Coping with environmental stress:
From the individual and population perspective

Johanna Gardeström
To my grandmothers
Natural stress and disturbances are important factors affecting the structure and function of ecosystems. However, the magnitude of stress has escalated due to anthropogenic activities. Environmental monitoring and toxicity assessments try to protect ecosystems from unwanted human alterations. The major aim of this Doctoral thesis was to increase the understanding of the complex effects that environmental stress has on individuals and invertebrate populations. The low saline environment in the Baltic Sea is perceived as stressful for most organisms living there. In Paper I, it was found that Baltic blue mussels living in the less saline northern Baltic Proper (~5 psu) had lower basal metabolism and were more susceptible to toxic exposure than the mussels in the south (~7 psu). Paper III used microsatellites developed in Paper II to show that there was no genetic differentiation between the mussels from the northern and southern areas. There were however genetic differences between mussels from sites within the respective areas, indicating that there is not a simple relationship between the health of the mussels and genetic diversity in the microsatellite loci studied. In Paper IV it was confirmed that the heat tolerance of the intertidal dogwhelk *Nucella lapillus* is oxygen dependent. Increased oxygen levels led to a higher survival rate of the whelks. Accordingly, the protein expression profiles of dogwhelks exposed to high temperature and high oxygen levels were more similar to those of the control, compared to those exposed to high temperature and normal oxygen levels. In Paper V and VI stress responses were studied at both the individual (RNA content and/or individual length) and population level (abundance, proportion of respective life stages, genetic diversity, and genetic differentiation). Exposure to a single toxicant (the brominated flame retardant BDE-47 in Paper V and copper in Paper VI) for more than one generation decreased the genetic diversity in exposed copepod populations, however abundances remained unaltered. In Paper VI, exposure to naturally contaminated sediments, which contained of a mixture of toxicants, did not decrease genetic diversity. However the genetic divergence ($F_{ST}$) within the treatments was very high, probably due to small effective population sizes in the replicates. In Paper III, two of the three sites that diverged from the rest were located in the northern area. The very low blue mussel abundance in the north together with the stressful environment suggests a small effective population in the northern Baltic Proper. The results of both Paper III and VI hence indicate that a tough environment can reduce the effective population size, possibly by selection against sensitive genotypes, increasing the effect of genetic drift.

In conclusion, my studies show that stress may affect various levels of biological organization differently, and although effects are not detected in individuals and obvious population endpoints, genetic diversity can still be substantially affected by toxicant exposure. Consequently, measuring effects on several levels, including both functional and structural endpoints will increase the sensitivity of the tests as well as their ecological relevance.
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LIST OF PAPERS

This thesis is based on the following six papers, referred to in the text by their Roman numerals

**Paper I**  

**Paper II**  

**Paper III**  
Gardeström J., Lilja K., Prevodnik A., Elfwing T., Bollner T., Tedengren M. No differences in neutral genetic loci between blue mussels from the northern and southern Baltic Proper. Manuscript.

**Paper IV**  

**Paper V**  

**Paper VI**  

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My contribution to the papers: (I) - collected the mussels, performed the experiment, participated in writing. (II) and (III) - conducted all laboratory work, data analysis and the main part of the writing. (IV) - collected the dogwhelks, performed the experiments and the proteomics work in the laboratory. Main part of the writing. (V) - performed the genetic analysis (AFLP) and did the main part of the writing. (VI) - participated in all the experimental work and performed the genetic and data analysis together with a Masters student. Main part of the writing.
INTRODUCTION

We have witnessed increasing pressure on both aquatic and terrestrial ecosytems during the past centuries (Crutzen and Steffens 2003, Millennium Ecosystem Assessment (MEA) 2005, Lewins 2006). The anthropogenic impact on the environment is still escalating and we have now reached what scientists refer to as ‘the Anthropocene’ (Crutzen 2002), a time period when a third to half of all land is exploited by humans, ecosystems lose productivity, and toxic substances are released into the environment. Although stress is a normal ingredient in the environment and species have always gone extinct, anthropogenic activities are escalating and elevating stress levels, not only for individuals but also for entire populations. Biodiversity loss has occurred more rapidly during the last fifty years than under any other time in human history (MEA 2005), and is now referred to as “the sixth extinction” (Lewins 2006).

A central part of ecotoxicology is studying and/or predicting anthropogenic effects on the environment. Typically, studies are often performed on individual organisms, using high concentrations of toxicants and short-term lethal effects as endpoints (Calow and Forbes 2003). As there are problems associated with extrapolating from the lower to higher levels of biological organization, it has been recommended that ecotoxicological test methods should move beyond tests with endpoints at the individual level, and instead identify population-level endpoints to provide a more comprehensive indication of adverse biological effects of exposure (Calow and Sibly 1990, Walthall and Stark 1997, Breitholtz et al. 2006).

