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# Genetic Aspects of Environmental Disturbances in Marine Ecosystems

## Studies of the Blue Mussel in the Baltic Sea

Josefine Larsson

SÖDERTÖRN DOCTORAL DISSERTATIONS

# Genetic Aspects of Environmental Disturbances in Marine Ecosystems

## Studies of the Blue Mussel in the Baltic Sea

*Josefine Larsson*

Subject: Environmental Science
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To Ilon & Stefan

*Nothing in biology makes sense except in the light of evolution*

Dobzhansky, 1973

**Abstract**
Anthropogenic environmental changes can serve as drivers for evolutionary responses in wild populations. To predict the long-term impact of anthropogenic changes on populations, it is crucial to understand the genetic effects caused by these disturbances. The Baltic Sea is considered to be one of the world's most contaminated seas, and the increase of anthropogenic chemical pollution is a major threat to its ecosystems. This thesis assesses the impact of harbors and sewage treatment plants on physiological traits and genetic structure of resident populations of blue mussels at replicated sites in the Baltic Sea.

The initial evaluation of the overall genetic pattern in blue mussel populations in the Swedish West Coast, the Baltic Proper and the Bothnian Sea found genetic differentiation between the three water basins and a low genetic differentiation within each basin, especially within the Baltic Proper. Despite the low genetic differentiation among blue mussels within the Baltic Proper, a parallel genetic differentiation associated with sewage treatment plant effluents was found in this basin. This included genomic regions with a high degree of differentiation between reference sites and sites affected by sewage plants effluent. This genetic differentiation is suggested to be due to post-dispersal selection acting in each generation. In contrast, no parallel genetic differentiation was associated with harbors. We identified five genomic regions in blue mussels, showing strong signs of selection, shared among three out of four replicated reference sites and sites affected by sewage effluents in the Baltic Proper i.e. Askö, Tvärminne and Karlskrona. An initial characterization of these genomic regions revealed functions related to immune and endocrine responses, oxidative stress and shell formation. Our results indicate that selection caused by sewage effluents involves multiple loci. The same genomic regions are found across different locations in the Baltic Proper but there are also unique genomic regions at each location. No genotoxic or histopathological effects was found among blue mussels from sewage effluent-affected areas but a higher frequency of histological abnormalities in the digestive gland were observed in mussels from harbors. However, mussels from both sewage effluent-affected sites and harbors, had a higher body mass index, gonadosomatic index and lipid content (mussels from harbors only) compared to mussels from reference sites. The strongest toxicity effects were found in resident mussel populations in the Gulf of Gdańsk, Poland regardless of pollution type. A generally higher genetic differentiation between the reference and sewage effluent-affected site as well as higher genetic differentiation between the Polish sites and the other sites was found which indicates that higher levels, or more complex mixtures, of pollution is present in the Gulf of Gdańsk.

**Keywords:** Blue mussel, Baltic Sea, anthropogenic disturbance, pollution, sewage effluents, harbors, genetics, genomics.

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# List of papers

This thesis is based on the following papers referred to in the text by the Roman numerals (I–IV):

**Paper I:** Larsson, J., Lind, E.E., Corell, H., Grahn, M., Smolarz, K. and Lönn, M., 2017. Regional genetic differentiation in the blue mussel from the Baltic Sea area. *Estuarine, Coastal and Shelf Science*, *195*, pp.98–109.

**Paper II:** Larsson, J., Lönn, M., Lind, E.E., Świeżak, J., Smolarz, K. and Grahn, M., 2016. Sewage treatment plant associated genetic differentiation in the blue mussel from the Baltic Sea and Swedish west coast. *PeerJ*, *4*, p.e2628.

**Paper III:** Larsson, J., Smolarz, K., Świeżak, J, Turower, M., Czerniawska, N., Grahn, M. Multi biomarker analysis of pollution effect on resident populations of blue mussels from the Baltic Sea (Submitted to *Aquatic toxicology*).

**Paper IV:** Larsson, J., Grahn, M., Wheat C.W. A population genomic analysis of blue mussels identifies genomic regions associated with sewage treatment plant effluents in the Baltic Sea. (Manuscript)

## Contributions of the author to the different manuscripts

Planning and designing the study (Paper I–IV), collecting the data (Paper I–IV), performing genetic analyses (Paper I, II and IV), analyzing data (Paper I–IV) and writing the papers as main author (Paper I–IV). Papers I and II are reprinted with the kind permission from the publisher.

# Abbreviations

| | |
|---|---|
| Amplified fragment length polymorphism | (AFLP) |
| Basic Local Alignment Search Tool | (BLAST) |
| Body mass index | (BMI) |
| Cetyl trimethylammonium bromide | (CTAB) |
| Cochran–Mantel–Haenszel | (CMH) |
| Constrained principal coordinate analysis | (cPCoA) |
| Gonadosomatic index | (GSI) |
| Harbor | (HAR) |
| Helsinki Commission | (HELCOM) |
| Isolation by distance | (IBD) |
| Markov Chain Monte Carlo | (MCMC) |
| Mining Exons in Scaffolding-Poor Assemblies | (MESPA) |
| Polymerase chain reaction | (PCR) |
| Principal component analysis | (PCA) |
| Reference | (REF) |
| Sewage treatment plants | (STP) |

# Introduction

Human activities are constantly contributing to environmental changes in almost all natural environments, including large-scale changes, such as a global increase in temperature and ocean acidification together with activities that release nutrients and chemicals into different ecosystems (Vitousek et al., 1997; Palumbi, 2001). Over the course of the last centuries, the increase of activities releasing anthropogenic aquatic pollutants has created a stressful environment for many organisms, and the primary source of such pollutants include industries, sewage water treatment plants, shipping, oil drilling, aquacultures, and land use changes (van Straalen, 2003; Walker et al., 2006). The consequences of anthropogenic pollutants can be severe both on the population level and the ecosystem level. Depending on the strength of an anthropogenic pollutant, it can cause population decline leading to extinction, population persistence mediated by physiological or behavioral plasticity, or adaptation through natural selection (Chevin et al., 2010; Banks et al., 2013; Whitehead, 2014). The effects on the ecosystem level can include elimination of sensitive species due to direct toxic effects, replacement of a sensitive species with a more resistant species, or food web shifts due to changes in grazing/predation patterns among organisms (Medina et al., 2007). There are several examples where anthropogenic environmental changes have served as drivers for evolutionary responses in different taxa (Whitehead, 2014). To understand the long-term impact of anthropogenic disturbances on populations, it is crucial to gain knowledge of how these changes affect the genetic structure in populations and that in the end may cause evolutionary changes and ecosystem shifts. This thesis assesses the impact of environmental pollution on physiological traits and the genetic structure in resident and naturally occurring populations of blue mussels in the Baltic Sea.

## Phenotypic plasticity and natural selection driven by anthropogenic pollution

To survive in a changing environment, organisms must have the ability to effectively regulate their biochemical and physiological traits, and these responses can be affected by phenotypic plasticity and/or the genetic constitution of the individual (Bickham et al., 2000). Phenotypic plasticity, defined as the capacity of a genotype to give rise to different phenotypes depending on the environment, allows individuals in a population to handle different environmental changes without genetic change (West-Eberhard, 2003) and to survive and reproduce in a wider range of environments (Thibert-Plante & Hendry, 2011). Natural selection acts on genetic variation,

and the most successful alleles and combinations alleles in a certain environment will increase in frequency, and the genotype carrying these alleles will have a higher viability and fecundity and thus contribute more to subsequent generations. Without natural selection, all genotypes would contribute randomly to the next generation (Allendorf & Luikart, 2009). The ability of a population to respond to environmental changes is in most cases dependent on the standing genetic variation within the population because high genetic variation in a population makes it more likely that alleles beneficial in a changing environment are present in the gene pool (Lowe et al., 2004; Bell & Gonzales, 2009; Allendorf & Luikart, 2009). Strong selective pressure can cause an overall reduction in genetic variation, thus reducing the ability for a population to handle further changes in the environment (Ma et al., 2000; van Straalen & Timmermans, 2002). There are several examples where evolutionary responses in wild populations have been linked to anthropogenic environmental changes (reviewed in Palumbi, 2001; Smith & Bernatchez, 2008). One of the classical examples of rapid evolution driven by anthropogenic pollution is the adaptation of plants living in soils contaminated by heavy metals (Antonovics & Bradshaw, 1968; Smith & Bernatchez, 2008). Studies in the aquatic environment have documented genetic differentiation and/or difference in genetic variation between pollution-affected sites (for example harbors/industrial sites or sites contaminated by heavy metals) and non-polluted reference sites (Ma et al., 2000; Williams & Oleksiak, 2011; Giantsis, et al., 2012; Bach & Dahllöf, 2012; Mussali-Galante et al., 2014; Laporte et al., 2016; Reid et al., 2016).

Connectivity and gene flow between populations play an important role in shaping the genetic structure of a species as well as in the process of local adaptation by natural selection (Palumbi, 1994). Natural environments are heterogeneous and have strong spatial variation in terms of both biotic and abiotic factors. Natural selection calibrates a population to their local environment resulting in resident genotypes with a fitness advantage in local conditions, which is referred to as local adaptation (Sanford & Kelly, 2011). However, high connectivity, migration and gene flow will counteract effects of local adaptation as the immigration of non-adapted genotypes from populations outside the local conditions tend to mix with and swamp out locally adapted genotypes (Lenormand, 2002; Sanford & Kelly, 2011). Local adaption has mostly been studied in terrestrial and limnic environments, because these are often characterized by restricted connectivity and gene flow (Sanford & Kelly, 2011). In contrast, local adaptation in the marine environment has raised relatively little attention because the traditional view of marine populations has been that they are demographically open populations that are interconnected by high gene flow. This view mainly stems from the fact that connectivity barriers in the marine environment are less apparent compared to barriers on land and in limnic environments and because many marine organisms have long planktonic stages, with free-swimming larvae that spend hours to weeks in the water column (Paris et al., 2007; Sanford & Kelly, 2011). This, together with passive dis-

persal by ocean currents and large populations, has traditionally been assumed to result in high connectivity and low genetic differentiation over long distances in marine environments (Bohonak, 1999; Kelly & Palumbi, 2010; Sanford & Kelly, 2011). However, gradients in environmental factors such as temperature, salinity, pollutants, etc. can have significant impacts on the connectivity and local adaptation. Over the last decade, several studies have revealed genetic structuring on small geographical scales even in marine organisms with a long pelagic larval stage (Barber et al., 2002; Gilg & Hilbish, 2003; Knutsen et al., 2003; Kelly & Palumbi, 2010; Sanford & Kelly, 2011) and in populations of species experiencing high gene flow (Lamichhaney et al., 2013; Guo et al., 2016; Laporte et al., 2016).

