Protein Expression in Baltic Sea Blue Mussels Exposed to Natural and Anthropogenic Stress

The use of stress inducible proteins in ecotoxicological studies

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Doctoral thesis in Marine Ecotoxicology

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Stockholm 2005
Abstract

The focus of this thesis is the early detection of stress in the environment. It has been proposed that studies on the cellular level would detect stress reactions earlier in time compared to common physiological methods. In a series of experiments it was investigated how different stress factors, both natural and introduced by man, affect levels of stress proteins. One- and two-dimensional gels were used to determine individual proteins and families of proteins. The two-dimensional gels were also used in a proteomic approach, where the presence and absence of proteins after treatment was observed, and the protein expression signatures (PES) were identified.

Baltic Mytilus edulis was used in all experiments and it is evident that earlier observed differences in physiological rates and pollution sensitivity, compared to marine mussels, is also manifested as lower concentrations of stress proteins after exposure to copper and cadmium. When the Baltic mussels were allowed to acclimate for one month the difference decreased, suggesting an environmentally induced difference (paper I). Pre-exposure to heat before exposure to either a second heat-shock or cadmium was found to enhance the levels of HSP70 and thus tolerance, significantly (paper II). Exposure to a mixture of stress factors (PCB (Aroclor 1248), copper and lowered salinity) revealed synergistic, additive as well as antagonistic effects in induction of 6 different stress proteins. When analyzing a large number of proteins with two-dimensional gels and the MELANIE II 2D software it was shown that it is possible to identify PES with this technique, and hypothesized that it could be possible to separate responses to mixtures of different stress factors (Papers III and IV).

Pre-exposure to heat before exposure to either a second heat-shock or cadmium was found to enhance the levels of HSP70 and thus tolerance, significantly (paper II). Exposure to a mixture of stress factors (PCB (Aroclor 1248), copper and lowered salinity) revealed synergistic, additive as well as antagonistic effects in induction of 6 different stress proteins. When analyzing a large number of proteins with two-dimensional gels and the MELANIE II 2D software it was shown that it is possible to identify PES with this technique, and hypothesized that it could be possible to separate responses to mixtures of different stress factors (Papers III and IV).

It is concluded that measuring the induction of stress proteins is a reliable way to detect stressful conditions. Proteins visualized on a one dimensional gel give a “gross” picture of an organism’s condition. The major challenge is to identify the origin and severity of the elucidated stress response. Further mapping of two-dimensional gels suggested that protein patterns are specific to type and level of stress.

A most important future step is to establish links between sub-cellular protein response to well known physiological effects. This should include long term experiments where altered protein expression signatures are linked to life history characteristics like survival, growth and reproductive success.
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III. Olsson, B., Shepard, J. L., Zhou, L., Bradley, B. P., Tedengren, M. (Submitted manuscript). Co-induction of hsp 70 isoforms and four other stress proteins in Baltic Sea mussels (*Mytilus edulis* L.) exposed to reduced salinity, copper and a PCB.


Introduction

Background

Society has become a complex matrix of energy flowing or being momentarily in solid state before transformed into further states, including processes like communication, transport, farming, construction, pleasure, combustion, waste, etc. This is a development that has been going on since humans started to colonize earth. The activities have always had an impact on the surrounding environment, increasingly so as populations grew and urbanization took place with the rapid development beginning with the start of the industrial era in the 17th centuries. Not much attention was paid to potential negative influences on the environment, but occasionally unexpected negative effects were realized. In the capitol of the Roman Empire it became evident that lead is not an appropriate metal for construction of pipes for drinking water. Several Emperors like Queen Cleopatra got some insight in this field as they realized the advantage of testing food and liquids before consumption. Much later in the British Empire it became obvious that coal miners got sick from something in their working environment.

Acceptance of human disturbances on the environment in a broader perspective was not achieved until the late 1960s, with a political wake up in the United Nations conference in Stockholm 1972. At this point it also became evident that the disturbances had reached a magnitude that were no longer local but global, and that it was necessary with international cooperation to seek solutions. This also put the environment on the political agenda.

This brief retrospective outlook is meant to show that the development of a complex society has created an even more complex disturbance matrix in the environment. This is aggravated by the late realization of the negative impact in combination with the lack of knowledge of how materials, chemicals and processes that have been used/are in use, alone or in combinations, affect the environment including man. This situation emphasizes both the needs for methods that can dissolve and connect reactions in the environment to a complex pollution situation and the use of precautionary screening methods to prevent further disturbances.
Stress

The term stress is commonly used to describe an adverse situation in the environment but there is no consensus definition. In ecotoxicological research the focus is often to quantify the condition or health status of organisms in situ. Health can be regarded as the capacity to withstand stress, the more stressed; the less capable is the organism of withstanding further stress (Bayne et al. 1985). In this thesis “stress” is used as a term describing reduced Darwinian fitness (“any environmental change that acts to reduce fitness of an organism”), and a close definition was coined by Brett (1958), “a state produced by an environmental or other factor which extends the adaptive responses of an animal beyond the normal range, or which disturbs the normal functioning to such an extent that the chances of survival are significantly reduced”. In this thesis I include survival of the genes in this definition at the level of organisms, and on higher levels survival (or persistence) of ecosystems in different demarcates.