Objectives of the thesis

Current environmental monitoring and toxicity assessments aim to protect ecosystems from unwanted anthropogenic effects. However, since ecosystems are so complex to base regulatory studies on this level of biological organization, extrapolations from studies on lower orders of biological organization are commonly used to assess the effects of e.g. pollution. Such extrapolations are generally based on short-term effects at the individual level (often related to mortality), whereas little effort has been put on long-term effects at the population level.
The main aim of the present thesis was therefore to make use of a diversity of techniques to increase the understanding of the complex effects that environmental stress has on individuals and invertebrate populations covering both functional and structural responses. Some more specific objectives were to:

i) assess if natural stress affects physiology, tolerance to additional (anthropogenic) stress and population genetics.

ii) assess if there is a correspondence between some lower level responses (cellular and individual) with higher level effects (populations).

iii) assess if toxic exposure affects genetic diversity.

Defining stress

In general, stress is defined as a condition that disturbs the normal function of the biological system or a condition that decreases fitness (Sorensen et al. 2003). There is nothing absolute about stress so it has to be referred to a normal state of the biological system being studied. Van Straalen (2003) therefore concludes that stress should most correctly be defined as “a condition evoked in an organism by one or more environmental factors that bring the organism near and over its ecological niche”. A stressful condition will result in a reduction of net energy balance and reduce the Darwinian fitness (Fry 1947). From an individual perspective, stress can be dealt with by returning to the niche by either behavioral mechanisms (i.e. avoidance) or by suppressing the stress (biochemical and physiological responses, Fig. 1) (Futuyma 1998, Van Straalen 2003). The latter is referred to as acclimation, at the cost of many fitness traits such as growth and fecundity (Futuyma 1998). These types of responses (i.e. at the individual level or below) are often referred to as biomarkers. These are either up-regulated to protect the cell from the toxicological damage by e.g. detoxification or they can be a result of toxicological damage (Walker et al. 2001). If the negative impact persists or increases, a sequence of alterations within higher levels of biological organization is initiated. It may eventually have such an impact on individual health that it causes mortality, which results in structural changes of the populations (Fig. 1). From a population perspective, the niche can be adjusted by genetic adaptation through selection for the right genotypes and / or against the less successful genotypes (Van Straalen 2003). While metabolic and molecular processes may return to normal within days or weeks after the source of the stress is removed, genetic diversity may take many generations to recover (Bickham and Smolen 1994).
Figure 1. A model describing the different stress responses in an ecosystem as a function of the amount of stress or its duration. Initially behavioral changes are triggered which, if stress persists, will initiate functional changes. These may in turn be followed by large-scale structural changes in the ecosystem, which may be irreversible.

Baltic Sea: a naturally stressful environment

Although stress is a natural ingredient of all types of ecosystems, some systems may generally be considered more stressful for its inhabitants than others. The Baltic Sea is an evolutionary young and geographically peripheral sea. The semi-enclosed nature of the Baltic Sea, with freshwater inflow in the north and saltwater inflow in the south, in combination with a positive water balance, has resulted in a stable salinity gradient with decreasing salinity in a south north direction. The relatively young age (7500 years since the connection to the North Sea was established), together with its brackish nature, makes it an extreme environment for most marine and freshwater species living there (Remane and Schlieper 1971, Elmgren and Hill 1997).

The long-term adaptation of the organisms to the brackish state has resulted in many morphological differences between populations within the Baltic Sea compared to their counterparts in the more marine North Sea (Remane 1934, Kautsky et al. 1990). Many of the populations have further been reported to deviate genetically in the Baltic compared to the North Sea, and many species
constitute unique evolutionary lineages (see Johannesson and André 2006, and references therein). The Baltic blue mussel, *Mytilus trossulus* is one such unique evolutionary lineage. It is a keystone species in the Baltic Sea in the sense that it performs many ecological services e.g. it “vacuum cleans” the Baltic Proper by filtrating the coastal waters, provides a link between the pelagic and benthos by cycling nutrients and matter, and is prey for fish and birds (Kautsky and Evans 1986). Hence, the sustainability of the blue mussel population is of critical importance for the Baltic Sea ecosystem. The scientific name of Baltic blue mussels has changed over the years, and still does (including in this thesis), largely because the DNA consists of parts from both *Mytilus trossulus* and *M. edulis*. It was recognized as *M. trossulus* in 1988 from allozyme analysis (Varvio et al. 1988). It has been argued that the step cline in allozyme allele frequencies through Öresund and the Danish Straits is best explained by primary differentiation of *M. edulis*, due to adaptation to the low salinity conditions of the Baltic Sea (Bulnheim and Gosling 1988, Johannesson et al. 1990). This does not however explain the fact that the Baltic blue mussels have high frequencies of *Mytilus trossulus* alleles. Today, most scientists agree that Baltic blue mussels have alleles characteristic of both *M. edulis* and *M. trossulus*, and the debate is mostly concerned about which scientific name is most appropriate to use for this unique evolutionary lineage.