## Studying responses to anthropogenic pollution

In order to understand the ability of natural populations to respond to different environments, experimental approaches ranging from standardized tests and single species experiments (Breitholz et al., 2006) to multi-biomarker approaches in field-based experiments have been used. These multi-biomarker approaches often include biochemical, morphological, and physiological measurements representing different biological functions and levels of organization (Brooks et al., 2009; Lam 2009; Bignell et al., 2011; de los Rios et al., 2012; 2013; Turja et al., 2013; 2014; 2015). However, because the susceptibility or tolerance of an organism to environmental stressors is altered by phenotypic plasticity and/or genetic constitution of individuals and populations (Chevin et al., 2010; Banks et al., 2013; Whitehead, 2014), a combined approach including both phenotypic measurements and genomic data (including genetic composition and genetic variation) will yield the most informative results (Savolainen et al., 2013).

Anthropogenic changes can induce genetic effects ranging from a few loci that are strongly affected by selection contributing to a large part of the genetic variation for a certain trait, both within and between populations (Nosil et al., 2009; Schluter et al., 2010; Jones et al., 2012), to polygenic traits based on many loci each with small effects (Yeaman, 2015; Pavey et al., 2015; Laporte et al., 2016). Recent theoretical and empirical studies have shown that even in the face of considerable gene flow and no differentiation at neutral loci, selection from environmental heterogeneity can still result in adaptation (Nosil et al., 2009; Michel et al., 2010; Yeaman & Whitlock, 2011; Feder et al., 2012; Guo et al., 2016). This is because different regions across the genome will show patterns of variability where some genomic regions are more affected by genetic drift and gene flow, and less by selection, while other regions (or regions linked by linkage disequilibrium) are more strongly influenced by selection (DeFaveri et al., 2013; Nosil et al., 2009).

To understand the genetics of different traits involved in adaptation, genetic markers such as microsatellite markers and/or candidate genes are widely used. However, most microsatellite markers are selectively neutral and mainly provide

insights about neutral processes such as reduced gene flow and connectivity disruption (Stinchcombe & Hoekstra, 2008) and might therefore be less suitable for studies of selective processes. The use of candidate genes, i.e. genes with known important functions for the particular selection regime, is a useful method to study selection. Although a lack of genomic information in many organisms makes it difficult or even impossible to develop such markers, advances in sequencing technology have increased the opportunities for studies scanning a large proportion of a species' genome. This has increased the opportunities to identify genomic regions that are under selection, and as the proportion of the genome that is scanned increases, the chance of finding genomic regions that differ also increases. Several population genomic studies of marine fish have provided more in depth sequence data that have provided new insights into adaptive differentiation and the footprints of selection across habitats in wild populations (Nosil et al., 2009; Bradbury et al., 2010; Gagnaire et al., 2012; Lamichhaney et al., 2012; Corander et al., 2013; Hess et al., 2013; Karlsen et al., 2013; Ulrik et al., 2014; Jackson et al., 2014; Guo et al., 2015; Laporte et al., 2016; Guo et al., 2016). The high throughput sequencing techniques create opportunities to better understand the evolutionary impact of pollutants in the marine environment, but still few studies has used genomic approaches in this context (Laporte et al., 2016; Reid et al., 2016).

# Aims of the thesis

The aim of this thesis was to assess the impact of environmental pollution on the physiological traits and population genetic structure of resident and naturally occurring populations the blue mussels in the Baltic Sea. The thesis focuses on two common types of point sources of pollution, harbors and sewage treatment plants, in the Baltic Sea and Skagerrak. This work was carried out through a multidisciplinary approach combining elements of ecology, evolutionary biology, oceanography, and ecotoxicology, including a nested sampling scheme (using sites from reference habitats, geographically paired with sites from sewage treatment plants and harbors), a multi-biomarker approach and both genetic and genomic analyses.

The specific objectives were to investigate:

i. The genetic structures and barriers to gene flow in blue mussels within the Baltic Sea and between the Swedish West Coast (Kattegat and Skagerrak) and the Baltic Sea.
ii. How two types of point sources of environmental pollution - sewage treatment effluents and harbors – affect the genetic diversity and differentiation in resident blue mussel populations in the Baltic Sea and the Swedish West Coast (Skagerrak and if the point source of pollution affects the condition of the resident blue mussels and if it has genotoxic effects on the mussels. An additional question is whether geographic effects not explained by differences in pollution type can be identified.
iii. The effect of the species introgression pattern of *Mytilus trossulus* and *Mytilus edulis* on the overall genetic structure and on the frequency of micronuclei and histological lesions in combination with pollution type.
iv. The detailed genetic effects of sewage effluents by using a pool sequencing approach to identify genomic regions that are potentially involved in the observed genetic differentiation.

# Study system

## The Baltic Sea

The Baltic Sea is an extension of the Atlantic Ocean spanning from Öresund and the Danish Belt in the southwest to Northern Finland and Sweden in the north (Figure 1).

The Baltic Sea is a shallow (mean depth of 57 m), inland, semi-enclosed sea, resulting in limited water exchange with the ocean. It receives inflow from over 200 rivers creating large-scale environmental gradients ranging from temperate marine conditions to subarctic limnic conditions (Leppäranta & Myrberg, 2009). The Baltic Sea is one of the largest brackish water bodies in the world (Snoeijs-Leijonmalm & Andrén, 2017). The history of the Baltic Sea began ~20,000 years before present (BP) and has included one period of total glacial cover, and since the end of the last glaciation, ~12,000 years BP, the Baltic Sea has gone through several alternating freshwater and brackish-water stages (Andrén et al., 2011; Snoeijs-Leijonmalm & Andrén, 2017). The largest inflow of salt water occurred ~8000 years BP and was the start of the stage called the Littorina Sea, and since then the salinity has gradually been diluted by freshwater inflow from rivers. The current brackish stage of the Baltic Sea was established approximately 2000–3000 years BP and has been more or less stable since then (Andrén et al., 2011; Snoeijs-Leijonmalm & Andrén, 2017).

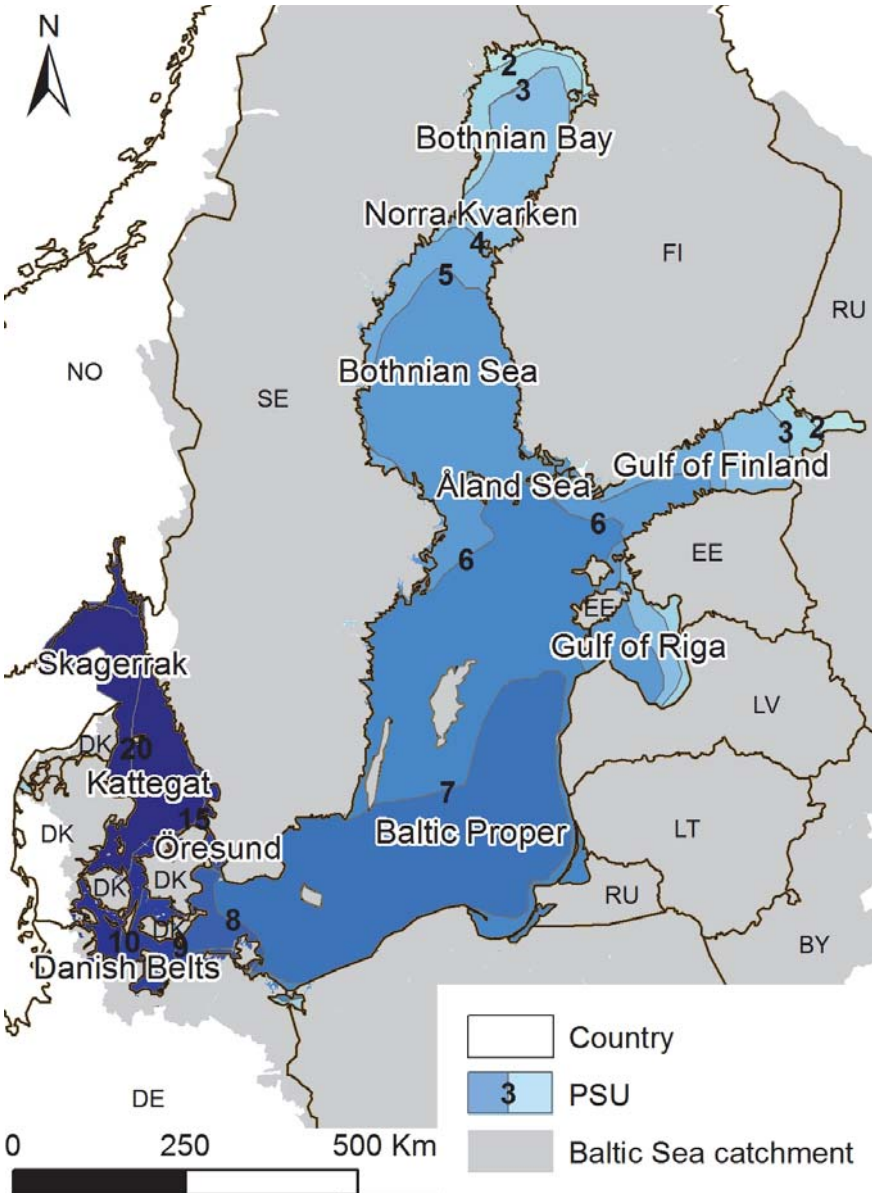


**Figure 1:** The Baltic Sea with the basins, landmarks and the surface water salinity gradient (PSU). The catchment area indicated in light grey. Countries are denoted with country codes, Norway (NO), Sweden (SE), Finland (FI), Russia (RU), Estonia (EE), Latvia (LV), Lithuania (LT), Belarus (BY), Denmark (DK). Data source: Country data 2014 (@EuroGeo–graphics); Baltic Sea basin data (ICES Dataset on Ocean Hydrography, 2014); Catchment area data (HELCOM); salinity data (modified from SHMI modeled data (1989–2015) using E.U. Copernicus Marine Service Information).

The Baltic Sea consists of four major sub-regions. The Baltic Sea Proper (Baltic Proper) is located from the entrance at Öresund and the Danish Belts up to the Åland Sea, the Bothnian Sea is located between the Åland Sea and Norra Kvarken, Bothnian Bay is located from Norra Kvarken northwards, and the Gulf of Finland is an eastern going extension of the Baltic Proper (Figure 1). The sub-regions are separated by sills (except for the Gulf of Finland) and narrow extensions, and the salinity spans from 17 PSU to 25 PSU along the Swedish West Coast, to 5.5 PSU to 7.3 PSU in the Baltic Proper, approximately 5 PSU in the Bothnian Sea, and to 2 PSU to 4 PSU in the Bothnian Bay and Gulf of Finland (Fig 1). The salinity gradient is caused by inflow of marine water from the North Sea in the southwest through the narrow and shallow sills in Öresund/ the Danish Belts together with the freshwater inflow of large river inputs (Elmgren, 2001; Leppäranta & Myrberg, 2009; Snoeijs-Leijonmalm & Andrén, 2017). Various factors such as long-term tectonic movements and glacial processes together with on-going wind and coastal processes create different coast types along the coasts of the Baltic Sea. Sandy coasts dominate the southern Baltic Sea while in the northern areas the coats are dominated by an with rocky shores (Snoeijs-Leijonmalm & Andrén, 2017). These features together with the history of the Baltic Sea affect the distribution of species and influences the evolutionary processes in the Baltic Sea.