The pathway of stress

A hard lesson that mankind has already learned is that an adverse impact detected late in time has often reached a magnitude or complexity that makes it difficult or even impossible to reverse. But if we regard stress as a course of events that develop from a “mild” start-level until a severe distant endpoint is reached, it should be possible to detect the stress and identify the inducers at several points on the way. A theoretical pathway, independent of speed and character of the stress factor as well as the target of the stress, is shown in (Fig. 1).

![Levels of biological organization](redrawn from Stegeman et al. 1992)
Biomarkers at the suborganismal level of organization have started to gain interest as early warning signals, including biochemical, physiological and histological endpoints. Very often the stressor is a chemical toxicant and since the toxicological response to a chemical is caused by the interaction between the toxicant and a biochemical receptor, biochemical responses would therefore occur before responses are observed at higher levels of organization (Mayer et al. 1992). If this is true, biomarkers should respond more rapidly than the whole organism.

A biomarker used in monitoring should preferably be on the organism level or earlier to be regarded as both early and sensitive. Several alternatives of biomarkers are more or less developed already like energetic rates, growth rates, metabolic products (xenobiotics), DNA alterations, histopathology changes, immunological changes, and enzyme and protein synthesis (Huggett et al. 1992).

This thesis focuses on the cellular level, on protein alterations in response to stress.

**Stress proteins**

A class of proteins frequently termed stress proteins presents an early response on the intracellular level. This group actually consists of two major groups; HSPs (heat shock proteins) and GRPs (glucose regulated proteins), and these groups are both structurally and functionally closely related (Welch 1990, Hightower 1991). The HSPs are called so because the first known factor to induce the synthesis of those proteins was heat. In 1962, Ritossa reported that heat induced puffs in chromosomes from salivary glands of *Drosophila melanogaster* larvae, later on Tissieres et al. (1974) found the synthesis of a set of new proteins in other cells of *Drosophila*. The proteins became well known as heat shock proteins. In the following decades it was shown that HSPs are induced by a wide variety of other stimuli including elevated temperatures, anoxia, heavy metals, organo-chlorines, ethanol, wounding and viral infections, this also rendered the more general expression; stress proteins. It also became evident that when cells or whole organisms are exposed to external stress they always respond by synthesizing this small group of highly conserved proteins. Numerous articles have reported a significant correlation between stress proteins and enhanced tolerance to stress in terms of survival and the physiological status of organisms (Krause et al. 1986, Lindquist, 1986, Bosch et al. 1988. Sanders 1988, Bansal et al. 1991, Sanders et al. 1991, Stegeman et al. 1992, Krebs & Löeschcke
1994, Bond & Bradley 1995, Brown et al. 1995; Tedengren et al. 1999a). The HSPs are mostly divided into five major groups by molecular weight. These include HSP90, HSP70, HSP60 (often called chaperonins), named after their respective weight in kilo Dalton (Sanders 1990, Stegeman et al. 1992, Sanders 1993). The GRPs are a group of proteins involved in glycoprotein synthesis; the synthesis of these proteins is significantly stimulated by glucose deprivation but only slightly by other stressors (Welch 1990, Hightower 1991). Paper I, II, III and part of paper V deals with HSPs, whereas paper IV has a broader perspective. This is partly explained below and in more detail in paper IV.

Terminology the HSPs stress proteins is somewhat misleading since many of those proteins actually occur in a cognitive form as well (often referred to as HSC’s; heat shock cognates), especially in cells of young growing individuals (Lindquist 1986, Milarski et al. 1989, Deshaies et al. 1988). A common function for all those proteins are that they, independently of the cognitive or stress induced form, are chaperones and thus involved in protein folding. The stress factors that induce those proteins are all in some way proteotoxic (Hightower 1991). When using common one-dimensional electrophoresis technique the HSP60, HSP70 and HSP90 are easily detected, and also the ones we have focused on.

The picture of stress protein knowledge today would not be complete without mentioning that a lot of other proteins are more or less affected in response to stress. Confusingly, some of them are sometimes also referred to as stress proteins. Some of the most known are metallothioneins, heme oxygenase and cytochrome P450 monooxygenases, (Sanders 1990, Welch 1990, Nover 1991).