**Experimentally studying the effects of anthropogenic stress**

In their natural environment, organisms are generally exposed to multiple stressors. Anthropogenic-released toxicants are usually found in mixtures, and the toxic effects usually interact with natural stress. Typically, all these types of stressors can act either sequentially or simultaneously. When working in controlled laboratory environments, interactive features of different anthropogenic and/or natural stressors are often intentionally avoided or unintentionally overlooked. However, the toxicity of mixtures is often due to several chemicals and is not necessarily the summation of the toxicities of its individual components (i.e. additive), but can also be interactive resulting in synergistic or antagonistic effects (Walker et al. 2001). Ignoring this can decrease the ecological relevance of the results when extrapolating to real-world effects.

Toxicant exposure in the environment is often chronic, acting in low concentrations over long time. Yet, short-term studies are still commonly used to predict effects at higher biological level effects since they are relatively easy, inexpensive and quick to perform (Calow and Forbes 2003). However, it is definitely not clear-cut that results from short-term high exposure studies can be meaningfully extrapolated to long-term, low concentration exposures, which is what the organisms often experience in their natural habitats (Eggen 2004). Exposure studies with low dose and long exposure time are further the most useful ones when trying to identify effects at higher biological levels such as populations (e.g. abundance, population structure, and genetic diversity) since is
it usually takes longer time before effects are manifested at higher levels (compared to cellular and individual effects).

Harpacticoid copepods generally have short generation times which makes it possible to study long-term effects of toxicant exposure over a full life cycle. These organisms live in the most superficial layer of bottom sediments where they are the second most abundant group of species after the nematodes (Huys et al. 1996, Coull 1999). Their main food source is particulate organic material and presumably associated bacteria (Dole-Olivier et al. 2000). These animals therefore represent a vital link between primary producers (bacteria and algae) and secondary consumers such as fish and larger crustacean species (Hicks and Coull 1983). Harpacticoid copepods are due to the above mentioned criteria often used to experimentally study the effects of anthropogenic stress. The effects will further not only cover many crucial life stages, such as larval developmental rate, reproduction etc., but will also address population effects over several generations (Breitholtz et al. 2006).

Genetic diversity and ecosystem resilience

Biodiversity is a broad concept involving the diversity – and complexity – within and among all levels of biological organization, i.e. genetic resources, species, ecosystems and landscapes (Convention of Biodiversity 1992), where the properties of one level also set the limits of performance at other levels. Biodiversity provides a vast amount of ecosystem services and represents unexplored options for the future (MEA 2005). Furthermore, there seems to be a strong relationship between diversity and ecosystem resilience, i.e. the ability to absorb and to cope with disturbance while maintaining ecosystem functions (Holling 1973, Hughes 1994, Nyström et al. 2000, Rönnbäck et al. 2007).

Most biodiversity studies deal with effects of species diversity, and until recently very little attention had been directed to the importance of genetic diversity, which includes diversity among individuals within populations as well as variation among different populations within a species (Bagley et al. 2002). A diploid individual that has a high frequency of heterozygote alleles is considered more genetically diverse in the studied loci compared to an individual with homozygote alleles in those loci. The genetic diversity in the population is the sum of all allelic variants in the studied loci in the population. As presented further below, recent studies have indicated that genetic diversity play an important role by enhancing ecosystem recovery after exposure to environmental stress (e.g. Hughes and Stachowicz 2004, Reusch et al. 2005).

Four main mechanisms set current levels of genetic diversity and indirectly affect the resilience of populations and ultimately ecosystems, namely, mutation, migration, genetic drift, and selection (Futuyma 1998). The ultimate
source of genetic diversity is mutation, an outcome of normal cellular process or interactions with the environment (Hansen 2006). The other three forces work on what is provided by mutations. Migration generally increases genetic diversity within populations and homogenizes it between populations (Pages and Holmes 1998). The extent to which this occurs is tightly linked to the species dispersal ability. Genetic drift represents a random change in gene frequencies in each generation due to a limited number of breeders producing each new generation, which normally decreases genetic diversity within populations and increases genetic diversity among populations. Random changes in allele frequency are larger in small populations and are hence common phenomena associated with sampling and experiments (Lowe 2004). Selection is the primary force that is responsible for adaptation. If environmental conditions cause a set of genes to be advantageous, selection is expected to decrease genetic diversity at selected loci (i.e. directed selection) (Lowe 2004). It is today well documented that chemical pollution can act as a strong selective force. For instance, metal tolerance in plants has been established in areas with mine waste (Bradshaw and McNeilly 1982) and pesticide resistant insects have been documented in crop-land areas (e.g., Devonshire et al. 1998). However, the outcome on total genetic diversity as a result of toxicant exposure can theoretically be in both directions, with an increase by mutation or decrease by selection and/or drift (for reviews, see Bickham 2000, Belfiore and Andersson 2001, Staton 2001). If the conditions in the environment change, for natural or anthropogenic reasons, a population requires genetic diversity in order to deal with the ensuing stress by adaptation (Reed and Frankham 2003). Generally speaking, a population with greater genetic diversity renders a higher chance that some individuals possess genes required to cope with new environmental conditions as compared to a population with lower genetic diversity (Futuyma 1998, Frankham et al. 2002).