## Organisms in the Baltic Sea

Many of the organisms with marine origin were introduced to the Baltic Sea during the Littornia stage and have since adapted to the decreasing salinity. Organisms inhabiting the Baltic Sea are all post-glacial immigrants that originate from either marine or freshwater environments and have entered the Baltic Sea either with currents from Kattegatt or from rivers and streams connected to lake systems (Snoeijs-Leijonmalm & Andrén, 2017). The species distribution follows the salinity gradient with more species with marine origin in the southern Baltic Sea and around the belts, and more freshwater species in the Bothnian Bay (Snoeijs-Leijonmalm & Andrén, 2017). However, recent molecular data have shown a more complex pattern, where some taxa evolved in isolation in the Atlantic or Pacific Ocean or in different freshwater refugia's during the glacial times. These populations might represent early post-glacial arrivals or recently introduced cryptic species co-existing either with early arrivals or other introduced species (Väinölä & Johannesson, 2017). Nevertheless, the adaptation of species to the brackish conditions (i.e. to be able to live on the edge of their natural niche) in the Baltic Sea has caused both morphological and genetic differences compared to their saltwater or freshwater ancestors (Kautsky & Evans, 1987; Johannesson & André, 2006; Johannesson et al., 2011). The area around Öresund (Figure 1) represents the transition zone between the Baltic Sea and the Danish Belt Sea/Kattegatt. The salinity

decrease dramatically in this zone, and this zone represents the distributional limit for many marine species (Väinölä & Johannesson, 2017). Many Baltic Sea populations constitute unique evolutionary lineages with genetic differentiation between populations in Skagerrak/Kattegat and the Baltic Sea and with lower genetic diversity among Baltic Sea populations (Johannesson & André, 2006; Johannesson et al., 2011). Due to the salinity gradient, comparably few species have adapted to the Baltic Sea, and a few key species dominate their respective functional groups making the Baltic Sea a vulnerable ecosystem with low resilience (Snoeijs-Leijonmalm & Andrén, 2017; Johannesson et al., 2011). Examples of key species are the piscivorous Atlantic cod *Gadus morhua* and two planktivorous clupeids – the Atlantic herring *Clupea harengus* and the European sprat *Sprattus sprattus*. In the benthic zone, examples of key species are the habitat-forming macrophytes, the bladderwrack *Fucus vesiculosus*, and the common eelgrass *Zostera marina* as well as the filter feeders the blue mussel *Mytilus* sp. and the sand gaper *Mya arenaria*. Even if these species run limited risk of become extinct, threats including e.g. over-exploitation of the fish stocks by humans, habitat destruction in coastal areas, and chemical pollution are increasing (Snoeijs-Leijonmalm & Andrén, 2017).

## Anthropogenic impact on the Baltic Sea

The Baltic Sea has always been an important resource for humans, and the human population increase in the region has been accompanied by the expansion of agriculture, forestry, urbanization and industrialization. The Baltic Sea has one of the largest drainage areas (relative to its surface area) in the world at 1.7 million km$^2$, and more than 200 rivers discharge into the Baltic Sea (Snoeijs-Leijonmalm & Andrén, 2017) (Figure 1). Today approximately 85 million people live in the Baltic Sea drainage area, 10% in the northern part, 15% in the eastern and 75% in southern part of the Baltic Sea. Almost 18% live within 10 km of the coast (HELCOM, 2010). The large population within the drainage area and on the coast has led to an increased discharge of nutrients and anthropogenic chemical pollution (Ducrotoy & Elliott, 2008; Rönnberg & Bonsdorff, 2004; Jansson & Dahlberg, 1999). The increase in nutrients causes eutrophication, or accelerated primary production, that in turn causes increased frequency and intensity of algal and cyanobacterial blooms and reduced water transparency. The increased eutrophication has led to changes in food web composition and caused hypoxia and even complete anoxia in large areas of the Baltic Sea (Laamanen et al., 2017; Snoeijs-Leijonmalm & Andrén, 2017).

The Baltic Sea is considered one of the most contaminated seas in the world, and the increase of anthropogenic chemical pollution has been identified as a major threat to the Baltic Sea ecosystem (Lehtonen et al., 2017). The main sources of hazardous substances include atmospheric deposition, industrial waste, diffuse run-off from land, river discharge, shipping accidents, leakage from ship hulls, and discharges from sewage treatment plants. The enclosed position, large discharge

volume, and shallow nature, together with the long water retention time (30–40 years) makes the Baltic Sea particularly susceptible to these disturbances (Elmgren, 2001; Lehtonen et al., 2017). Many hazardous substances bind to the sediment and are not removed from the Baltic basins by water exchange (Lehtonen et al., 2017).
Sewage treatment plants are one of the primary point sources of both eutrophication and anthropogenic pollution in aquatic environments in general and particular in the Baltic Sea (Lehtonen et al., 2017). Sewage treatment plants process wastewaters from industry and households (Walker et al., 2006), and although the efficiency of the sewage treatment plants has increased over the last 20 years (Bolong et al., 2009; HELCOM, 2010) several compounds such as pharmaceuticals, personal care products, and surfactants are still not fully eliminated from the effluents (Rule, 2006; Bolong et al., 2009; Rosi-Marshall & Royer, 2012; Lehtonen et al., 2017). Many of these substances cause sub-lethal toxic effects at low concentrations in invertebrates and fish (Nentwig, 2007; Corcoran et al., 2010; Brodin et al., 2013). Exposure to sewage waters has been shown to cause several negative biological responses including, genotoxic effects, lysosomal responses (Turja et al., 2015; Lehtonen et al., 2017), and effects on the immune system (Akaishi et al., 2007). Sewage effluents have been shown to cause endocrine disrupttive effects (Bouchard et al., 2009; Gagné et al., 2011; de los Rios et al., 2013), including feminization in freshwater mussels (*Elliptio complanata*) (Gagné et al., 2011) and in roach (*Rutilus rutilus*) (Jobling et al., 1998). Effects on the genetic composition caused by sewage effluents have been addressed by a few studies. Puritz and Toonen (2011) showed that runoff from sewage treatment plants and storm water act as a barrier to larval dispersion, resulting in genetic differentiation among populations of bat star (*Patiria miniata*) in the Southern California Bight (Puritz & Toonen, 2011). In contrast, in a study of roach (*R. rutilus*) living in rivers that are highly polluted by sewage effluents, Hamilton and colleagues (2014) found no pollution-associated population genetic effects despite the high feminization rate of males (see Wedekind, 2014; Hamilton et al., 2014).

Other important and common sources of pollutants are harbors and shipping routes. The Baltic Sea has one of the busiest shipping routes in the world with approximately 10,000 unique vessels registered in 100 countries traveling through the Baltic Sea every year (Snoeijs-Leijonmalm & Andrén, 2017). Harbors and shipping routes are often affected by high concentrations of heavy metals and various kinds of persistent organic pollutants (i.e. polycyclic aromatic hydrocarbons, polychlorinated biphenyls, polychlorinated dibenzodioxins/furans, polybrominated biphenyls, and polybrominated biphenyl ethers) (Ma et al., 2000; Walker et al., 2006; Smolarz & Berger, 2009; Turja et al., 2013; 2014). Due to their ubiquitous occurrence, slow biodegradation, high toxicity, bioaccumulation, biomagnification, and mutagenic and carcinogenic features, these substances they are dangerous for the aquatic ecosystem (Baumard et al., 1998; Ahlf & Heise, 2005). Several ecotoxicological field studies have been carried out in different coastal areas of the Baltic

Sea, and these studies have shown that negative effects in multiple biomarkers coincide with observed concentrations of environmental chemicals, including DDTs, PCBs and heavy metals (Schiedek et al., 2006; Höher et al., 2012; Dabrowska et al., 2013; Turja et al., 2013; 2014). Also, genetic differentiation and/or differences in gene diversity between organisms inhabiting industrial sites, harbors, and shipping routes compared to reference sites have been correlated to substances found at these sites (Ma et al., 2000; Williams & Oleksiak, 2008; Giantsis et al., 2012; Bach & Dahllöf, 2012; Reid et al., 2016).

## Study organism: blue mussel

One of the key organisms in the Baltic Sea ecosystem is the filter feeding blue mussel (*Mytilus* sp.) (Väinölä & Strelkov, 2011). Their distribution is limited by the salinity and the blue mussel can be found up to the northern part of the Bothnian Sea (salinity around 4 PSU) and into central parts of the Gulf of Finland (salinity around 4.5 PSU) (Westerbom et al., 2002). The blue mussel is highly abundant along the western coast of the Baltic Proper and biomass of approximately 300 g dry weight/m2 is common (Jansson & Kautsky, 1977). The blue mussel serves several ecologically important functions for example, filtering the water, forming habitats for other species, and acting as a food source for many fish and bird species (Kautsky & Evans, 1987; Koivisto, 2011). The filtering capacity of one blue mussel individual have been estimated to be 5 liters of sea water per hour, hence, theoretically, the blue mussel population could re-circulate the entire water column of the Baltic Sea four times a year. The filtering of water is an important link in the food chain because it connects the benthic and the pelagic zones and recirculates nitrogen and phosphorus (Kautsky & Wallentinus, 1980; Kautsky & Evans, 1987).

The blue mussel also serves as a bio-indicator species that is important for research and bio-monitoring (Viarengo & Canesi, 1991). Mussels are good bio-indicators because they are i) sedentary (i.e. reflect local conditions), ii) abundant and widely distributed, iii) filter-feeders and thus take up pollutants, iv) bioaccumulators, v) responsive to sub lethal levels of pollutants, vi) suitable for both field- and experiment-based studies and vii) relatively easy to sample and handle. Due to their sedentary lifestyle, mussels will be exposed to pollutants and at the same time be unable to move away from the contamination (other than temporarily by closing their shell) (Widdows & Salkeld, 1993).

The blue mussel is gonochorous, i.e. it has separate sexes and fertilization is external. The reproduction of Baltic Sea blue mussel populations follows a seasonal pattern, with gametogenesis during winter and spawning that takes place between May and July. The timing of reproduction is also correlated to environmental factors such as temperature and food availability, and a delayed spawning has been observed in northern populations (Kautsky & Evans, 1987). The blue mussel has an

expected lifespan of up to 12 years and a generation time of approximately 1–2 years (Kautsky, 1982).