**Functions of the 60, 70 and 90 kilo Dalton stress proteins**

In papers I-III and V up to seven different isoforms of the chaperonins HSP70 and HSP90 families have been detected. They have related cellular functions, and three isoforms from these families (hsp56, hsp70 and hsp90) even exist in a heterocomplex (Czar et al. 1994). The chaperonins and HSP70 families have also been reported to co-vary in response to elevated temperature (Sanders et al. 1992). Members of the Chaperonins and HSP70 families are molecular chaperones. Chaperonins are found in the mitochondria of eukaryotes, where they bind to target proteins and facilitate folding and assembly in an oligomeric complex. Under normal conditions, this complex binds incompletely folded proteins, which prevents aggregation and
facilitates correct conformation. During stressful conditions, when an increase in protein denaturizing occurs, the chaperonins have the additional role of facilitating refolding of damaged proteins (Horwich et al. 1991, Sanders 1993). The proteins of the HSP70 family have been highly conserved during the course of evolution and are found in several compartments of the cells of all organisms studied so far. Under normal conditions the constitutive forms of HSP70 bind to target proteins and facilitate folding transport and repair. Under adverse conditions the HSP70 has similar functions, like binding to pre-ribosomes and other protein complexes to protect them from denaturizing and prevent the formation of insoluble aggregates. These proteins also have the ability to break up already aggregated proteins (Sanders 1993, Morano et al. 1998). The proteins of the HSP90 family are found in the cytosol, where they bind to target proteins including enzymes, hormone receptors and components of the cytoskeleton. Their functions include activation and inactivation of kinases and nuclear receptors, and they are thus important in signal transduction. When an organism is under stress, HSP90 has functions redirecting cellular metabolism to enhance tolerance (Yahara et al. 1991, Kimura et al. 1995, Morano et al. 1998).

Initiation of the stress protein synthesis

More is known about functions than initiation of stress protein induction. The mechanisms that sense changes in the environment and transform them to a protein response are still in the model construction stage (Kültz & Burg 1998, Kwast et al. 1998, Owen & Hofmann 1998). An interesting model for the activation of the HSP gene has been proposed by Craig & Cross (1991) and Morimoto (1993) (Fig. 2). The basis of the model are two well described features in the activation of HSPs, the transcriptional factor (HSF) and the target sequence on the inducible genes called heat shock element (HSE) (Sorger & Pelham 1987, Sorger & Pelham 1988). It is established that HSF under normal conditions occurs in a monomeric form and undergoes a conformational change in response to stress, trimerizes and binds to the HSE that activates transcription. In the model (Fig. 2) it is further proposed that under normal conditions HSP binds to HSF in the cytoplasm and prevents trimerization. According to the model, exposure to stress will lead to elevated levels of denaturing proteins that compete for cytoplasmic HSP and also removes it from the complex with HSF. Monomeric HSF is then free to move into the nucleus, trimerize and activate the expression of the HSP gene. The model was tested by comparing HSP70 levels with
HSF levels in intertidal mussels (*Mytilus galloprovincialis*), and with the levels in the same mussels acclimated to a stable temperature, and by comparing threshold temperatures for HSP70 synthesis. The results were found to support the model (Owen & Hofmann 1998, Hofmann 1999).

Figure 2. Proposed model for the activation of HSP genes (redrawn from Hofmann 1999).

Nevertheless, irrespective of the model used, known facts show that the stress protein response is an early response and thereby interesting as an environmental monitoring tool. Especially the fact that the induction is directly regulated on the genetic level, but also including induction, function and the close connection to stress induced synthesis makes them suitable as biomarkers (Sanders 1988, Sanders et al. 1991, Bradley 1993, Sanders 1993).
The proteome approach

When realizing that the complexity in terms of HSP induction was as complex as the surrounding mixture of stress factors it was obvious that we had to look for a more selective response. Together with our co-workers at the UMBC, USA, we began to develop an extended protein approach in the field of proteomics.

The study of the entire protein content of an organism, the proteome, is increasingly being used to understand key molecules involved in normal physiological pathways as well as disease (Wilkins et al. 1996). Advances in the methods developed by O'Farrell (1975) for separation of proteins as well as those for the analysis of the protein maps has led to a resurgence of protein expression research. Two-dimensional electrophoresis opens a “window” into the proteome, visualizing between 800-3000 proteins of the approximately 10000 found in cells. By using those methods in proteome analysis it is possible to develop novel, sensitive and specific bio-indicators.

As was mentioned above an organism undergoes major changes on the cellular level that all share the fact that they are prior to higher order effects. Environmentally stressful conditions thereby cause changes in the levels of many proteins in the proteome not just those termed "stress proteins". Using many of these proteins together may provide an indicator more specific to each stressor than found using single proteins. Given the degree to which proteins are known to be regulated by the environment, the next step is to examine the many proteins present in an organism simultaneously to identify “signatures” of stress that will be able to compensate for much of the background variability found in protein expression.