As mentioned, there are an increasing number of studies supporting the importance of genetic diversity for stability of populations and ecosystems (e.g. Hilborn et al. 2003, Hughes and Stachowicz 2004, Reusch et al. 2005). Two of the first studies that tested the effect of intraspecific variation in the marine environment were conducted on the seagrass Zostera marina. It was found that more genetically diverse seagrass beds displayed faster rate of recovery both after grazing (Hughes and Stachowicz 2004) and thermal stress (Reusch et al. 2005) than less genetically diverse ones. Interestingly, higher resistance to water temperature was not attributed to selection but to niche differentiation or complementary effects e.g. facilitation of less fit genotypes in the presence of other genotypes (Reusch et al. 2005). In a laboratory study, Gamfeldt et al. (2005) found that settling success of the barnacle Semibalanus improvisus was superior in more genetically diverse groups as compared to those with less genetic diversity. Studies from terrestrial biology have further shown that higher intraspecific diversity also may give advantages beyond the populations of a single species, e.g. diverse plant assemblages have higher diversity of associated fauna compared to less diverse stands (Wimp et al. 2004, Crutsinger et al. 2006, Johnson et al. 2006). By using quantitative genetics, Lankau and Strauss (2007) showed
that heritable differences in the concentration of a secondary compound (a glucosinolate allelochemical) in *Brassica nigra*, is necessary for the coexistence of *B. nigra* and its competitor species. They found that changes in the mean level of the secondary product could lead to changes in total plant community structure that, in turn, affected selection on the secondary product. This results in an upheld genetic and species diversity simultaneously, indicating a clear dependence between the levels. Thus, these studies pinpoint that genetic diversity is important for the functionality and adaptive capacity in a wide number of organisms and that it affects higher levels, i.e. species, communities and ecosystems.

### Proteins and fitness

How well an individual copes with stress can be dependent on the plasticity of its physiological response, which may be related to the heterozygosity (i.e. genetic variation) within the individual (Mitton 1993), if none of the homozygotes possesses superiority under all environmental conditions. A more diverse set of genes can produce a more diverse set of proteins, resulting in a wider possibility of responses.

The study of proteins in relation to fitness is relevant since an organism’s proteome is a snapshot of the molecular phenotype expressed at that particular moment, and on which selection is acting (Diz and Skibinski 2007). Feder et al. (2000) summarize the relationship between genes and proteins as follows: “The phenotype determines the performance of organisms in natural environments in response to ecological or evolutionary stimuli, the performance determines the evolutionary fitness of alternatively genotypes, and the fitness determines the frequency of genotypes in the next generation, in recursive fashion”.

The analysis of individual proteins and/or the proteome (the total set of proteins expressed by a genome in the tissue or organism studied), comparing stressed and non-stressed conditions, allows the detection of changes in the induction and repression of proteins in response to environmental stress. If there is a well established link between the proteome response and effects at higher biological levels, such as individual health, these types of analysis will be of great value. Not only could it help in giving a mechanistic explanation to the response but also specify good biomarkers suitable for environmental assessments (Eisenbrand 2002).
The stress responses illustrated in Fig. 1 can be measured at different levels by various methods (presented in Fig. 2). The response variables studied in this thesis encompass both functional (e.g. protein modification and scope for growth) and structural (e.g. abundance and genetic diversity) changes. Biomarkers are considered to be tools that assess the early impact caused by stress (Huggett et al. 1992). The biomarkers used in the studies presented in this thesis have measured both changes in the level and structure of macromolecules (proteins in Paper I and IV and RNA in Paper V and VI) and in individual health (Paper I). Proteins can be used as biomarkers, as single proteins detected by various methods (e.g. western blotting and immunohistochemical staining) or as global protein expression, analyzed by a proteomic approach. Molecular activities cost energy and if the energy requirements are high it will result in decreased metabolic surplus (Koehn and Bayne 1989). The sum of all energy requirements can be measured at the individual level as scope for growth (SFG) (Bayne et al. 1976). A reduction in the net energy balance is usually a sign of stress and will eventually result in less production (less energy for growth and/or reproduction). Fig. 1 also shows that structural changes can occur at higher levels in the biological hierarchy if the stress persists. In this thesis, these types of changes have been measured as abundance alterations, i.e. the survival frequency (Paper IV, V and VI) and changes related to population genetics (genetic structure, and genetic diversity; Paper V and VI), analyses for which genetic markers are needed.

