55) to March (5.28 ± 0.97) and then decrease in August (2.39 ± 0.62) (Fig. 19B).

Regression analysis of samples from 1996-1997 showed a correlation between HSP70 levels and temperature \( (F=24.19, p<0.05, r^2=0.56) \). A regression model for the 1999 samples including temperature, salinity and disease prevalence was also significant \( (F=6.68, p<0.05, r^2=0.30) \). It showed again temperature \( (p<0.05) \) as the main factor influencing the HSP70 levels in these oysters. Salinity \( (p=0.85) \) showed no influence on the HSP70 levels and the infection prevalence \( (p=0.08) \) showed some influence. Temperature and infection prevalence are highly correlated and the contributions from each one are not easily separated. The regression model for the samples of January, March and August 2001 showed no statistically significant difference in HSP70 \( (r^2=0.35, p=0.38) \).
For the condition index a regression model including temperature, salinity and infection prevalence showed that temperature ($p<0.001$) was the factor with the largest influence. The model was highly significant ($F=21.65$, $p<0.001$, $r^2=0.58$). Salinity ($p=0.15$) had no influence on the CI.

**Discussion**

Few studies have attempted to characterize the HSP70 levels in relation to environmental temperature and salinity in field organisms. In the mussel, (*M. californianus*) Roberts et al. (1997) observed higher mean HSP70 levels during summer (June and August) compared to winter (February) for individuals collected in the high intertidal zone. These authors proposed that high levels in HSP70 might provide the mussels with an “anticipatory protection” against the likelihood of increased thermal stress in the summer months. Dietz and Somero (1992) have suggested that variation in stress protein levels in goby fishes (genus *Gillichthys*) could be associated to the acclimatization conditions. Summer acclimatized fishes have fundamentally higher levels of stress protein (Hsp90) than winter acclimatized fishes. Likewise, in the mussel, (*M. trossulus*) higher endogenous levels of HSP70 in summer than in winter were reported (Hoffman and Somero, 1995). A study by Feder et al. (1994) followed the HSP70 levels in freshwater fishes (*Pimephales promelas, Salmo trutta, Ictalurus natalis* and *Ambloplites rupestris*) sampled in a winter (February), spring (May), summer (August) and fall (October) months. These authors showed a relationship between seasonal ambient water temperatures and HSP70 levels in fishes. HSP70 levels increased from winter to spring as temperatures increased. As temperatures continue to increase in
summer and fall however, HSP70 levels decreased. The authors suggested that changes in HSP70 might be more dependent upon the relative increase in temperature rather than upon the absolute temperature. The authors speculated that the high levels of HSP70 observed in the spring month “might have been providing protection against the continued raise in temperature.” Consequently no additional synthesis of HSP70 to higher levels was necessary with further water temperature increases. In other words, these authors were proponents of the “anticipatory response” in HSP70 in the studied fish. In the present study, variations in the HSP70 levels apparently related to ambient water temperature in the eastern oyster were observed. Higher mean HSP70 levels observed happened in months with lower ambient water temperature (January 1999, 2001, February 1997 and March 2001) rather than in warm water temperature months (May 1999 and August 1997, 1999, 2001). Other factors (e.g. periodic anoxic conditions, hormonal changes associated with reproduction, energy status, metabolic rates) in addition to the relative water temperature experienced by the oysters might also be involved in the stress protein level variations observed.

There are few studies reporting on the effects of salinity in the stress protein expression. In a study by González and Bradley (1994) changes in salinity affected the expression of proteins in the copepod, *(Eurytemora affinis)*. Proteins of 30 kDa and 37 – 39 kDa were newly synthesized in copepods exposed to salinities of 2, 5 or 20 ppt. Werner et al (2000) proposed that changes in salinity might have affected the HSP70 response in the Asian clam, *(Potamocorbula amurensis)*. Increases in mean HSP70 levels in clams were noticed as contaminant concentration (cadmium) in the samples decreased,
simultaneously with salinity increases. Under controlled laboratory conditions the authors have observed increases in HSP70 related to increases in salinity. In the present study, no relationship between salinity and variations in the HSP70 levels was observed in oysters sampled in spring, summer, fall or winter months. In 1999 salinity ranged from 6 ppt to 14 ppt and in 2000 ranged from 8 ppt to 12 ppt (Ragone-Calvo and Burreson, 1999, 2000). The changes in salinity experienced might not be sufficient to appreciably stress oysters beyond their compensatory capabilities or HSP70 might not be involved in osmoregulation.