In 1975 O’Farrell published his method for the separation and visualization of several proteins using two-dimensional electrophoresis. During the last four decades this method has been used in several cases to study the effect of toxicants on the expression of many proteins simultaneously. (Jellum et al.1983, Blom et al.1992, Kultz & Somero 1996, Bradley et al. 2002, Pineiro et al. 2003, Martinez & Jakobsen Friis 2004).
Our approach was to seek sets of proteins induced and repressed by the environment in an attempt to find highly specific indicators of stress. This would result in distinct protein expression signatures (PES), based on the presence or absence of proteins at a given position on a 2D gel. Induction of different proteins dependent upon toxicant exposure have previously been shown in *E. Coli* and human tumor cell lines exposed to a variety of stressors, including PAHs, PCBs, heavy metals and osmotic stress, using very simple analysis techniques (Jellum et al. 1983, Blom et al. 1992, Gonzalez & Bradley 1994). Proteomics as a preliminary screening to track changes in protein expression caused by Aroclor 1254, copper, tributyl tin and arsenic, was reported by Rodriguez-Ortega et al. (2003). The identification of a PES focuses attention on key proteins possibly involved in toxicity mechanisms and thus identifies candidate proteins for further study. Biomonitoring using analysis of the PES may allow the identification of specific stressors in the system and may allow identification of key proteins leading to functional linkages to higher order effects.

**Stress proteins as biomarkers**

**Stress proteins in the Baltic Sea**

Many aquatic organisms live in naturally harsh environments, such as brackish water and intertidal zones, close to their physiological tolerance limits. The Baltic Sea is a young environment, created after the latest ice age. The present low salinity regime has been unaltered only for some 3000 years. Evolutionarily this is a very short time. This means that marine species like *Mytilus edulis* that have recently immigrated to the Baltic Sea are not yet fully adapted but tolerant enough to withstand the harsh salinity conditions of the Baltic Sea.

When an anthropogenic stress is added to such systems the biological effects can be more dramatic than those found in studies from areas with lower natural background stress. Organisms in the Baltic Sea live in an environment characterized by low salinity and poor water exchange, and many species in the Baltic Sea have to allocate energy for adaptation to harsh environmental conditions instead of growth. The marine mussel *Mytilus edulis* is one example where both growth rate and maximum size are significantly reduced compared to mussels in more marine environments (Remane 1971, Kautsky 1982, Kautsky et al. 1990). Their response to organic pollutants and heavy metals in the laboratory is also more pronounced than responses
seen in *M. edulis* from marine environments (Tedengren & Kautsky 1987, Tedengren et al. 1999b). It is well known that *Mytilus* from these two environments differ in their genetic composition and physiological behavior (Tedengren et al. 1990).

**The Baltic Sea: a different stress protein response (paper I)**

Mussels (*Mytilus edulis*) from the brackish Baltic Sea and the marine North Sea were acclimated to an intermediate salinity followed by exposure to the heavy metal cadmium for one week (Paper I). Tissue accumulation of the metal, physiological parameters and the level of HSP70 were measured. It was found that the North Sea mussels more rapidly induced and also reached higher levels of HSP70 compared to the Baltic Sea mussels. The North Sea mussels also survived to a greater extent than did the Baltic Sea mussels (2 Vs 8% mortality) during the exposure period. In a second experiment juvenile mussels from the Baltic Sea were transplanted in the field to North Sea conditions and allowed to acclimate for one month. After this the mussels originating both from the North Sea and the Baltic Sea were exposed to copper in the laboratory for 24 hours at North Sea salinity. It was found that the differences in physiological response and HSP70 induction between the populations decreased, suggesting that at least part of the difference in stress protein induction is due to environmental factors. Those rather simple studies of a species inhabiting two different aquatic environments revealed that the differences in physiological response were also present on the cellular level. Furthermore, the results also indicated plasticity in the stress protein response of the Baltic mussels that resembled earlier studies on the organismal level (Kautsky et al. 1990, Tedengren et al. 1990). In 2004 Halpin et al. noticed similar plasticity when measuring HSP72 in *Mytilus californianus* from different microhabitats. Those results opened new perspectives of the response that to our knowledge had been largely unexplored.