## The blue mussel in the Baltic Sea

The blue mussels arrived across the Bering Strait from the Pacific Ocean to the Atlantic Ocean approximately 2–5 million years ago. The subsequent isolation of the lineages has accumulated differences that allow the blue mussels to be genetically distinguished as Atlantic Ocean (*M. edulis*) Pacific Ocean (*M. trossulus*) species. However, indications of repeated trans-Arctic invasions of Pacific mollusks into the Atlantic basin, even post-glacially, have been found (Väinölä & Johannesson, 2017). Pacific *M. trossulus* have been found along the coast of Norway, Scotland and Northern Russia and in the Baltic Sea. In the Baltic Sea area (Baltic Sea, Kattegat and Skagerrak) both sister species of Mytilus occurs, the Atlantic *M. edulis* is the dominating species in the Kattegat and Skagerrak and the Pacific *M. trossulus* is the dominating species in the Baltic Sea (McDonald et al., 1991; Väinölä & Hvilsom, 1991; Johannesson & André, 2006). Both morphological and genetic differences between Baltic Sea blue mussels compared to their North Sea counterparts have been observed (Theisen, 1978; Johannesson et al., 1990; Kautsky et al., 1990; Tedengren et al., 1990; Väinölä & Hvilsom, 1991; Stuckas et al., 2009; 2017; Kijewski et al., 2011; Väinölä & Strelkov, 2011; Zbawicka et al., 2012; 2014). Morphological differences include a smaller size, thinner shell, weaker byssus treads, and reduced metabolic efficiency in blue mussels from the Baltic Sea (Kautsky et al., 1990; Tedengren et al., 1990). Recent studies have shown that the genome of the Baltic blue mussel has an extensive mixing of the *M. trossulus* and *M. edulis* genomes (Stuckas et al., 2009; Kijewski et al., 2011; Väinölä & Strelkov, 2011; Zbawicka et al., 2012). Most nuclear genes are from *M. trossulus* while, some genes have equal contributions of alleles from both species and the mitochondrial genome is from *M. edulis*. Today, the Baltic blue mussel behaves genetically as an independent local species that is distinct from the adjacent North Sea *M. edulis*, although in the transition zone they still interbreed (Zbawicka et al., 2014; Väinölä & Johannesson, 2017) and the best label to describe them are Baltic Mytilus or Baltic *M. trossulus edulis* (Väinölä & Strelkov, 2011). This phenomenon makes this species complex particular interesting for evolutionary and speciation research and quite extensive studies have been carried out regarding this issue (Stuckas et al., 2009; 2017; Kijewski et al., 2011; Väinölä & Strelkov, 2011; Zbawicka et al., 2012; 2014).

## Genetic differentiation within the Baltic Sea

The steep salinity change in the area around Öresund (Figure 1) represents the distributional limit for many species. For most species inhabiting the Baltic Sea, a genetic differentiation between the Baltic Sea and the Danish Belts/Kattegat (in the extension the North Sea) has been observed, and in most cases this genetic differen-

tiation is greater than the genetic differentiation within the Baltic Sea. The main driver for the genetic differentiation has, in most cases, been isolation by distance (Väinölä & Johannesson, 2017). The patterns of genetic differentiation vary both among species and among genetic markers. Species with strong homing behavior such as salmon (*Salmo salar*) exhibit strong genetic differentiation both when using neutral and selective markers (Nilsson et al., 2001; Koljonen et al., 2001; 2006). In other species, for example, the Atlantic herring (*Clupea harengus*) (Lamichhaney et al., 2012) and three-spined stickleback (*Gasterosteus aculeatus*) (DeFaveri et al., 2013), no genetic differentiation within the Baltic Sea has been identified using neutral markers, but when applying high resolution, non-neutral markers, geographic structuring can be seen. In the blue mussel, genetic differentiation among populations and basins within the Baltic Sea is less studied, and most existing studies have quite low resolution using few genetic markers and have looked at only one or a few sites, all within the Baltic Proper (Bulnheim and Gosling, 1988; Väinölä & Hvilsom, 1991; Johannesson et al., 1990). However, recent studies based on neutral microsatellite data (Gardeström et al., 2008) and neutral single nucleotide polymorphisms (SNPs) (Wennerström et al., 2013) suggests a generally high gene flow and low genetic differentiation between the different Baltic Sea regions i.e. Baltic Proper, Bothnian Sea and the Gulf of Finland (Gardeström et al., 2008; Wennerström et al., 2013).

# Material and methods

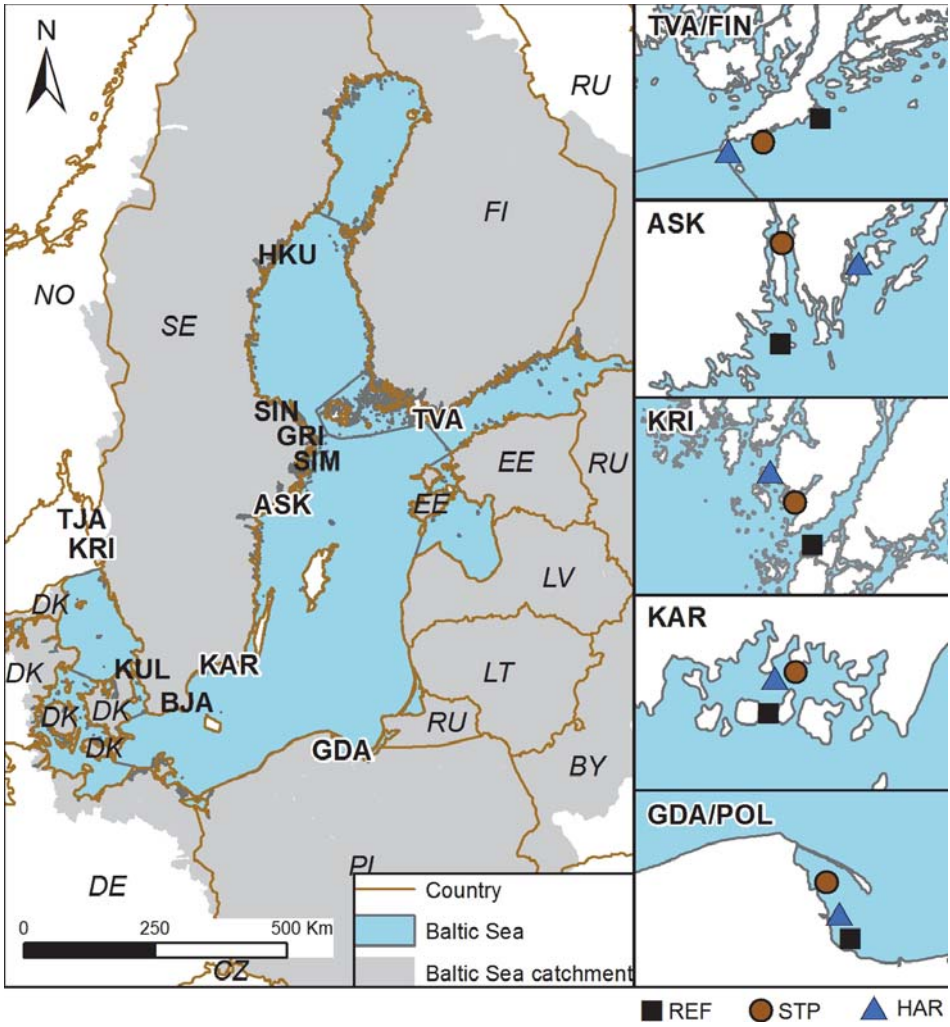


**Figure 2:** Sampling locations for Blue mussels within the Baltic Sea and off the Swedish West Coast. West Coast: Tjärnö (TJA), Kristineberg (KRI), Kullen (KUL). Baltic Proper: Bjärred (BJA), Karlskrona (KAR), Gdansk, Poland (GDA/POL), Askö (ASK), Tvärminne, Finland (TVA/FIN). Bothnian Sea: Simpnäs (SIM), Grisslehamn (GRI), Singö (SIN), Höga kusten (HKU). At location ASK, TV/FIN, KAR and GDA/POL three sites representing different pollution types were sampled; reference (REF) sites (black squares), sewage treatment plant (STP) effluent-affected sites (brown circles), and sites in the vicinity of harbors (HAR)(blue triangle). The light grey area indicates the catchment area of the Baltic Sea. Countries are denoted with country codes, Norway (NO), Sweden (SE), Finland (FI), Russia (RU), Estonia (EE), Latvia (LV), Lithuania (LT), Belarus (BY), Poland (PL), Denmark (DK). Data source: Country data 2014 (@EuroGeographics); Baltic Sea basin data (ICES Dataset on Ocean Hydrography, 2014); Catchment area data (HELCOM).

## Sampling

This thesis comprises four studies using blue mussels sampled from the Baltic Sea and the Swedish West Coast (Skagerrak).

In Paper I blue mussels were sampled at 12 sites along the Swedish West Coast, in the Baltic Proper and in the Bothnian Sea during June, July and August of 2012 and 2013. The West Coast sites were Tjärnö, Kristineberg, Kullen and Bjärred. The Baltic Proper sites were Karlskrona and Askö in Sweden, Tvärminne in Finland and the Gulf of Gdańsk in Poland. The Bothnian Sea sites were Simpnäs, Grisslehamn, Singö and Höga Kusten in Sweden (Figure 2).

In Papers II, III and IV individuals from the same sites were used. In this thesis, a nested sampling approach was used, including sites from reference (REF) habitats geographically paired with sites located close to a sewage treatments plants (STP) and in the vicinity of a harbor (HAR), i.e. within each location three sites representing different pollution types were chosen based on information from reports and local authorities' assessment schemes. All REF sites are situated within 5–30 km from the STP and HAR sites. The geographical distances between sites were estimated using the most direct marine route in Google Earth version 7.1.2.2014 (Google, 2014). Temperature regimes and salinity differ between the different regions and locations but are similar among the sampled sites in each of the regions (Leppäranta & Myrberg, 2009). Mussels were sampled during June 2012 and 2013. For Paper II we sampled mussels from three pollution types i.e. REF, HAR and STP at five locations, one location at the West Coast of Sweden, Kristineberg and in four locations in the Baltic Proper: Askö and Karlskrona, in Sweden, Tvärminne in Finland, and the Gulf of Gdańsk in Poland. In Paper III mussels from all three pollution types and all locations in the Baltic Proper except Karlskrona were used. In paper IV only the REF and STP sites from all the locations in the Baltic Proper (including Karlskrona) were used (Figure 2) and genetic data from mussels from the reference site in Tvärminne, Finland is used as reference in the Askö comparison. For the genetic studies (Papers II and IV), DNA from the same individuals was analyzed using different approaches.

In Papers I and II approximately 30 individuals from at each site were analyzed. In Paper III, 30 individuals/site were sampled for micronuclei and histology analyses and 90 individuals/site were sampled for physiological index and lipid content analyses. In Paper IV, 24 individuals were pooled from each site. The mussels were sampled using either a benthic sledge, a triangular bottom scraper or by hand, from a depth of between 1 and 13 meters depending on local distribution. The age of each individual was estimated by counting growth rings (Haskin, 1954) and all individuals included in this study had an estimated age of 2–5 years (3–5 years, Paper III).