The effects of parasitism by the oyster parasite, (P. marinus) on the HSP70 levels in the eastern oyster have not been investigated, thus are not known. Tirard et al (1995) reported that this parasite synthesized HSP70 at temperatures higher than their host, suggesting its capacity to function at temperatures that are stressful to the oyster. The observation of higher parasitic infection concomitantly with higher ambient temperatures has been well documented (Soniat, 1985; Fisher et al., 1992; Volety, 1995; Burreson and Ragone, 1996; Soniat, 1996). Because the infection prevalence is highly related to temperature (i.e. higher infection at higher temperatures) the present study cannot separate the effects of parasite infection from those of temperature on HSP70 levels.

Seasonal reproduction activities as well as metabolic demands due to temperature and diseases (Lee et al., 1960; Haven, 1962; Sakuda, 1966; Giesy and Pearse, 1974; Bayne, 1976) may alter the levels of biochemical components (glycogen, protein, lipids) (Giesy and Pearse, 1974; Bayne, 1976; Giesy and Kanatani, 1987; Volety, 1995) thus,
affecting the condition index (CI) in oysters. Decreases in CI observed in August (1999) and October (1999, 2001) might represent reproductive events in the oysters as this index is highly affected by the reproductive cycle. In the Chesapeake Bay reproduction season can extends from June to late September (Andrews, 1979). As seasons move on to winter the oyster begin to accumulate energy reserves and improve their condition (Chipman, 1948; Engle, 1951) reflected as increases in the condition index. This pattern has been generally observed and reported in many marine invertebrates suggesting that condition index is, in part, reflecting the reproductive state of the organisms (Engle, 1958; Menzel and Hopkins, 1952; Lee et al., 1960; Giesy and Pearse, 1974; Bayne, 1976).

Variations in the timing and magnitude of HSP70 levels due to local conditions, especially in a species with such a wide geographical distribution like the eastern oyster would not be unexpected. Unfortunately such a study has not been reported yet. In response to local conditions oysters reportedly show variability in physiological parameters (condition index and gametogenic cycle) and biochemical components (Walne, 1970; Sidwell, 1979; Deslous-Paoli and Héral, 1988; Barber et al., 1988). Walne (1970) described increases in condition index with the latitude. Populations from cooler water generally exhibited better condition than populations from warmer waters. Gametes develop earliest at the southern end of the species’ distribution (Kennedy and Battle, 1964; Andrews, 1979; Hayes and Menzel, 1981; Kennedy and Krantz, 1982). In the present study differences in the levels of the HSP70 in different years were observed. Natural variations in the timing and duration of changes in physical and chemical parameters might have affected the stress protein levels in organisms. The year 1996 was
unusually cold with water temperatures more than 2°C below long-term average (1980-1996) (Ragone-Calvo and Burreson, 1997). November 1996 and January 1997 showed water temperatures 2 – 3°C below long-term average. Since then, temperatures above the long-term average have been reported. Above average temperatures were reported for March through April and from June to September 1997 (Ragone-Calvo, 1997). For 1999 and 2000 winter temperatures were 2 – 6°C above the long-term average and 1 – 3°C above long term average for spring and summer (Ragone-Calvo and Burreson, 1999, 2000). Possibly, the higher HSP70 values recorded in 1996-1997 samples compared to 1999 and 2001, resulted from water temperatures below long term average. The HSP70 levels in eastern oysters presented here represent the southern Chesapeake Bay and specifically the James River system. Extrapolation to other geographical areas must be carefully considered in light of the geographical variations present in many oysters' physiological responses (Walne, 1970; Sidwell, 1979; Deslous-Paoli and Héral, 1988; Barber et al., 1988). Future studies should sample more often, in different localities and in different years. This will improve the detection of changes associated with season in HSP70 levels. Additionally, it is important to determine what effects the timing and duration of changes in physical and chemical factors have on the HSP70 levels to better understand the significance of any changes observed.