According to the results of paper I, the stress protein induction in Baltic mussels seems to be suppressed compared to marine living *Mytilus*, although possible to trigger. Other studies of closely related species with a common ancestor inhabiting different habitats show that when enough time is given for speciation processes, also the stress protein response gets diversified. Hightower et al. (1999) investigated several species in the fish genus *Poeciliopsis*. Stemming from a common ancestor those fish species inhabit a wide range of habitats in the USA. The habitats include as well stable tropical rivers with a narrow temperature range and small streams with
rapid temperature changes (as much as +/- 20°C within a few hours). When *Poeciliopsis* from those habitats were exposed to higher temperatures, it became evident that they expressed several isoforms of the HSP30 and HSP70 in different ways. The experimental design enabled recognition that the induction threshold temperature of the isoforms was linked to the most frequent temperatures in their natural habitat. Furthermore, a comparison of two species of amphipods, the rock pool (variable temp.) living *Gammarus duebeni* and *Gammarus oceanicus* living in the littoral zone (more stable temp.), showed differential induction of stress protein, accompanied with significantly different survival rates at higher temperatures (Brown et al. 1995). The findings of differentiated ability of cellular response in different habitats were brought further by Bosch et al. (1988) who examined thermo tolerance and stress protein synthesis in six closely related species of hydra from different habitats. They found a strongly suppressed cellular stress response in the four species from stable habitats and conversely a pronounced stress protein reaction in the two species from variable habitats. When HSP70 isoforms, induced in response to heat treatment, were measured in northern living *Mytilus trossulus* and southern living *Mytilus galloprovincialis*, it became obvious that the northern living species had a higher level of protein damage and also expressed significantly higher levels of one HSP70 isoform (Hofmann et al. 1996). This strengthens the picture of genetic selection that tunes the cellular response with the environment if enough evolutionary time is given. Stress protein as an ecological and evolutionary force is discussed in a review article by Sørensen et al. (2003). It also raises questions, however, if stress proteins are reliable biomarkers, as they seem to differ in those aspects.

**Acquired tolerance and energetic costs (paper II)**

In paper II the focus was turned to the Baltic Sea environment in an attempt to look into the stress protein reaction of Baltic *Mytilus* in more detail. With the earlier results in mind we were interested in the speed of induction and levels of stress proteins in response to stress. This rendered an experiment designed to investigate the idea of acquired tolerance. The mussels were exposed to cadmium only, or together with a contemporary heat shock (a rapid raise in temperature from 4°C to 20°C). Their response was compared to that of mussels exposed to a previous heat shock (of the same magnitude) followed by four days of recovery and then exposed to cadmium. In order to validate the results we also measured physiological rates such as oxygen
consumption, ammonia excretion, clearance rates and absorption rates. From this we calculated energy available for somatic growth and reproduction. We found a positive relationship between physiological parameters indicating “good health” and stress protein levels also in Baltic Sea mussels. We also found that pre-exposure to a heat shock enhanced the speed of induction and also the final stress protein levels. This supported our previous findings that the difference in stress reaction between marine and brackish water mussels is mainly due to environmental factors. Acquired tolerance, where a “mild stress” confers tolerance to subsequent stress that would otherwise be lethal, is a commonly reported feature in stress protein research (Lindquist 1986, Lindquist & Craig 1988, Veldhuizen-Tsoerkan et al. 1990, Sanders 1993).

Acquired tolerance in the laboratory suggests that the phenomenon might occur naturally. Roberts et al. (1997) sampled Mussels (Mytilus californianus) at different times of the year and found different endogenous levels of different isoforms of HSP70 depending on season. Following this they treated mussels with elevated temperatures in the laboratory and found that the mussels sampled during summer season with the highest endogenous levels of HSP70, had a dampened induction response compared to the winter sampled mussels. Roberts et al. (1997) also argued that the reason for this phenomenon is to be found in the cost of the protein synthesis. This lends support from other work that has predicted the metabolic cost of protein synthesis for mussels and fish to range from 18 to 26% of total metabolic heat losses. This cost represents an additional energy burden because the stress proteins do not directly contribute to increase in growth and reproduction. Furthermore, those proteins may be synthesized preferentially under stressed conditions, such that other proteins critical for normal functioning of the organism are either synthesized at lower rates or not at all (Hawkins 1985, Hawkins 1991, Houlihan 1991, Martin et al. 1991). These findings are in concert with earlier showings that the stress protein response really means a cost on the energy budget resulting in higher chance of survival but at a cost of reduced fecundity (Krebs & Loeschke 1994, Coleman et al. 1995, Köhler et al. 1998). The highly suppressed, but after pre-treatment enhanced, stress protein synthesis of the Baltic Sea Mytilus might be explained within this context, since they were already suffering from high energetic costs under natural osmotic stress (Kautsky 1981, 1982, Kautsky et al. 1990).
This adds some further complicating factors to earlier findings and suggests careful investigations of background variability in stress protein levels if the intention is to use them in environmental monitoring.