# Genetic analyses

## Preparation of samples

The adductor muscle was dissected from each individual. The tissue was snap frozen and immediately stored at -80°C prior to DNA isolation (Papers I–IV). To avoid batch/site bias, all samples were randomly ordered prior to DNA isolation. Total genomic DNA was isolated from a small piece of the muscle tissue of each individual using the E.Z.N.A. Mollusc DNA Kit (OMEGA Bio-Tek, Norcross, GA, USA) with minor modifications of the manufacturers' protocol (Papers I, II, III) and using a CTAB extraction protocol by Zuccarello and Lokhorst (2005) with modifications (Paper IV).

## Amplified fragment length polymorphism (Papers I and II)

Amplified fragment length polymorphism (AFLP) is a multi-locus DNA fingerprinting technique developed by Vos et al. (1995). The AFLP procedure was performed by digesting DNA of high quality and high molecular weight using restriction enzymes resulting in thousands of DNA fragments. Double-stranded DNA-adaptors were ligated onto the fragments, and two PCR amplifications were performed using complementary PCR primers. The first amplification, pre-amplification, included one selective nucleotide that extends into the restriction fragment, this to reduce the number of fragments. The second amplification, selective amplification, included three selective nucleotides extending into the restriction fragment (i.e. only fragments with the three chosen nucleotides will be amplified), this to further reduce the number of fragments (Vos et al., 1995; Bonin et al., 2007). The preferred numbers of fragments are 200 to 400 and the number of fragments generated depends on the sequence and the number of selective nucleotides in the primers. Fragments were separated using a DNA sequencer (e.g. ABI3730XL DNA analyzer). The presence of an allele (fragment) of a certain length was scored as 1, and the absence of the same allele was scored as 0, resulting in matrices with 1/0 for every individual and for all scored alleles. A particular AFLP fragment can represent either a heterozygote (1/0) or a homozygote (1/1) for an allele, while the absence of a particular AFLP fragment represents a homozygote (0/0), making it a so called dominant marker with every locus less informative compared to multi-locus markers such as microsatellites. However, the large number of markers together with the mainly random distribution in the genome compensate for the dominant character of AFLP. The random distribution of AFLP fragments including markers from both neutral and non-neutral genetic regions gives, high resolution and makes AFLP suitable for genetic studies (Mueller & Wolfenbarger, 1999; Bensch & Åkesson, 2005; Bonin et al., 2006). The resolution and relatively short start up time also make it an appropriate method for investigating small-scale genetic structuring between sites with different disturbance histories (Lind & Grahn, 2011; Bensch et al., 2002). Size homoplasy, i.e. co-migration of fragments representing different loci

in the genome, is a potential problem with AFLP. This will have four main consequences for population genetic inferences, including (1) an overestimation of the frequency of the presence of an allele, (2) an underestimation of the differentiation between populations, (3) an overestimation or underestimation of the heterozygosis depending on marker frequency, and (4) reduction in the power to detect selective loci (Caballero et al., 2008). The risk of size homoplasy increases for very short fragments (Vekemans et al., 2002), so to reduce the risk of size homoplasy we have excluded short fragments from the analysis.

## Introgression analyses (Papers I, II and III)

Due to the complex system with the occurrence of both *M. edulis* and *M. trossulus* in the studied area and the extensive mixing of the *M. edulis* and *M. trossulus* genomes (Väinölä & Strelkov, 2011) within the Baltic Sea, the species origin needed to be considered. The species marker Glu-5' (ME 15/16), (Inoue et al., 1995) was used to evaluate any remaining species identity effects on the genetic structure (Papers I and II) and on micronuclei and histological data (Paper III). This species marker is commonly used and distinguishes between the three Mytilus taxa *M. edulis*, *M. trossulus* and *M. galloprovincialis* (Kijewski et al., 2006). All individuals were genotyped at locus Glu-5' (ME 15/16) (Inoue et al., 1995), and each individual was coded as homozygous *M. edulis*, *M. trossulus* or *M. galloprovincialis* or as heterozygotes between them.

## Pool-sequencing (Paper IV)

To further investigate the genetic effects of sewage effluents and to identify the genomic regions potentially under selection, a whole-genome pool-sequencing approach was applied in Paper IV. Sequencing of pooled DNA samples allows a cost-effective comparison of population samples on a genomic scale (Futschik & Schlötterer, 2010). The technique has been used for a range of applications, including genomic comparisons between wild populations (Begun et al., 2007), characterization of genes involved in heavy metal tolerance (Turner et al., 2010), and in experimental evolution to identify genomic regions that show differentiation between different selective treatments (Burke et al., 2010; Parts et al., 2011; Turner et al., 2011). Mussels were sampled from two pollution types (REF and STP) at four locations (Askö, Tvärminne, Finland, Karlskrona and Gulf of Gdańsk, Poland) i.e. four replicated REF/STP pairs. Twenty-four individuals, from each site were pooled and sequenced using an Illumina HiSeq2500 (350bp insert size and 100bp paired end reads) at SciLifeLab, Stockholm. A *de novo* genome assembly was generated from the combined reads of the pools and together with a *de novo* transcriptome assembly based on *M. edulis* transcripts deposited at the NCBI. The Mining Exons in Scaffolding-Poor Assemblies (MESPA) approach (Neethiraj et al., 2017) was used to build gene models based on the genome assembly together with predicted protein

sequences inferred from the transcriptome data. The predicted gene models, i.e. scaffolds, were then used as a template genome for downstream analyses, including read mapping and variant calling.

## Statistics for genetic analyses

### Genetic diversity and differentiation (Papers I, II and IV)

Genetic variation is often divided into genetic diversity (the amount of genetic variation) and genetic differentiation (how the genetic variation is distributed between populations). There are four factors influencing genetic variation in natural populations, including genetic drift, gene flow (migration), mutation, and selection (Lowe et al., 2004; Allendorf & Luikart, 2009), and anthropogenic activities can have an impact on all of these processes (Smith & Bernatchez, 2008; Banks et al., 2013).

Genetic diversity is introduced to a population by mutation, gene flow from other populations, and hybridization/introgression from other taxa. Genetic diversity is lost by natural selection (selecting for well adapted alleles) and by genetic drift, i.e. the random loss of genetic diversity (alleles) in each generation. Population size influences genetic diversity because large populations can harbor more genetic variation and are less affected by genetic drift (Lowe et al., 2004; Allendorf & Luikart, 2009). Genetic diversity can be measured in different ways and in this thesis heterozygosity (the fraction of individuals in a population that are heterozygous for a particular loci) for the AFLP (Papers I and II) data and nucleotide diversity (the nucleotide polymorphism within a population) (Paper IV) for the SNP data have been used. The measure of nucleotide diversity is here measured by $\pi$, which is defined as the average number of nucleotide differences per site between two DNA sequences (Nei & Li, 1979).

Genetic differentiation is focused on how the genetic variation is distributed between populations. Movement between populations (in terms of gene flow) reduces differences between populations, while a lack of gene exchange cause population to diverge at neutral loci. This is even more prominent in small populations. The general and well-established way to study genetic differentiation between populations is F-statistics, developed by Sewall Wright in the 1930s. F-statistics is a mathematical model to quantify the genetic variation between subpopulations and individuals in diploid organisms using locus with two alleles. It is based on different coefficients, $F_{IS}$, $F_{IT}$ and $F_{ST}$ that measure the partitioning of diversity at hierarchical levels within and among subpopulations.

$F_{ST}$ estimates differences in allele frequencies between the subpopulations according to formula:

$F_{ST=} H_T - H_S / H_T$ (Nei, 1973)
$H_T$ = Total heterozygosity
$H_S$= The average heterozygosity in the subpopulations

If the allele frequencies are the same in all subpopulations, then $F_{ST}$ =0 and the subpopulations will be considered one large population. A difference in the allele frequencies, $F_{ST}$ >0, indicates limited gene flow between the subpopulations, and $F_{ST}$ =1 indicates complete isolation (Lowe et al., 2004; Allendorf & Luikart, 2009).

The dominant nature of AFLP i.e. not be able to separate if an individual is heterozygote or homozygote for a particular loci scored as 1, can be problematic in estimations of genetic diversity using F-statistics in the traditional way. To overcome this problem different approaches have been developed (Bensch & Åkesson, 2005; Bonin et al., 2007). In Papers I and II, $F_{ST}$ was calculated using AFLP-surv, a program developed especially for dominant AFLP data (Vekemans et al., 2002). Here, the Bayesian method by Zhivotovsky (1999) that assumes non-uniform prior distribution of allele frequencies and Hardy-Weinberg equilibrium was used to estimate allele frequencies (Vekemans et al., 2002). The program estimates genetic diversity ($H_j$ that is equivalent to $H_e$) within each population, global $F_{ST}$ and pairwise $F_{ST}$ values between populations, using the approach developed by Lynch and Milligan (1994) (Vekemans et al., 2002).

In paper IV the pooled sequence data were analyzed using PoPoolation. This software is tailored for population genetic inference analysis of pooled samples (Kofler et al., 2011a). The pool sequencing data have other challenges than the AFLP data. When mapping pool sequencing data onto a reference genome, the population pool might contain several different alleles and/or alleles that are different from the reference genome. To avoid variability due to sequencing errors and mapping errors, quality control and read depth control are important (Kofler et al., 2011a). In Paper IV, a sliding window approach was used to characterize genome-wide patterns using of a minimum read depth of 20 and a maximum read depth of 300. A sliding window calculates an average for a given window size of, for example, nucleotide diversity and $F_{ST}$ across the window. The main goal with this approach is to reduce the noise of a single marker by combining the effect of several markers (Hartl & Clark, 2007). Genome-wide nucleotide diversity ($\pi$) was calculated for each pool in PoPoolation v1.2.2 (Kofler et al., 2011a), and allele frequency differences and $F_{ST}$ values between REF/STP among all replicated pairs were calculated in PoPoolation2 v1.201 (Kofler et al., 2011b) using non-overlapping sliding windows of 250 bp.

## Genetic structure

To reveal the genetic structure among sampled populations various methods have been developed, including methods that summarize components of genetic variation (PCA, cPCoA), cluster samples in groups based on their genetic make-up (STRUCTURE) and methods that represent the relationship by population in a tree (TREEMIX).

Principal component analysis (PCA) and Constrained principal coordinate analysis (cPCoA) was used in Paper I, II and IV. Principal component analysis

(PCA, Pearson, 1901) is a useful tool to explore and visualize data with large numbers of measurements or dimensions as in the case for large genomic datasets. The PCA analysis reduces the dimensions to principal components, PCs (Hotelling, 1933), and these components can be used to explore main patterns in a dataset. A PCA was used in Paper IV to visualize patterns in the genomic data, where the sample sites were grouped based on their genetic differentiation, i.e. on $F_{ST}$ values. Sites with the least genetic differentiation are grouped together. A PCA in itself is not a statistical test but rather a tool to visualize the data, find patterns, and get a better understanding of the data.