The widespread use of allozyme markers in the 1970s led to dramatic progress in molecular genetics (Liu and Cordes 2004). DNA marker technologies have developed quickly over the last 20 years, resulting in a wealth of genetic methods. Two different genetic markers have been used in my studies; microsatellites and amplified fragment length polymorphism (AFLP). Microsatellites are simple repeated sequences of non-coding DNA that are distributed more or less evenly in the genome (Chistiakov et al. 2006). This repetitive characteristic makes the microsatellite areas very prone to mutations since the polymerase that adds nucleotides when DNA replicates can easily lose track when there are many repeats of the same sequence and consequently either add too many repeat units or leave some out (Maxson and Wills 1999).
The result of the polymerase “slippage” is different repeat numbers in the parental DNA strand and the new strand and this results in a large variation of microsatellite alleles in the population. Microsatellites are co-dominant markers, which means that homozygotes are distinguishable from heterozygotes. This property, together with its mutation-prone characteristic, makes microsatellites highly polymorphic and has consequently made this marker type very popular for intraspecific genetic studies. Further, since a mutation in a microsatellite is assumed to not affect the reproduction success, it is a neutral marker. The sequence areas before and after the microsatellite are called flanking regions. The disadvantage with microsatellite markers is that specific primers for the flanking regions of the microsatellites have to be developed anew for each species, which was something I did for the Baltic blue mussel, *Mytilus trossulus* (Paper II).

There is, to the best of my knowledge, no specific marker (e.g. microsatellites) developed for harpacticoid copepods. Hence, I used amplified fragment length polymorphism (AFPL) when working with these organisms (Paper V and VI). The advantage of AFLP is that no prior information is needed about the DNA sequence of the organism analyzed, so there is no need to design specific primers for respective species. In essence, it is a fingerprinting technique that allows

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<th>B. Methods used to assess effects of stress</th>
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Figure 2. The different levels analyzed and methods used in the respective papers. The copepods shown here serve to illustrate the different levels of biological organization, however, other organisms were studied in Papers I-VI.
for simultaneous detection of polymorphisms in different genomic regions (Vos et al. 1995, Mueller and Wolfenberger 1999). The use of restriction enzymes of known cutting sites enables the design of double-stranded adapters, which provide the primer sites for the following PCR amplification. A drawback with AFLP is that it is a dominant marker, which means that heterozygotes can not be directly distinguished from homozygotes with the bands (Zhitovovsky 1999). Instead the expected heterozygosity is calculated from the matrix of plus-alleles (the presence of a band) and null alleles (no band).
SUMMARY OF THE PAPERS

Paper I. The salinity of the local environment influences the stress response of blue mussels

The aim of the first study was to investigate if mussels originating from three areas in the Baltic Proper with different salinities (south, middle and north Baltic Proper, with 5.1, 6.1 and 7.1-7.4 psu, respectively) showed corresponding differences in stress response when exposed to copper (35 µg/l) or 95 octane petroleum (0.3 percent by volume). We investigated responses both at the molecular level (i.e. activities of glutathione transferase (GST) and catalase (CAT) and the amount of disulphides and carbonyls) and at the individual level (scope for growth). The mussels were exposed for 10 days at the salinity of their origin. Among the control (non-exposed) mussels, those from the southern population (7.1-7.4 psu) showed the highest scope for growth (SFG) values, while the northern population (5.1 psu) showed the lowest. Hence, mussels from higher salinity areas experienced less background stress than those from areas with lower salinities. The absolute carbonylation levels were higher in mussels from the southern and middle Baltic Proper. Since carbonylation are structural modifications of proteins, commonly as a result of oxidative stress, the high levels might be related to higher metabolic rate, and hence production of ROS (reactive oxygen species) in these mussels. Exposure to petroleum affected both SFG and carbonylation levels, and the southern mussels showed the greatest decrease of SFG in this treatment, indicating higher energy availability to counteract the stress. Conversely, the northern mussels showed the lowest SFG and highest levels of carbonyls in response to stress, which, taken together, signals severe oxidative stress. Differences in disulfide profiles were also detected between the sites at which they were analyzed (one sampling site in the north and south, respectively). No significant effects of treatment were found for enzyme activity. In conclusion, the results from Paper I show that there are differences in stress susceptibility on a regional scale in blue mussels within the Baltic Proper.

Paper II and III: The genetics of Baltic blue mussels

Previous genetic studies on the Baltic blue mussel have mostly been performed using allozymes or mitochondrial DNA sequences. Despite the wide distribution and extensive scientific interest for this species complex, there were only seven microsatellite markers available for Mytilus prior to our contribution,
of which only two showed satisfactory amplification in Baltic blue mussels. It was therefore necessary to construct novel primers to facilitate genetic analysis of this species. **Paper II** is a technical note summarizing the procedures for construction of *Mytilus* microsatellite primers. These primers were then used in the study presented in **Paper III**. We experienced similar problems with blue mussel DNA as many other mollusk studies have reported, i.e. null alleles. This means that the primer does not bind satisfactorily to the flanking sequence surrounding the microsatellite, resulting in failed amplification of the microsatellite. Null alleles occurred frequently with all our designed primers but this was not a problem after correction using the software FreeNA (Chapuis and Estoup 2007). We found that the flanking regions of the microsatellites were frequently affected by mutations, decreasing the chances of good primer annealing, which likely explains the high frequency of failed amplification. Further, we found that four of the six primers amplified well in North Sea *Mytilus edulis* and can hence also be used in genetic studies of this species.