The response to mixtures of stress factors (paper III)

Our knowledge this far, from our own experiments and literature studies, both supported and complicated the idea of using stress proteins in environmental monitoring. The fact that stress protein synthesis is a cellular response with direct linking to DNA and RNA and that it was possible to correlate to higher order effects confirmed the idea of stress proteins as ecologically relevant early warning signals. But the nature of stress protein induction as depending on environmental parameters also raised new questions. If brackish water was enough to cause differences within species, what other circumstances would be enough to do this? Further questions were raised regarding the proteins themselves as endpoints, is the appearance or level of one isomer or group of isomers enough to characterize an environment as stressful or should several isomers from many stress protein families be taken into account? Furthermore, it was unknown whether several different stress factors acting at the same time would obscure the pattern of stress protein induction.

In paper III we designed an experiment where we intended to investigate the effect on stress protein induction from exposure to mixtures of different classes of stress factors, and also study the importance of sex and age of the mussels. In order to achieve this we sampled a large amount of mussels and picked out 720; the idea was to collect mussels of both sexes and of different age. We used three different stress factors; one strictly natural (lowered salinity), one semi-natural (ionic copper Cu\(^{2+}\)) that occurs naturally at low concentrations in marine systems and at somewhat higher concentrations in the Baltic, and PCB as a entirely human introduced stress factor. In this experiment the protein assay was not accompanied by physiological measurements.

When the mussels were analyzed we found an age span between 1-7 years and equal proportions of females and males. We analyzed two isomers in each of the three common stress protein families HSP60, HSP70 and HSP90. The levels of induced stress proteins indicated no difference due to sex and age or a combination of them. Almost all proteins from treated mussels reached significantly higher levels than
found in the control mussels. When the mussels were exposed to the three stressors as single stress factors the protein levels showed a clear picture. The PCB treated mussels reached the highest levels followed by lowered salinity but not differing significantly in any case. Both of those treatments caused significantly higher protein levels than copper. The protein level in response to two- and three-factorial combinations of copper, PCB and lowered salinity showed a much more complicated picture. All of the combinations reached higher levels of stress proteins than any single factor treatment. The combination of copper and lowered salinity was the weakest inducer and PCB and lowered salinity the strongest inducer of all combinations. The three-factorial combination generally reached lower levels than the strongest two-factor combination, indicating that the mussels reached levels of stress where stress protein synthesis no longer continued to increase.

The results emphasize the fact that the same protein has a differentiated response to different stress factors as well as to combinations of stress factors. Furthermore it was clear that the protein concentration levels only continue to increase until a certain stress threshold level is reached, where the levels instead start to decrease. The reason for this is most likely found in the same context of energetic cost discussed above. The differentiated response phenomenon to stress has been reported elsewhere. Werner et al. (1998) when investigating the levels of HSP60 and HSP70 in amphipods exposed to contaminated sediments came to the conclusion that chemicals in complex mixtures sometimes interact to inhibit stress protein expression. Werner and Hinton (1999) found a site dependent decrease in the level of HSP72 and HSP76 in response to cadmium exposure in Asian clam (Potamocorbula amurensis). Dilworth et al. (2000) investigated the response of HSP25 and HSP72/73 in rat hepatocytes exposed to hydrazine and CdCl₂ and found up- and down regulation both due to toxicant and measured protein. Radlowska and Pempkowiak (2002) also found a differentiated HSP70 response in Mytilus edulis exposed to cadmium, copper and lead, with cadmium being the strongest inducer.

The results of paper III point out that measurements of isomer levels of several stress proteins by 1-D electrophoresis might be satisfactory when looking for stress signals compared to a defined control level. When it comes to distinguishing between different amplitudes of stress or different stress factors it is not satisfactory due to the
differentiated expression phenomenon described by us and others. It also underscores how important it is to know the recent exposure history of the study organism as well as taking season and habitat variability etc into account.

**Stress proteins in a proteomic perspective (IV)**

When continuously discussing the idea of using stress protein induction as a sensitive monitoring tool, we decided (together with our colleagues at UMBC, USA) to try an approach extended beyond the stress inducible proteins. Protein expression changes with the state of development, type of tissue and the internal and external environmental conditions of an organism. The entire protein complement expressed at a given time, the proteome, is increasingly being studied to identify key molecules involved in normal physiological pathways, as well as in disease development (Williams & Hochstrasser 1997). There is growing evidence that sets of proteins, up- and down regulated by exposure, are stressor specific (Jellum et al. 1983, Blom et al. 1992, Bradley et al. 1996, Kultz & Somero 1996, Kimmel & Bradley 2001, Hogstrand et al. 2002). Techniques have been developed to analyze large numbers of proteins simultaneously to discern subtle changes in protein expression (Herbert et al. 1997). Applied to environmental toxicology, proteome analysis may be used to isolate chemical-specific protein expression signatures (PES). In paper IV specific PES were isolated in *Mytilus* from the Baltic Sea subjected to the same treatments as in paper III (copper (70ppb), Aroclor1248 (1ppb) and lowered salinity from 6.3‰ to 4.3‰). Whole body tissue was homogenized and separated using two-dimensional gel electrophoresis. The protein gels were scanned to Tiff files and compared using MELANIE II 2D-gel analysis software (BioRad). Protein expression signatures including proteins induced and repressed by exposure were isolated for each treatment group.