In summary, seasonal variation in the HSP70 levels was exhibited in oysters sampled from the southern Chesapeake Bay in three different years. However, because under field conditions it is difficult to separate the effects of various environmental factors (e.g. temperature, photoperiod, food availability) completely, at this time we
cannot unequivocally describe the variations in HSP70 levels to be the result of changes in ambient water temperature. Likewise the effects of endogenous factors (e.g. hormones, metabolic rates, energy status) on HSP70 levels are not known. Future studies should investigate separately endogenous and exogenous factors to ascertain their possible effects on stress protein levels.

Moreover, the use of stress proteins (HSP70) as a biomarker is predicated on being universally present in organisms and inducible by multiple agents (Lindquist and Craig, 1988; Morimoto, 1993; Sanders, 1993), especially proteotoxic compounds. Roberts et al (1997) showed that mussels from subtidal areas had higher HSP70 (high molecular mass isoforms range 69 – 73 kDa) levels in winter than in summer. The low molecular mass isoforms did not show statistically significant difference between seasons in subtidal populations. In the present study the eastern oyster did show seasonal changes in HSP70 levels. Thus, the use of HSP70 as a biomarker of environmental contamination must be accompanied by the appropriate controls for seasonal modulation in the levels of this protein family. Knowledge about the physiological changes in the organism associated to the seasons for any particular locality is necessary to properly interpret the results.
References


Engle, J.B. 1958. The seasonal significance of total solids of oysters in commercial


the biology of heat shock proteins and molecular chaperones. In: The Biology of Heat
Shock Proteins and Molecular Chaperones. Morimoto, R. I., Tissieres, A. and
31p.


tolerance: degradation and reactivation of damaged proteins. Annu. Rev. Genet. 27:437-
496.

Price, J.L., Lyons, C.E. and Huang, Ru Chih C. 1990. Seasonal cycle and regulation by
temperature of antifreeze protein mRNA in a Long Island population of winter flounder.

Virginia Institute of Marine Science. Gloucester Point Va.

Ragone-Calvo, L and Burreson, E. 1998. Status of the major oyster diseases in Virginia --
Virginia Institute of Marine Science. Gloucester Point Va.


Walne, P.R. 1970. The seasonal variation of meat and glycogen content of seven populations of oysters Ostrea edulis L. and a review of the literature. Fish. Invest., Lond. Ser. 2. 26:1-35.


Figure 17: Disease prevalence (% infected) by the parasite, (*Perkinsus marinus*) in oysters collected in (A) May, August and October 1999, and (B) January, March and August 2001 from Point of Shoals in the James River, VA. Infection prevalence was highly associated to the ambient water temperatures (n= 7 – 9).
Figure 18: Condition Index in oysters collected from Point of Shoals in the James River, Virginia, in (A) 1999 and (B) 2001. Bars with different letters denote significant difference (p < 0.05) (Mean ± SD, n = 7 - 9).
Figure 19: HSP70 levels in the eastern oyster, (Crassostrea virginica) under natural conditions sampled in (A) November 1996, January, March and August 1997, (B) May, August and October 1999 and (C) January, March and August 2001. Numbers above bars indicate water temperature on sampling date. Mean ± CI (95% Confidence Interval), n = 7 - 9, NR = not recorded.
Chapter 6

The HSP70 response in the eastern oyster exposed to various contaminants: Summary of findings and future research
Introduction

Filter feeders play an important role in the ecosystem. These species filter water, trapping suspended matter and in the process accumulating contaminants. Resuspension of particles back into the water column might expose these organisms to contaminants after they are no longer present in the water. Thus, filter feeders are exposed to contaminants not only through the water but also through their food, affecting bioaccumulation up the trophic levels and posing a health concern when consumed by humans. Because many bivalve species are sessile and accumulate contaminants they are considered good indicators of exposure (Zaroogian, 1980; Obana, 1983; Sheehan and Powers, 1999).