Another, similar, analysis that both visualize patterns of, for example, genetic structure and test these patterns is a constrained principal coordinate analysis (cPCoA). The cPCoA is a supervised model where a variable, such as site, is used as an explanatory variable, and this allows for the testing of the significance of the effect using a permutation-based and order-dependent ANOVA (Oksanen et al., 2015). Most cPCoA is limited to Euclidian distances, but the capscale function implemented in the vegan package (Oksanen et al., 2015) in R 3.2.1 (R Development Core Team, 2015) allows other distance measures other than Euclidian. In Papers I and II, Jaccard distances were used, which are more suitable distances for binary AFLP data (1/0). The cPCoA approach was used to assess the significance of the explanatory variables (constraining variables) of site, location, pollution type and species identity in Papers I and II. The order-dependent ANOVA makes it possible to test the significance of the explanatory variables in a sequential, manner i.e. the variation explained by the preceding variables is removed.

A different way to perform cPCoA is to remove the effects of variables by using a condition (Oksanen et al., 2015). This makes it possible to test and visualize the effect of the remaining constraining variables. In Paper II, this approach was used to remove the effects of location and species identity in order to be able to analyze pollution type separately, i.e. to analyze differences between the different pollution types (REF/STP and REF/HAR) in the Baltic Proper.

The STRUCTURE 2.3 was used to assign each individual to a cluster based on its genetic makeup independent of prior information such as sampling site (Paper I). A Markov Chain Monte Carlo (MCMC) simulation was conducted to find the most probable number of genetic clusters under Hardy–Weinberg equilibrium. The program calculated the optimal number of clusters (K), where the K with the highest probability was indicated as having the lowest Pr(X|K) (Pritchard et al., 2000).

In Paper IV, Treemix (Pickrell & Pritchard, 2012) was used to investigate the gene flow between the sampled populations and to infer the potential for admixture. The analysis was based on genome-wide pairwise allele frequency estimations.

## Isolation by distance, connectivity and salinity (Paper I)

Isolation by distance (IBD) is the relationship of genetic similarity between populations over a geographic distance based on the stepping stone model. The model predicts that the further apart two populations are from each other, the more genetically different they should be. This test can be used for different types of distances. In Paper I, the effects of the geographical distance, salinity, and connectivity (derived from the model) were tested using Mantel tests and partial Mantel tests of correlation in the vegan package (Oksanen et al., 2015) in R 3.2.1 (R Development Core Team, 2015). The geographical distances between each site were estimated using the most direct marine route in Google Earth version 7.1.2.2014 (Google, 2014). The salinity measurements were from the field sampling, so for this data the "isolation by salinity" was tested. The connectivity data were derived from a biophysical model, where connectivity between the sampled sites, measured as dispersal of planktonic larvae, was tested rather than actual geographic distance. The dispersal was estimated with a biophysical particle-tracking model using flow fields from the BaltiX 3D ocean circulation model (Madec, 2010). A particle-tracking model (TRACMASS; Vries & Döös, 2001) was used to calculate dispersal trajectories. To mimic the dispersal of *Mytilus* sp., 49 virtual larvae (evenly spread within each grid cell) were released in all coastal grid cells and cells with depth ≤ 15 m, for 8 years. The drifting depth was set to a distribution of 25% surface drifters, 50% 10 m drifters and 25% 30 m drifters, and the spawning season was set to 75% in June and 25% in July following survey data (Helminen, 2002; Corell et al., 2012). In total, about 180 million trajectories were calculated and the virtual larvae drifted for 30 days. The average of the between-site directional connectivity from the multi-generational connectivity matrix was used to produce a non-directional connectivity matrix. Three different datasets were tested, one including all sites, one only including the West Coast sites, and one only including the Baltic Sea sites (Bothnian Sea and Baltic Proper).

## Identifying genomic regions potentially under selection (Papers II and IV)

One of the goals when studying genetic effects from anthropogenic changes is to identify genomic regions showing signs of selection. In Paper II, the use of AFLP provided an opportunity to identify alleles at AFLP loci associated with different pollution types (Williams & Oleksiak, 2008; Nosil et al., 2009; Lind & Grahn, 2011). In Paper IV the aim was to identify genomic regions differing between the REF sites and STP sites in the replicated pairs.

In Paper II the association between pollution type and genetic markers was explored using different approaches; two of them, DFDIST (Beaumont & Nichols,

1996) and BayeScan (Foll & Gaggiotti, 2008; Fischer et al., 2011), use an outlier approach, while varSelRF (Diaz-Uriarte, 2007) is a classification method. DFDIST is a version of FDIST that can be used for dominant markers. This program uses coalescent simulations to generate a null sampling distribution of estimates based on neutral expectations. Loci that do not fit neutral expectations are identified as putative outliers (Beaumont & Nichols, 1996). The Bayesian approach implemented in BayeScan (Foll & Gaggiotti, 2008; Fischer et al., 2011) uses the multinomial Dirichlet model, including an island model in which the allele frequencies of each subpopulation are correlated through a common migrant gene pool and population-specific and locus-specific components of the $F_{ST}$ coefficients are estimated. This approach compares neutral models with models including selection and then estimates Bayesian factors in support of selection over neutrality for each locus (Foll & Gaggiotti, 2008). The package varSelRF (Diaz-Uriarte, 2007) is based on the ensemble classifier of the decision tree-based program Random Forest (Breiman, 2001). The principle behind Random Forest is to build a large number of decision trees (a "forest") using a bootstrap technique (bagging). AFLP loci that are strong predictors of class (here pollution type) will occur in many trees resulting in a higher importance rank (Holliday et al., 2012; Brieuc et al., 2015).

In both Paper II and Paper IV, the Cochran–Mantel–Haenszel (CMH) test for repeated independence (Cochran, 1954; Mantel & Haenszel, 1959; Mantel, 1963) was used to test if there was a consistent difference in proportions of alleles between pollution types across locations (Paper II) and if there were consistent changes in allele frequencies between REF/STP pairs across locations (Paper IV). For Paper II, one contingency table for each locus including both pollution type and location was analyzed using package "stats" in R 3.2.1 (R Development Core Team, 2015). The CMH test implemented in Popoolation2 was used for the pool-sequencing data in Paper IV. Here the allele frequency change was calculated at SNPs with a minimum count of 24 and minimum coverage of 24, and only SNPs with a significance level of $p < 0.001$, after Holms correction was applied, were retained.

In Paper IV, genomic regions showing signs of selection, i.e. genomic regions with elevated $F_{ST}$ estimates combined with a reduced nucleotide diversity $\pi$ in STP sites. Each replicated pair of REF/STP was analyzed separately. Delta nucleotide diversity ($\Delta\pi$) was calculated as $\pi$REF $-\pi$STP for each sliding window in all REF and STP pairs. We identified genomic regions showing signs of selection as non-overlapping 250 bp windows that fell within the 95% tails of $F_{ST}$ distribution combined with 95% tails of $\Delta\pi$ distribution. The relationship between delta $\pi$ and $F_{ST}$ was visualized by plots, generated in the ggplot2 package (Wickham, 2009) in R-studio v.0.99.902 (RStudio team, 2015). Because we wanted to know if genomic regions showing signs of selection in one of the REF/STP comparisons were shared among the other pairs, we made Venn diagrams (overlapping diagrams). The genomic scaffolds containing windows showing signs of selection were also compared in Venn diagrams. Only genome scaffolds containing genomic windows of interest and con-

taining >1 SNP identified by CMH and that was shared between at least three repeated pairs were considered an "outlier scaffold" and was used for annotation and functional analyses. These genomic scaffolds were annotated using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). This tool compares nucleotide similarities (BLASTn) between a sequence and sequences in a database. The estimation was done with default settings and an expected threshold of 10 using megablast. It can also compare nucleotide sequences with translated protein similarities (BLASTx) (estimated with default settings and threshold of 10). To find other translated protein similarities and to infer functions, i.e. gene ontology terms (GO), the Uniprot database was used (using default settings with E-threshold of 10) (Uniprot consortium, 2017). If no GO-term was found based on the sequence, a manual ortohology search, based on protein id, for putatively function was conducted.

## Physiological and ecotoxicological analyses (Paper III)

### Physiological analyses

Environmental stress assessments are usually based on multiple biomarkers representing biochemical, morphological, and physiological changes (Lam, 2009; Bignell et al., 2011). The physiological conditions of a given organism can be measured by variations in weight and in physiological indexes. In this thesis, the gonadosomatic index (GSI) and body mass index (BMI) were used. GSI measures the amount of energy allocated to reproduction compared to the building of somatic tissue. BMI represents the general condition of an individual. Lipids are an important energy factor for mussels, and different lipid contents in mussels from different populations can indicate an energy imbalance associated with increased stress (Mouneyrac et al., 2012). Gonad development (GD) is correlated with lipid content, BMI and GSI, and GD was therefore estimated for each individual. To test if any location or pollution type deviated in GD, a site-based GD index (GI) was calculated for each site. Approximately 90 individuals per site were used for index calculations and lipid content determination. Length was measured to the nearest 0.01 mm with a digital caliper.

Indexes were calculated as BMI, expressed as the total body mass (somatic + gonad tissue) divided by shell length$^3$ [mg/cm$^3$] and GSI, which is the relative investments in reproduction expressed as gonad dry mass divided by total dry mass (Roff, 1993).

GD stage was established according to (Wenne, 1985; Gosling, 2008):

Stage 1: immature gonads recovering after spawning.

Stage 2: active gametogenesis with previtellogenic oocytes and larger and thicker follicles.

Stage 3: maturity, gonadal follicles filled with mature gametes and packed together, ongoing oogenesis and spermatogenesis.

Stage 4: spawning after gonads reach the maximum size.

Stage 5: resting, ongoing lysis of un-discharged gametes, and restoration of the gonad tissue.

Based on established GD, the GI was calculated for each site using the following formula: $GI= [n_1*1+n_2*2+n_3*3+n_4*4+n_5*5]/N$

Where "$n_i$" is the number of individuals in a particular stage of development and "N" is the total number of all individuals at the particular sampling site.

Sex was determined by examining a small subsample of gonadal tissue under a light microscope.

## Histological analyses

To identify genotoxic effects and/or signs of chronic stress on populations inhabiting sewage effluent-affected sites and harbors the micronucleus test (Bolognesi & Hayashi, 2011) and histological analyses (Smolarz et al., 2005; Kim & Powell, 2006) were used. For the micronucleus test, a cell suspension of gill tissue was prepared by homogenization of gill tissue in a drop of ethanol:acetic acid (3:1) solution and then smeared on a slide, stained with Giemsa solution and analyzed under a light microscope. Approximately 300 nuclei per mussel were examined following the criteria described by Fenech et al. (2003). For histology, soft tissue from each individual was fixed in formaldehyde, stained with Harris' hematoxylin and eosin stain and examined under a light microscope for lesions in the digestive gland, gonads, and gills.