Our intention was to seek sets of proteins induced and repressed by the stressors in an attempt to find highly specific indicators of stress. Induction of different proteins dependent upon toxicant exposure have previously been shown in *E. Coli* and human tumor cell lines exposed to a variety of stressors, including PAHs, PCBs and heavy metals, and osmotic stress using very simple analysis techniques (Jellum et al. 1983, Blom et al. 1992, Gonzalez & Bradley 1994). Computer analysis using the MELANIE II system was used to show induction of proteins due to osmotic and temperature stress in fish gill epithelial tissue (Kultz & Somero 1996). Analysis of the separated
and visualized protein spots in paper IV allowed identification of unique protein expression signatures in the overall pattern for each stressor used. The PES for Aroclor1248 included 23 protein spots. An expression signature of 23 protein spots (induced and repressed) was as well found for Copper treatment. Lowered salinity caused a PES of 26 protein spots. This way we found that the three stressors produce three distinct protein expression signatures (PES), based on the presence or absence of proteins.

At this point we were conducting experiments and analyses, that to our knowledge never had been performed neither in aquatic nor terrestrial environments. When summarizing our experiences so far we found that a proteomic approach definitely is interesting when trying to develop proteins as monitoring instruments. When confronted with complex mixtures of stress factors, the use of proteomics seemed more promising when it came to distinguishing between stress factors. The specificity of Protein Expression Signatures (PES) shows promise in bioindication, toxicity testing and in identifying possible toxicity mechanisms. Biomonitoring of environments using analysis of the PES may allow the identification of specific stressors in the system and may as well allow identification of key proteins leading to functional linkages to higher order effects.

**Stress proteins compared to physiological changes in a monitoring situation (paper V)**

At this point we had reached some insight in the integrated field of stress proteins and ecotoxicology. The feeling though was still that we had created more questions than answers. It was also evident that the idea of proteins as monitoring tools or stress indicators can be viewed in more than one way. If accepted as general indicators of stress, some families of proteins like HSP70 may be used as non-specific indicators of stress, but if a more detailed resolution regarding the identity of the stress in for instance mixtures is requested an approach like the proteomic one might be more appropriate. Any method involving proteins though still has to be further developed as many of the changes on the cellular level might be naturally occurring. A protein response also has the disadvantage of being more difficult to understand than obvious physiological changes.
In paper V, we designed an experiment where we wanted to compare the results of physiological measurements with both one-dimensional and two-dimensional (proteomic) proteins assays in the same experiment. The idea was also to mimic realistic conditions of the Baltic Sea by using PAH and PCB extracted from natural bottom sediments and dispersed back into the water column to simulate bioturbation. The sediment was sampled at a coastal site impacted by a pulp and paper mill industry (Iggesund) and at an offshore location in the middle of the Northern Baltic Proper (SR5). Mussels were then exposed to the waterborne extracts in a flow through system in the laboratory. In this relatively short-term experiment (6 days) we found that nitrogen excretion and oxygen consumption were not affected but there was a great impact on the clearance rate and absorption efficiency, which in turn negatively affected the overall energy budget. Seven protein isomers of three different stress protein families (HSP60, HSP70 and HSP90) were identified and quantified. We found that all protein concentrations except HSP73 and HSP90 increased significantly compared to control. This was an even stronger indication of stress than what was suggested by the physiological measurements, since the variation within treatment was smaller. Snyder et al. (2001) obtained similar results when isoforms of HSP60, HSP70 and HSP90 in mollusks were measured in response to increased temperature and microbial degradation products of weathered crude oil.

When running 2D gels and using the proteomic approach, we found a strong response in terms of absent and present proteins compared to control organisms. As in paper IV we tried to use the proteome analysis to isolate chemical-specific protein expression signatures (PES). The PES (induced and repressed proteins) for Aroclor1248 included 39 protein spots, whereas 36 and 99 spots were found in the SR5 and Iggesund PCB treatment respectively. As Aroclor1248 is a synthetic PCB reference it was possible to identify some key proteins that were specific in response to PCB exposure in the field samples. When the 39 spots of Aroclor1248 was compared with the 36 of the SR5 and the 99 spots of the Iggesund extract it was found that they shared 18 and 23 spots respectively. PAH extract from the Iggesund location caused a PES of 80 protein spots and the PAH extract from the SR5 location caused 79 spots, of which 29 were shared between treatments.