In **Paper III** we applied five of the primers from **Paper II** plus two constructed by others (Preza et al. 2002) to assess the genetic structure of mussels from two areas in the Baltic Proper (north and south) on which physiological measurements were done in **Paper I**. We genotyped mussels from three sites within the northern and southern area, respectively. Statistical analyses using AMOVA (Arlequin) showed that there was no genetic structuring between mussels from the northern and southern area, hence the physiological differences seen in **Paper I** between these two areas cannot be explained by genetic differences in these neutral loci. However, we cannot say if those physiological differences are due to differences in traits under selection, unlinked to these neutral loci, or to phenotypic plasticity. Further, there was some differences between sites, with three sites being genetically diverged from the others (high $F_{ST}$). Two of these diverging sites were within the northern area. It is possible that the harsher environment in the north reduces the effective population size, which increases the risk for genetic drift, and/or that the variability in environmental harshness prevents reproduction and larvae import in some sites some particular years while not in others.

**Paper IV**: The protein expression profile mirrors the health of the organism

High temperature is of great concern for intertidal organisms. For instance, the survival rate of Arctic invertebrates at high temperatures depends on oxygen availability (Pörtner et al. 2000, Pörtner 2001, 2002). A similar link has been reported for the dogwhelk *Nucella lapillus* (Davenport and Davenport 2007), a common predator on marine temperate intertidal hard bottoms. In **Paper IV** we extended these previous findings by examining if higher-level effects (i.e.
survival rate) are accompanied by molecular changes. Dogwhelks collected on the west coast of Ireland in June 2004 were exposed (24 hours) to 16 °C (ambient) under normoxic conditions and 26.5°C and 30 °C under normoxic (100% oxygen saturation in the water) and hyperoxic conditions (150 % oxygen saturation), respectively. Survival rates were recorded and protein expression profiles (PES) were analyzed in surviving organisms. Mortality, which was 40-50% in the high temperature: normoxic conditions, decreased by approximately 75% (to about 10%) when oxygen was added (i.e. high temperature: hyperoxic conditions). In line with Davenport and Davenport (2007), these results clearly suggest a positive relationship between hyperoxic conditions, high temperature and survival in dogwhelks. Accordingly, the protein expression profile of dogwhelks exposed to high temperature: hyperoxic conditions was more similar to that of the control, compared to the PES of those that were exposed to high temperature under normoxic conditions. This confirms that oxygen availability determines the temperature tolerance of dogwhelks, and also demonstrates that the positive response to added oxygen detected on a higher level (higher survival rate) correspond to a positive response on the individual level (a PES like that of the controls).

Paper V: Population recovery at the cost of genetic diversity

In Paper V we attempted to identify effects of toxicant exposure at a range of biological levels, using several endpoints, to untangle the complex relationship between long-term exposure (ca. 1 generation) and biological effects. The aim of the study was twofold: (1) to examine if toxicant exposure can alter genetic diversity (expected heterozygosity and genotype composition) and (2) to compare the response in this endpoint with other population- and development-related measures (e.g. RNA content, abundance). Harpacticoid copepods (Nitocra psammophila) were randomly exposed to four treatments: 1 control and 3 different concentrations of the brominated flame retardant BDE-47 (2,2’,4,4’-tetrabrominated diphenyl ether) (0.1 µg, 1.1 µg, and 11 µg BDE-47 mg⁻¹ food) in four replicates. The control and the treatments initially contained 15 ovigerous females per replicate. The exposure period was 24 days. In the highest concentration all animals died and was thus omitted from the analysis. In the remaining treatments, abundance was not affected but population structure changed (i.e. proportion of respective life stages in the samples) and RNA content decreased significantly in both 0.11 µg and 1.1 µg BDE-47 mg⁻¹ food. Heterozygosity was lower and there were significant differences in genotypic composition in the populations exposed to 1.1 µg mg⁻¹ food compared to the controls. This was not due to large reductions in individual numbers, since no effects could be seen in population abundance, but might instead be due to higher reproduction efficiency in resistant genotypes. In conclusion, the num-
ber of individuals is not a sufficient endpoint to capture population level effect and it highlights the importance of also following changes at the genetic level.