In the early 1960's Ferruccio Ritossa described a chromosomal puffing pattern in the salivary glands of *Drosophila* sp. subjected to a heat shock (Ritossa, 1962). This observation marked the chance uncover of what latter became known as stress proteins. Stress proteins can be found constitutively in cells not experiencing stressful conditions (Pelham, 1986). Under these conditions two members of the HSPs, Cpn60 (mainly mitochondrial) and HSP70, act as chaperones helping in the correct folding and transport of proteins, stabilize and/or unfold proteins, disaggregate protein aggregations, and translocate newly synthesized proteins across membranes (Craig *et al.* 1987; Chirico *et al.* 1988; Rothman, 1989; Welch, 1990). Generally, they help to maintain cellular homeostasis. The HSP70 family is highly conserved across taxa and accounts for much of the translational activity in cells responding to environmental stress. Members of this family of proteins are inducible and highly conserved among various phyla of marine
invertebrates. They have been induced in mussels (*Mytilus edulis*) exposed to Cu$^{2+}$ (Sanders *et al.*, 1993), Pacific oysters (*Crassostrea gigas*) exposed to elevated temperature (Clegg *et al.*, 1998), hemocytes of the eastern oyster (*C. virginica*) exposed to 41°C for 1 hour (Tirard *et al.*, 1995a) and echinoderms (*Strongylocentrotus purpuratus*) exposed to a heat shock (25°C, 1 hour) (Stegeman *et al.* 1992; Sanders, 1994).

The stress protein response has been proposed as a general indicator of exposure to stress (Sanders *et al.*, 1993) as their expression might integrate overall biological impact and interactions among multiple stressors (Sanders, 1994). A major advantage is the premise of higher sensitivity over other indices such as condition index, scope for growth, cell viability and survival (Sanders, 1993; Ryan and Hightower, 1994; Steinert and Pickwell, 1995). However, the role of the stress proteins in environmental toxicology depends on the outcome of studies examining their responses under environmentally realistic conditions. In this dissertation, a series of experiments were performed to evaluate the levels of the stress protein (HSP70) under continuous sublethal stress.

**Summary**

**Exposure to suspended field contaminated sediments (SFCS) and PAHs sorbed to suspended clay particles**

Field contaminated sediments were collected from the Elizabeth River, a highly contaminated estuary in Virginia. Laboratory exposure to SFCS elicited a stress protein response in the eastern oyster, (*C. virginica*). The SFCS is a mixture of different
contaminants and the stress response probably resulted from the combined effect of various contaminants including PAHs, metals and trace amounts of PCBs present in the suspended sediments. To further investigate if particular classes of contaminants were responsible for the response observed, other studies exposed oysters to individual contaminants (PAHs, PCBs and cadmium) sorbed to suspended matter.

Exposure to suspended clay particles spiked with a mixture of PAHs (fluoranthene, pyrene, benzo(e)pyrene and benzo(a)pyrene) caused an increase in the levels of HSP70. No changes in the condition index or mortality were observed. B(e)P was observed to accumulate to higher concentrations than any of the other compounds in oyster tissues, including its isomer B(a)P. B(e)P seems to be preferentially accumulated, retained or to have a slower degradation rate. Evidence from Bender et al. (1988) in the eastern oyster, (C. virginica) indicates that B(e)P has a depuration rate constant slower than B(a)P and may be retained longer in the oyster tissues. Likewise, Baumard et al. (1998) have reported preferential accumulation of B(e)P in mussels, (M. edulis). It seems the fate of a compound cannot be necessarily predicted a priori even in cases of closely related compounds.

This study appears to indicate that changes in HSP70 levels provide a sensitive indicator of exposure to PAHs. However, although there is a dose dependent accumulation of PAHs in tissues of the exposed oysters, no dose dependency was observed in the stress protein response. Is possible that similar bioavailable doses in all treatments, as the contaminants desorbed from the sediments, were responsible for the
lack of a significant change in stress protein response. Alternatively, the range of dosage used was too small to generate differences in the stress protein response. In principle the finding of a stress protein response is promising in establishing the stress proteins as a tool to evaluate sublethal exposure to organic contaminants.