Kin et al. (2004) identified more than 1000 protein spots as a response to high molecular weight PAH exposure, in Mycobacterium vanbaalenii. Several of the spots
were identified and found to be specifically associated with the PAH exposure. This confirms the result of paper V.

Our conclusion was that both one- and two dimensional protein assays are as well suited as physiological measurements to indicate environmental stress. The protein methods even seem to give a more distinct stress signal (smaller individual variation) in response to the exposure than did the physiological measurement approach.

Conclusions and perspectives

We have conducted a series of experiments over several years in an attempt to evaluate the idea of using stress protein induction as an environmental stress indicator. In order to reduce possible seasonal and yearly fluctuation in stress protein induction we have sampled mussels during the same time of the year, in the same area and also chosen mussels within the same size range. We have further ensured to sample in early springtime (April and early May), well before the spawning period, when winter temperatures still are dominant in the water. We have also looked for age and sex dependent differences and concluded that according to the conditions used, those parameters do not affect the results (Paper III and IV). In spite of those precautions we found that the stress protein response is not as general or as uniform as expected.

The findings that the same or closely related species from different habitats display differences in stress protein induction correlated to habitat limits the use of those proteins as monitoring tools (Paper I, Bosch et al. 1988, Brown et al. 1995, Hofmann et al. 1996, Hightower et al. 1999). The fact that acquired tolerance occurs is also a complicating factor since it seems to influence the level of stress proteins as well as higher order effects, like physiological status and survival (paper II). Furthermore, it is obvious that the proteins respond in different ways to different stress factors, which is even more obvious in response to different mixtures of stressors (Papers III and IV, Werner et al. 1998, Dilworth et al. 2000). The biphasic induction nature of some proteins also diffuses the possibility to a correct interpretation of the response, depending on whether the observation is correlated to the increasing or decreasing phase (Paper V, Clayton et al. 2000). The proteomic approach used in paper V is promising since it seems possible to discriminate between different stress factors, as
there are actually different numbers of induced and suppressed proteins in response to different environmental disturbances as well as combinations of them. However, little is know about the persistence and reliability of such protein expression signatures.

Our findings, together with those of others, thus raise many questions regarding the use of proteins as stress indicators. But the conclusions also suggest ways to develop the methods further:

- Investigations of background variability of stress protein levels. As far as we know today this is habitat- and species-specific.
- Investigations of the correlation between protein response and higher order effects. This should preferably include long-term experiments where altered protein levels are linked to life history characteristics like survival, growth and reproductive success.
- Improvement of the proteomic approach by detecting a greater part of the proteome and identification of the individual protein spots. Those results would enable links to the genomic level as well as enable determination of protein functions that might stretch to the organism level.

Along with the efforts of developing the stress protein response into a useful monitoring tool new perspectives open up as well. According to the present OECD guidelines for the testing of chemicals and the US EPA ecological risk assessment guideline the parameters typically include mortality, immobility, growth, maturation, reproductive success, sperm production and reproductive behavior. If the stress proteins become generally accepted as a response prior to adverse effects on higher levels of biological organization can be observed, they would fulfill criterions for both acute and chronic testing in risk assessment of new chemical substances prior to introduction on the market (the environment). The stress protein response would then be used as a sensitive complement to the parameters that are used today to define NOEC (no observed effect concentration), EC (effect concentration) and LC (lethal concentration) in risk assessment, and allow us to detect potentially harmful effects earlier in time than today’s environmental monitoring methods.
Acknowledgements


A long journey of findings and experiences reaches a kind of stop with this thesis. The beginning is in 1993 when I as a detective sergeant start to study as a Ph. D. student at the department of Systems Ecology at Stockholm University. A lecture by Michael Tedengren about proteins signatures and “finger printing” caught my interest. The work went on very quick in the beginning and then slowed down markedly. During the years I learned to know Olof Reimer, we performed several experiments together. Olof’s very sharp scientific mind really was a nice help in endless planning, discussions and complicating statistic conclusions. I financed my studies as detective lieutenant for several years and Olof actually acted as a police officer in a television soap opera, to increase his cash flow. Lianzhen was a nice friend for some years and a good coworker in the laboratory, before you left for USA. Thanks Michaels Gilek and Björk for interesting discussions. The work extended to UMBC, USA and new friends. Many thanks professor Brian for your encouraging ideas and patience in the writing. And also to Judy for nice housing during visits. Dear Jennifer, it has been a pleasure to work with you. I will never forget how well you and all Davids took care of me in Baltimore in 1997.

Thank you Michael Tedengren for a never ending optimism and professor Nils Kautsky for believing in my work. You who are not especially mentioned are not forgotten, I have really appreciated hints and skepticism over my work, but most your friendship Halldora, Max, Annika, Anders and Peter and many others.

It would not have worked without the never ending support from my family and the ♥ of my life; Pia.
References