Paper VI: Complex effects of complex mixtures

Since sediments accumulate large numbers of anthropogenic substances, they can represent a source of complex mixtures of toxicants. Paper VI focuses on the harpacticoid copepod *Attheyella crassa*, which has a generation time of 4-5 weeks. The major objective of the study was to increase the ecological realism in the test system by using sediments collected from polluted and clean sites, and having exposure times spanning over several generations. More specifically, we were interested to see if long-term exposure to contaminated sediments could alter genetic diversity, and genetically differentiate populations. The effects on population abundance and on two growth-related end-points (RNA content and cephalothorax length) were also analysed. Copepods were exposed for 60 days (>1 generation) and 120 days (2-3 generations) to sediments collected at two well-known contaminated sites in the Baltic Proper, Svindersviken and Trosa (each in two concentrations: 50% contaminated sediment mixed with 50% control sediment, from a reference site near Askö, and 100% contaminated sediment), and for 120 days to control sediment to which copper sulfate was added, corresponding to a concentration of 130 µg Cu/l water. There was no treatment effect on any of the two growth–related measures, but in the copper treatment there was a significant decrease in genetic diversity after 120 days, although abundance remained unchanged. This indicates a probable selection for copper tolerance. There was a significant decrease in total abundance after 60 days in both of the 100% naturally contaminated sediments. This abundance bottleneck recovered in the Trosa treatment after 120 days but not in the Svindersviken treatment. Neither of the naturally contaminated sediments (50% and 100%) affected genetic diversity after 120 days, despite genetic differentiation between replicates within these treatments (i.e. high *F*<sub>ST</sub>). In fact, the within–treatment *F*<sub>ST</sub> (divergence between replicates) showed a concentration-related pattern, with highest *F*<sub>ST</sub> in both 100% treatments. There seem to have been selective mortality against highly sensitive genotypes in these treatments. The high reduction in abundance caused a bottleneck resulting in higher genetic drift. In conclusion results from Paper VI show that there is more than one plausible effect on population genetics of toxicant exposure; one toxicant (copper) reduced genetic diversity even though abundance was unaltered while the contaminant mixture (i.e. the naturally contaminated sediments) had effects on abundance and genetic differentiation (*F*<sub>ST</sub>) within the treatments but not on genetic diversity.
DISCUSSION

This thesis covers both functional and structural effects of exposure to toxicants. The physiology (function) of e.g. blue mussels provides information about the current sensitivity of individuals, while the genetic diversity (structure) of e.g. a copepod population indicates the sensitivity of the population in the face of environmental change. Neither is more important, as they are dependent on each other (Snelgrove et al 1997). Taken together, the results show that responses at several levels of biological organization, covering both functional and structural changes, provide additional information to single level tests, insights that increases our ability to understand and interpret the complex relationship between exposure and adverse effects. Responses at the cellular and individual level are supposedly sensitive, specific and fast. However, many biomarkers need further investigations considering their natural variation and how well their responses correspond to plausible population effects, making it questionable to solely rely on biomarkers in risk assessment studies. Indirect effects, higher-order effects (Belfiore and Anderson 2001) or compensatory effects (Breitholtz et al. 2003) are not possible to track from the observed responses of individuals, but might be reflected at the population level. There is consequently a growing recognition that environmental risk assessments should rely more on tests that address alterations at higher levels i.e. populations and ecosystems (Admiraal et al. 1994, Belfiore and Anderson 2001, Breitholtz et al. 2006, Forbes et al. 2006) than on the common short-term single species test. While these tests at higher biological levels are more ecologically relevant, they usually require a longer time before responses are manifested (Fig. 1) and these responses can further be complex and difficult to link to the causative agent(s). Hence, to solely base risk assessment on these higher levels is not satisfactory either. The advantage of a multilevel approach, i.e. measuring both functional and structural changes, at different levels of biological organization, is that it will generate a broader picture, as well as improve predictions regarding the population. This approach will both increase the sensitivity of the tests and increase their ecological relevance.

Effects of natural stress on blue mussels

The Baltic Sea is considered a naturally stressful environment due to e.g. its brackish nature (Remane and Schlieper 1971, Elmgren and Hill 1997). The stable nature of the salinity gradient suggests a strong chronic stress pressure on the organisms living there. The results from Paper I show that mussels from the northern Baltic Proper had lower scope for growth (SFG) under normal condition (controls) than those in the south, a result indicating that the northern mussels spend more energy on coping with osmotic stress. The exposure
study in **Paper I** further suggests that the northern mussels experience severe oxidative stress in response to toxic exposure, with severest effects recorded in the petrol treatment. Hence, even though the salinity difference between the northern and southern Baltic Proper is only about 2 psu, the overall results of **Paper I** shows that it has large effects on the basal health in blue mussels and on their handling of additional stress. These physiological differences between mussels from the northern and southern Baltic Proper were not however manifested as genetic differences in neutral loci (**Paper III**). This highlights that there is not a simple relationship between the health of the mussels and genetic diversity, at least not in the 7 neutral markers analyzed in **Paper III**. However, markers of neutral loci only reveal the neutral variation, and not the other important part, which is adaptive variation (Lowe et al. 2004). Still, our studies show that the *degree* of ecological marginality and stress in the environment highly influences the tolerance of the mussels. Since Baltic blue mussels are so abundant and the amount of water that they filtrate per year corresponds to the total water volume above the halocline of the Baltic Sea (estimated from Kautsky and Wallentinus 1980, Kautsky 1981), they are the major significant contributors of the key function filtration in this ecosystem. Therefore, the difference in stress tolerance between mussels in the northern and southern Baltic Proper will have to be considered from a pollution management perspective along the Baltic salinity gradient. The higher susceptibility of mussels in the less saline north, implies the enforcement of a stricter pollution regulation in these more stressful, and consequently, more vulnerable areas.