**PCB sorbed to algal food**

Sexually mature oysters were fed 0.2 g algal paste containing 0, 0.10 or 1.00 µg PCBs for 15 and 30 days. Sexually immature oysters were conditioned before gametogenesis and fed 0.7 g algal paste containing 0, 0.35 or 3.50 µg PCBs daily for 56 days. Exposing oysters to PCBs (Aroclor 1242, 1254, and 1260) sorbed to suspended algal food did not produce statistically significant changes in the stress protein levels. Possibly the PCB concentrations used or the length of exposure might not have been sufficient to promote a significant stress response in the oyster.

**Cd sorbed to algal food and suspended clay particles**

Cadmium has been described as a common pollutant in estuaries and to accumulate in the biota (Zaroogian, 1980, Jenssen and Scholz, 1976; Hung, 1982). In previous studies exposure regimes ranging from 10ppb to 500ppb Cd in the water have been used to study the stress protein response in marine bivalves, particularly the mussel, *M. edulis* (Veldhuizen-Tsoerkan, 1990, 1991; Brown et al., 1995; Bradley, et al., 1998). Values above 40ppb are considered indicative of heavily polluted areas (Hung, 1982). Studies have shown an increase in the HSP70 levels of exposed organisms in concentrations as low as 10ppb Cd in the water for 7 days (Brown et al., 1995). In the current experiments however, moderate concentrations (15 - 30ppb Cd) in long-term
exposure to Cd sorbed to algal food and suspended clay particles, generally did not cause changes in stress protein levels in the oyster. In bivalves, possibly mechanisms other than the stress proteins may have major relevance in metal toxicity. The effective sequestration and removal of metals from the cell's cytosol might prevent further interactions with protein components or other structures, thus preventing stimulation of a stress protein response in chronically metal-polluted environments. These results might suggest that not all toxic compounds will elicit a stress protein response in oysters and the fate of a contaminant in the organism might influence a stress protein response. A large body of literature (Zaroogian, 1980, Jenssen and Scholz, 1976; Hung, 1982; Veldhuizen-Tsoerkan, 1990; Brown et al., 1995; Bradley, et al., 1998) refers to the high toxicity of cadmium, but mainly the metal is dissolved in solution. Nonetheless, exposure to moderate doses of Cd for a long term (11 months) did not elicit a stress protein response in mussels, (M. edulis) (Veldhuizen-Tsoerkan, 1990; 1991). The present study did not show significant changes in stress protein levels in oysters exposed via suspended matter (i.e. algae and suspended clay particles).

**Natural variations in stress proteins**

Oysters collected from Point of Shoals in the James River, Virginia, exhibited variation in the HSP70 levels which appear to be related to changes in water temperature. There seems to be an inverse relation between ambient water temperature and levels of stress protein (i.e. low temperature related to high levels of stress protein in gills). However, because under field conditions is difficult to separate the effects of various environmental factors, at this time we cannot unequivocally describe the variation observed in HSP70 levels as the result of ambient water temperature effects. This result
shows some similarity to a previous study by Fader et al. (1994) in which after an increase in HSP70 levels from winter to spring, decreases are observed as temperatures continue to increase in summer and fall.

Conclusions and limitations of the stress protein response (HSP70 family)

The stress protein response has potential as a biomarker of exposure to contaminants. Sanders (1993) pointed out that the stress protein response indicates deleterious effects at the molecular level and possesses high sensitivity. However, there are limitations to its application. Most relevant to the current studies was not been able to observe a response after exposure to contaminants in every case. Different contaminants conceivably can affect different targets in the organism, not necessarily implicating proteins and thus a stress protein response as evidence suggests that the stress protein response is mediated by the presence and accumulation of denatured proteins (Baler et al., 1996). In the oyster, when associated to suspended matter, PAHs demonstrated the capacity to elicit a stress protein response whereas contaminants such as PCBs and Cd did not. The response or lack of a stress protein response, could be the result of low levels of the contaminant or not enough exposure time. Alternatively, (1) the oyster could have mechanisms to more effectively cope with certain contaminants precluding a stress protein response, (2) the contaminant does not cause protein damage specifically or (3) the contaminant effects are more apparent when acting in synergy with other contaminants.
As most biological processes, the stress protein response could be dependent on the life history of the species under study. It has been hypothesized that organisms endemic to polluted areas might not exhibit a stress response when compared to individuals from an unpolluted area (Eckwert and Köhler, 1997; Salminen et al., 2001). For example, isopods, *Oniscus asellus* from a polluted site did not show a HSP70 response after exposure to 50ppm Cd in leaf litter material compared to the high response noted in individuals from an unpolluted site (Eckwert and Köhler, 1997). These authors speculated that metal tolerance had been selected in the population from the contaminated site. Adaptations to metal contaminated sites in the terrestrial isopod, *Porcellio scaber* were reflected as changes in life history traits including early reproduction and increased allocation of energy for reproduction (Donker, et al., 1993). This could lead to a selection process for decreases in development time and a shorten lifespan (demographic selection) of the individuals in the affected population (Chippindale, 1994; Chippindale, et al., 1994; Deckert-Cruz, 1997).