## Statistical analyses of physiological and histological data

The experimental design with a nested sampling, including repeated pollution types in different locations, made it possible to use models to evaluate both the effects of each variable and the effects of interactions. All of the statistical models included location and pollution type. Sex was included in models analyzing the index data. Species introgression pattern was included for the histological and micronuclei analyses in order to evaluate any remaining species effects. All models were evaluated using ANOVA type 2 tests, thus accounting for any cofounding effects by removing the variation explained by confounding variables. The main models were reduced using the step function as implemented in the car package (Fox & Wies-

berg, 2011). Pairwise differences among locations, pollution types, and sex were evaluated with *post-hoc* tests (FDR corrected) using the package Phia (Helios De Rosario-Martinez, 2015). Significant interactions between variables were visualized by effect-plots, generated with the use of the package effects (Fox & Hong, 2009). To analyze differences in GI among locations and pollution types, the calculated GI value for each site was used in a linear model with location and pollution type, as fixed explanatory variables. The variable GD was analyzed using a generalized linear model (GLM) with location, pollution type and sex included as fixed explanatory variables. The physiological variables (BMI, GSI, and lipid content) were analyzed using linear models with location, pollution type and sex as fixed explanatory variables. The histological and micronuclei data were analyzed using GLM with location, pollution type, and the species introgression pattern as fixed explanatory variables.

# Key results of the papers included in the thesis

## Paper I
## Regional Genetic Differentiation in the Blue Mussel from the Baltic Sea Area

To explore the genetic structure and potential barriers to gene flow in the blue mussel between the West Coast and the Baltic Sea as well as within the Baltic Sea AFLP was used yielding a large number of genetic markers randomly distributed across the genome, including both neutral and non-neutral regions. The genetic data were analyzed in combination with biophysical connectivity modeling and species hybridization data. A distinct genetic differentiation between the West Coast, Baltic Proper and Bothnian Sea regions was identified and a lower gene diversity in the Bothnian Sea. The genetic differentiation between the West Coast and the Baltic Sea (Baltic Proper and Bothnian Sea) could partially be explained by oceanographic connectivity, salinity, and to a certain extent species identity. The genetic differentiation between the Baltic Proper and Bothnian Sea could not be directly explained by oceanographic connectivity, species identity or salinity, while the lower connectivity to the Bothnian Sea might explain the observed lower gene diversity. A generally low genetic differentiation among the sites within each of the basins was found but especially within the Baltic Proper. This is indicative of strong gene flow, which can be attributed to the high oceanographic connectivity within the Baltic Proper but also the generally long planktonic larval phase, 5–6 weeks in blue mussels (Kautsky, 1982). In contrast to the genetic differentiation identified between the West Coast and the Baltic Sea, the genetic differentiation between the Baltic Proper and the Bothnian Sea could not be associated with species differences, which supports the presence of strong introgression in the Baltic Proper blue mussels.

## Paper II
## Sewage Treatment Plant Associated Genetic Differentiation in the Blue Mussel from the Baltic Sea and Swedish West Coast

This study aimed to understand how two types of point sources of aquatic environmental pollution, harbors and sewage treatment plants, affect gene diversity and genetic differentiation in the blue mussel in the Baltic Sea area and off the Swedish West Coast (Skagerrak). By using a nested sampling scheme with reference sites, geo-

graphically paired with sites from sewage effluent-affected areas and harbors together with the AFLP genetic markers we could evaluate patterns of genetic composition in relation to the pollution types. This is one of the first studies showing that exposure to sewage treatment plant effluents is associated with differences in genetic composition. The pollution effect from sewage effluents on the genetic composition of blue mussel populations acts in the same direction in all sites investigated. Mussel populations from harbors were, in contrast, genetically divergent from each other, suggesting an effect of pollution from harbors but that the direction is site specific. Due to the strong introgression and extensive mixing of the *M. edulis* and *M. trossulus* genomes (Väinölä & Strelkov, 2011) in the Baltic Sea, any remaining effects of species pattern on the genome-wide genetic structure and effects in combination with pollution type were also evaluated. Species identity, based on the Glu-5' marker, was not associated with genetic structure, thus supporting the presence of strong introgression in the Baltic Proper. No significant interaction between species identity and pollution type was observed, thus excludeing any remaining effects of species identity as an important factor on the pollution effect.

## Paper III
## Multi biomarker analysis of pollution effect on resident populations of blue mussels from the Baltic Sea

To assess the impact on resident populations of blue mussels from harbor sites and sewage effluent-affected sites the same nested sampling scheme as in Larsson et al. (2016, Paper II) together with a multiple-biomarker approach was used. Physiological condition measured as BMI and GSI, lipid content, histological analyses, and the micronucleus test were used to evaluate how the point sources of environmental pollution affect the bioenergetics and growth of the blue mussel. The results showed that mussels sampled in harbors and in sewage effluent-affected areas had a generally higher condition, indicated by a higher BMI, GIS, and higher lipid content compared to mussels from reference sites. Indications of increased stress in mussels sampled near harbors, as shown by a higher frequency of histological abnormalities in the digestive gland, were found. The strongest genotoxic effect was found in mussels sampled in the Gulf of Gdańsk area, regardless of pollution type, as indicated by higher frequencies of histological abnormalities and micronuclei. Despite the increased stress, blue mussels sampled in the Gulf of Gdańsk were characterized by higher condition (in terms of GSI and BMI) compared to the Askö and Tvärminne mussel populations. The species introgression pattern was included to determine if resident populations from different locations have different species introgression patterns and, if so, if this will give different responses to similar pollution types. Species identity was not associated with either micronuclei or histopathological lesions, which was supportive of the previous findings of a strong genetic

introgression in the Baltic Proper in Larsson et al. (2017, Paper I; Larsson et al., 2016; Paper II).

## Paper IV
## A Population Genomic Analysis of Blue Mussels Identifies Genomic Regions Associated with Sewage Treatment Plant Effluents in the Baltic Sea.

A population genomics approach was used to investigate the effects of selection imposed by sewage treatment plant effluents on wild blue mussel populations, first identified by Larsson et al. (2016, Paper II). The genome-wide distribution of $F_{ST}$ for all comparisons shows that only a small fraction of the genome exhibited even moderate levels of differentiation. However, genomic regions with high degree of genetic differentiation, indicated by high $F_{ST,}$ was found among all the replicated REF/STP pairs. The PCA, based on the average $F_{ST}$ values, revealed a pollution type ordering where sewage effluent-affected sites grouped together, indicative of a higher genetic similarity among blue mussels from these sites compared to mussels from the reference sites. The CMH analysis showed that the allele frequency changes in many SNPs are shared among the repeated pairs. A low number of genetic regions were shared among the replicated REF/STP pairs, but five genomic scaffolds were shared among the replicated REF/STP sites in Askö, Tvärminne (Finland) and Karlskrona. An initial functional characterization of these regions revealed functions related to immune and endocrine disruptive responses, oxidative stress and shell formation. These results suggest a within-generation selection that affects multiple loci, involving both parallel recruitment of the same genomic regions and the divergence of different genomic regions across the Baltic Proper.

# Discussion

This thesis shows that two common point sources of pollution, sewage effluents and harbors, affect resident blue mussel populations. Genetic differentiation associated with sewage treatment plant effluents was found among blue mussel populations in the Baltic Proper, indicative of within-generation selection. Mussels resident in harbors showed no congruent genetic differentiation but were more influenced by genotoxic pollutants with an increase in histological lesions in the digestive gland. The strongest genotoxic effects were observed among the sites in the Gulf of Gdańsk, regardless of pollution type. This, together with the higher genetic differentiation between the sewage treatment plant effluent-affected sites and reference sites in the Gulf of Gdańsk is indicative of higher levels and more complex pollution regimes in this area. The study design using a nested sampling scheme where sites from different pollution types were paired with reference sites at different geographical locations was necessary to allow simultaneous studies of the effects of pollution type and location.

## Genetic structure within the Baltic Sea area

To better understand the genetic structure of blue mussels in the Baltic Sea area, genetic differentiation was studied in relation to connectivity, salinity, and species introgression. The already identified genetic barrier between the West Coast and the Baltic Sea (Väinölä & Hvilsom, 1991; Väinölä & Strelkov, 2011; Johannesson & André, 2006; Stuckas et al., 2017) was confirmed. The genetic barrier between the West Coast and the Baltic Sea is located around the Öresund and coincides with the steep salinity gradient at this location. In Paper I, the genetic barrier is explained by limited connectivity/dispersal, changes in salinity, and to some extent species differences. A study of the Baltic Mytilus species along the southern Baltic coast using multi-locus genotyping in combination with a hydrodynamics model also localized the genetic barrier to between Öresund and the Darss area west of Rugen Island (Stuckas et al., 2017). In that study, connectivity could not fully explain the maintenance of this barrier. Selection driven by environmental adaptation and weak reproductive barriers as the main drivers of these differences, rather than dispersal, was suggested. These drivers, according to the authors, are capable of maintaining the species differences between the Baltic Mytilus species despite the strong introgression. Salinity has also been found to be an important selection factor shaping the genetic structure between the Baltic clam species *Macoma baltica* and *M. rubra* in the same area (Luttikhuizen et al., 2012). However, it is difficult to disentangle

the exogenous (selection) and endogenous (genetic incompatibilities between species) processes (Bierne et al., 2011). This might be reflected in the results in Paper I in which a strong correlation to salinity (selection, adaptation) and a weaker, though significant, correlation to species differences (genetic incompatibility) was observed. To understand this pattern, further experimental studies are needed that can correlate genome-wide genetic signatures of pure and hybrid specimens with performance in different salinity conditions, as proposed by Stuckas et al. (2017).

In Paper I, genetic differentiation was also found between mussels from the Baltic Proper and the Bothnian Sea. In contrast to the differentiation between the West Coast and the Baltic Sea, this genetic differentiation could not be explained by connectivity, salinity or species introgression pattern. Earlier studies have indicated both genetic differences (Gardeström et al., 2008; Wennerström et al., 2013) and differences in metabolic rates and stress (Prevodnik et al., 2007; Lilja et al., 2008) between blue mussels sampled in the Baltic Proper and the Bothnian Sea. These physiological differences were suggested to be caused by either phenotypic plasticity or to be linked to selected genes involved in physiological adaptations to the lower salinity in the Bothnian Sea. No significant correlation between genetic differentiation and salinity could be found in this study, implying that other selective forces than salinity are involved in the genetic differentiation. Because the dispersal model does not fully account for biophysical factors such as suitable habitat or post-settling mortality, local adaptation and founder effects are candidates for causing the observed genetic differentiation. These factors might include phytoplankton composition, which differs between the Baltic basins and can change due to anthropogenic influences (Lehtinen et al., 2015). Species-related pre- or post-zygotic genetic incompatibilities suggested by Bierne et al. (2011) were not suported by the results in Paper I.