**Correspondence of lower level response and higher level effects**

A major aim of environmental management is protecting populations and ecosystems. Hence, we are constantly searching for the optimal tools to assess the effect of anthropogenic-induced stress. In both monitoring and ecotoxicological tests for risk assessment it is optimal to include and measure signals that are early and sensitive, as well as ecologically relevant. A great deal of interest has been directed towards biomarkers (Huggett et al. 1992). In order to use these in risk assessment we initially have to establish whether there is an actual link between responses on the lower level of biological organization and effects on higher levels. In **Paper I** we found that this was the case for two out of the four molecular approaches used (cellular response), and for SFG (individual). We further established a link between the protein profile (individual) and survival rate (population) in the dogwhelk *Nucella lapillus* (**Paper IV**). The advantage of using a proteomic approach is that it has a much wider “effect window” than a few single biomarkers; the proteins are the phenotypic expression of the genes and the proteome, in a way, mirrors the functional genetic diversity of an organism. In **Paper V** it was further found that both the estimate of growth
(individual), i.e. RNA content in copepods, and genetic diversity (population) decreased due to toxic exposure during ca. 1 generation. However, in Paper VI we saw that exposure to other toxicants for 2-3 generations did not cause the same pattern between RNA content and genetic diversity in another copepod species. This emphasizes the importance of re-evaluating tools for each new species analyzed and the importance of investigating how the exposure time affects the studied endpoints.

Genetic effects of toxic exposure

My studies have shown that there is no linear relationship between stress and population genetic effects. Exposure to a single toxicant decreased the genetic diversity in exposed copepod populations (Paper V and VI), in line with many other exposure studies in aquatic systems (e.g. Murdoch and Hebert 1994, Street and Montagna 1996, Street et al. 1998, Ma et al. 2000, Ross et al. 2002). This was most likely due to directional selection for tolerant individuals. The results of these two papers further show that even though a single toxicant can reduce genetic diversity, abundance may still be unaltered. This implies that populations studied using standard course metrics such as abundance, could be perceived as unaffected by pollutant exposure, while they actually may have reduced adaptive capacity, thus having reduced resistance to further perturbations. Consequently, the gradual impoverishment of genetic variation could make the affected populations more fragile and less resilient, and unexpected ecological surprises could appear since no reductions in their abundances have been observed as early warning signals.

The naturally contaminated sediments to which the copepods were exposed in Paper VI contained a mixture of toxicants. This multi-toxicant exposure did not decrease genetic diversity, however, it increased the genetic divergence within the treatments ($F_{ST}$), meaning that the genetic partitioning analyzed in copepods collected at the termination of the experiment was very different between the replicates of the respective treatment. At the first sampling occasion there were very low abundances in the two 100% naturally contaminated sediments (Svindersviken100% and Trosa100%). These two treatments further had the largest within treatments differentiation ($F_{ST}$). High differentiation is often a result of a small effective population size, which is the number of individuals carrying a particular allele to the next generation and not the absolute population size (Newman 2001). It thus seems likely that high selective mortality against highly sensitive genotypes in these treatments decreased the effective population size, resulting in drift, which in turn caused the large differentiation recorded in these two naturally contaminated sediments. The copper treatment also had higher $F_{ST}$ compared to the controls but not as high as the treatments with naturally contaminated sediments. Possibly, the directional selection imposed by the copper was so strong that it shadowed the drift effects.
In **Paper III** we further suggest that one possible explanation to the genetic divergence in blue mussels seen between sites is also due to genetic drift. Two of the three divergent sites are located in the north, where the mussels were generally more stressed than in the south (**Paper I**). Although not statistically significant, the within area comparisons revealed a larger mean differentiation (mean $F_{ST}$ within respective area) in the north compared to the south. Since the null allele frequency was equally large in mussels from both these areas, these results could possibly also be explained by smaller effective population size. The very low absolute abundance of blue mussels in the north together with the stressful environment compared to in the south support this. The harsh northern environment could further prevent immigration of larvae and larvae survival at some years in some sites. **Paper III** and **VI** hence suggest that stress can differentiate populations.

**Conclusion**

Taken together, the studies presented in this thesis highlight the multidimensional nature of stress, affecting various biological levels differently. Therefore, risk assessment should cover effects in more than one level in the biological hierarchy. My studies imply that if abundance is solely used as a measure of a population’s sustainability, populations could be perceived as unaffected by pollutant exposure, while they might in fact be more fragile and less resilient due to altered adaptive capacity. However, assessments cannot solely be focused on the genetics of the organisms either since a populations ability to cope with toxicant exposure also depend on the background stress they experience in their environment, not necessarily detected in studied genetic loci.

Genetic diversity is the fundamental organizational component of biodiversity, since it sets the limits for the protein expression, i.e. the phenotypic response (function). The fitness of different phenotypes determines the frequency of genotypes in the next generation (structure). Thereby, the genetic level of biodiversity affects the sustainability of species and populations and ultimately ecosystems. The increasing biodiversity loss due to anthropogenic activities, referred to as “the sixth extinction”, is consequently likely underestimated since the focus has mainly been on species diversity and not on genetic recourses.
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