Adaptation to constantly fluctuating environmental conditions might predispose organisms to be tolerant and/or adaptable to environmental insults. Some of these adaptations could enhance coping mechanisms against certain pollutants precluding a stress response by effectively sequestering the offending compound in specialized structures (e.g. intracellular concretions) or by specialized proteins such as metallothioneins (Ruddell and Rains, 1975; Carmichael and Fowler, 1981; Zaroogian and Yevich, 1994; Engel, 1999). Thus energy might be devoted more into processes limiting toxicity, possibly contaminant detoxification mechanisms.
Experimental limitations

The exposure conditions employed in the present studies might have some contribution to the lack of an observable stress response in certain instances. Under exposure conditions to mixtures of contaminants, synergistic interactions between different contaminants could elicit a stress protein response. The conditions presently used testing single compounds might not have provided the right conditions under which to develop a stress response. Additionally, under moderate continuous stress, the HSP70 could be transient in nature (Sanders, 1994; Köhler et al., 1999). Sampling periods might have been spaced enough to miss an initial transient response. Also consideration must be paid to possible seasonal variations and its impact on stress protein levels. Seasons might affect the stress protein response to stressors and the overall levels of stress protein expressed. The seasonal effects might not be necessarily on the stress protein response but could affect the organisms overall condition and energy status thus limiting a potential response to stressors.

A limitation of the slot blot technique is the inability to distinguish various isoforms of the HSP70 (i.e., measures total HSP70). Improvements to the experimental design could include the concurrent characterization of proteins induced exclusively under stress or induced specific for particular contaminants. In addition to slot blot, studies employing other techniques (e.g., Western blot, protein synthesis studies) might help improve in detecting specific stress responses by isoforms missed by measuring total HSP due to their low proportional amounts in the cell. Sampling at shorter intervals
would potentially register any short term or transient response. Also analyze different tissues that may be more relevant to a particular contaminant either as a target or depot.

The stress protein response may still be useful as part of a suite of biomarkers. Combining markers at several levels of organization (e.g. field monitoring, diversity indices, behavioral measures, physiological measures, changes in gene frequencies) will facilitate data interpretation and identification of deleterious effects caused by stressors (Sanders, 1993; Forbes and Forbes, 1994).

Future research

Due to the limitations in the use of the stress protein response, more research is needed to address its utility as a biomarker. Future research areas might include; (1) examination of the response under realistic conditions of exposure using other known pollutants, or pollutants with known mechanism of action, (2) evaluating its relation to various other toxicity end points, (3) long-term studies of exposure to chronic stressor regimes, (4) comparison to species tolerance, habitat and prior exposure (put the response in ecological context), and (5) study the effects of endogenous factors on the stress protein response. The ultimate evaluation is the extent in which results of these researches can be used to monitor real-world environmental issues.
References


VITA

Luis A. Cruz Rodríguez

Born in the quiet city of Arecibo, Puerto Rico, on the shores of the Atlantic Ocean. Earned a Bachelor of Science in Marine Biology from the Universidad de Puerto Rico, Recinto Universitario de Humacao with honors in 1981. Received a Master of Science in Marine Science from the Universidad de Puerto Rico, Recinto Universitario de Mayagüez in 1987 and a Master of Arts in Biology from the University of California at Los Angeles in 1992. Entered the doctoral program at the School of Marine Science in 1996. Defended dissertation in September 13, 2001.