The low genetic differentiation among sites within the different basins, and especially within the Baltic Proper, is indicative of strong gene flow, which can be attributed to the high oceanographic connectivity as shown by the oceanographic model in Paper I. The connectivity is dependent on the long planktonic larval phase of 5–6 weeks, (Kautsky, 1982), during which the larvae can be transported far away from their release point. The connectivity model shows that larvae can reach around the Baltic Proper (from Gulf of Gdańsk to Karlskrona, Paper I Fig 4) within 12 generations, while for shorter distances fewer generations are sufficient (e.g. Askö and Karlskrona are connected in four generations). The main aim with this thesis was to understand the genetic effects of different pollution types in resident blue mussels. For this, the migratory capacity between of the mussels among replicates of reference site, sewage treatment plant effluent-affected sites and harbor sites needs to be sufficient. All of our sampling sites were selected to be located within 10 to 30 km, and this is within the distances that have been shown that most larvae settle in

(Stuckas et al., 2107). Large-scale geographical components were also taken into account when analyzing possible effects of pollution type in Papers II, III, and IV.

## Genetic differentiation among pollution types and geographical locations

Despite a generally low level of genetic differentiation and considerable gene flow among blue mussels in the Baltic Proper, genetic differentiation associated with sewage treatment plants was found (Papers II and Paper IV). In Paper II, the genetic differentiation was shown by the cPCoA and the pollution-type based $F_{ST}$ analysis. Several AFLP loci were identified to be under putatively selection. In Paper IV the PCA revealed a pollution type-ordered structuring where all the sewage effluent-affected sites are grouped together. This is indicative of genetic convergence among the mussels in these sites, while the reference sites showed a higher variation and did not cluster together. A consistent allelic frequency change in several SNPs was found among the replicated pairs, by the CMH analysis. Also, several genomic regions showed signs of selection with an elevated $F_{ST}$ between the reference and sewage effluent-affected samples combined with low nucleotide diversity in the sample from sewage effluent-affected area. These results support other studies showing genetic differentiation between populations at several loci even in the presence of considerable gene flow (Nosil et al., 2009; Michel et al., 2010; Yeaman & Whitlock, 2011; Lamichhaney et al., 2012; Feder et al., 2012; Guo et al., 2015).

No consistent genetic differentiation associated with harbors was observed in Paper II. Such genetic differentiation has been found to be both present (Ma et al., 2000; Williams & Oleksiak, 2008; Bach & Dahllöf, 2012; Reid et al., 2016) and absent (Štambuk et al., 2013) in highly contaminated areas, including harbors. The discrepancy in observations might be due to differences in pollution regimes among the sampled harbors resulting in differences in selection pressure.

The nested sampling scheme made it possible to differentiate between location and pollution effects. In Paper II, the AFLP data revealed a low genetic differentiation as indicated by a low $F_{ST}$ among all the studied sites. However, in Paper IV the genome-wide pool sequence data, which gave much higher resolution, showed that despite of the low degree of genome-wide genetic differentiation among the sites there was significantly higher pair-wise $F_{ST}$. These results are in line with studies of fish in the Baltic Sea, where the use of a small number of genetic markers revealed low or no genetic differentiation among populations, but high-resolution sequencing methods detected genetic differentiation among the same populations (Lamichhaney et al., 2012; DeFaveri et al., 2013; Guo et al., 2016). The increased resolution also revealed a higher genetic differentiation, with the highest mean $F_{ST}$, between the REF/STP pair from the Gulf of Gdańsk, Poland. This pair also had fewer shared genomic regions (on both scaffold and window level) and none of the scaffold indicated to be under selection were find among the Gulf of

Gdańsk replicate. These results are indicative of a stronger and more local selection regime compared to the other locations.

## Physiological differentiation among pollution types and geographical locations

No genotoxic or histopathological effects were found in mussels from sewage effluent-affected sites, but mussels from harbor sites had a higher prevalence of histological lesions in the digestive gland compared to mussels from reference sites and sewage effluent-affected sites, suggestive of a stronger influence of genotoxic pollutants in harbors. Mussels from both sewage effluent-affected sites and harbors had a higher BMI, GSI and mussels from harbors had a higher lipid content compared to mussels from references sites (Paper III). This in contrast to previous studies showing negative effects of harbor and industrial pollution also on condition index and lysosomal stability, and also an increased frequency of micronuclei formation, which we did not find in our study (Schiedek et al., 2006; Smolarz & Bradtke, 2011; Turja et al., 2013; 2014). The increased condition probably reflects the higher nutrient levels and suspended matter found at the sewage effluent-affected sites and harbor sites (Hellou et al., 2003; Bouchard et al., 2009; de los Rios et al., 2013; Turja et al., 2015). Most negative effects caused by sewage effluents have been related to endocrine disruption (Gagné et al., 2004; 2011; de los Rios et al., 2013; Turja et al., 2015) and negative effects on the immune system (Akaishi et al., 2007), and only weak effects on biomarkers related to general stress (Gagné et al., 2008; de los Rios et al., 2012) have been found. However, one study in the Baltic Sea using transplanted blue mussels found negative effects also on stress-related biomarkers caused by sewage treatment plant effluents (Turja et al., 2015). Most previous studies regarding effects of sewage effluents have been conducted on transplanted mussels and it is difficult to compare results from studies based on resident and transplanted mussels because the differences in response can be due to adaptation to the environment. Such adaptation has been observed in blue mussels sampled outside sewage treatment plants showing a higher recovery rate after exposure to pharmaceuticals compared to mussels from more pristine sites (Kumblad et al., 2015) and the higher condition in mussels sampled in sewage effluent-affected sites may perhaps be a sign of adaptation. Due to the fact that the measured biomarkers in Paper III were related to pollution-induced chronic stress, rather than immune- and endocrine responses, we have not fully evaluated possible effects from sewage effluents and future studies should include immune- and endocrine related biomarkers.

The strongest effect of pollutants was found in the Gulf of Gdańsk, Poland, regardless of pollution type. The mussels from the Gulf of Gdańsk had a higher prevalence of micronuclei and lesions in gills and the digestive gland. These results indicate higher levels, or a more complex mixture, of pollution in the Gulf of Gdańsk. Still, mussels from the Gulf of Gdańsk had a high condition with a high

BMI and GSI (Paper III). This might reflect the trade-off between growth, reproduction, and stress resistance. The complexity of the results in this study emphasizes the importance of including multiple sites in studies of environmental effects.

## Footprints of selection and functional analyses

The observed genetic differentiation associated with exposure to sewage treatment plant effluents in the Baltic Sea blue mussel is indicative of selection due to these effluents. An important sign of selection is genetic regions, which, compared to neutral loci, show elevated differentiation between populations inhabiting different environments (Via, 2002; 2009; Nosil et al., 2009; Nosil & Feder, 2012a; 2012b). Such regions are also expected to show reduced genetic diversity within populations (Nielsen, 2005). In Paper II, we found outlier AFLP loci that were indicated to be under selection, although only a few loci were verified across replicated pairs and different methods. In Paper IV, many SNPs showed similar allele frequency changes across the replicated REF/STP pairs indicating a similar response to sewage effluents across pairs. Also, several genomic regions showed signs of putative selection (elevated $F_{ST}$ in combination with decreased nucleotide diversity) within each of the REF/STP comparisons. None of these genomic regions were shared among all replicated pairs and only a small number were shared among three replicated pairs. Five genomic regions, scaffolds, were identified to be under putative selection both by having windows with elevated $F_{ST}$ in combination with decreased nucleotide diversity and > 1 SNPs identified by CMH. These genomic regions were all shared among the replicated pairs in Askö, Tvärminne, and Karlskrona and although most candidate regions for selection are specific to each sewage effluent-affected site also a convergent selection occurs at these locations. The convergence among these sites was supported by the Treemix analysis, which identifies a migration event, with gene flow from the sewage effluent-affected site in Karlskrona to the sewage effluent-affected sites in Askö and Tvärminne.

A greater percentage of shared genomic regions had strengthened the patterns of shared differentiation across replicated pairs. However, many recent genomics studies of adaptation investigating repeated selection have shown both shared and non-shared divergence among replicated environments or phenotypes in populations (Renaut et al., 2011; Perrier et al., 2013; Roda et al., 2013a; 2013b; Ravinet et al., 2016; Reid et al., 2016; Wagner et al., 2017). This as traits involved in adaptation probably follow complex genetic trajectories affecting multiple loci and that these might arise via multiple pathways (Hoekstra & Nachman, 2003; Jones et al., 2012; Laporte et al., 2016; Reid et al., 2016). It is therefore plausible that adaptation to different environments might involve not only selection on the same genetic regions, but also on different regions/genes that participate in the same biological functions. Sharing biochemical pathways or organismal functions might be much

more common than sharing the same changes at the gene level (Roda et al., 2013b; Ravinet et al., 2016).

The anonymous nature of AFLP markers prevented a functional analysis of the outlier loci in Paper II but in Paper IV a brief functional annotation and GO term analysis of genomic regions shared by at least three repeated pairs were performed (Paper IV), representing an initial characterization of the effects of sewage treatment plant effluents. The functional GO term and manual homologue search of the five scaffolds showing strong signal of selection found genes involved in functions important in the immune system (i.e. fibrinogen containing proteins) (Hanington & Zhang, 2010), in the endocrine system (i.e. SDR) (Zhang et al., 2014) and genes involved in female fertility as the regulate translation during oocyte maturation (i.e. pumilio protein 2) (Juliano et al., 2010). Both immune responses (Akaishi et al., 2007) and endocrine disruptive effects (Gagné et al., 2004; Gagné et al., 2011; de los Rios et al., 2013; Turja et al., 2015) have been shown in studies of mussels. Endocrine disruptive compounds and potentially pathogenic bacterial and viral contamination in post-treatment sewage water (George et al., 2002; Gagné et al., 2004; Gagné et al., 2011; de los Rios et al., 2013; Turja et al., 2015) have been linked to such effects. Although no data on either immune or endocrine disruptive effects exist from the sampled sites used in this study, it seems likely that these functions may be target of selection.

Furthermore, the anion exchange protein was also identified in the functional analysis and has been shown to be involved in anti-oxidative and developmental pathways in copepods (*Tigriopus japonicus*) exposed to manganese (Mn) (Kim et al., 2013). Although very few studies have shown effects on markers related to oxidative stress (Gagné et al., 2008; de los Rios et al., 2013) in mussels, a transplant study of blue mussels in the Baltic Sea suggests that changes on such markers can occur (Turja et al., 2015). Also, a gene encoding epidermal differentiation protein was found and the manual orthology search in mice (*Mus musculus*) revealed that it was a keratin-associated protein. This group of proteins has been shown to be involved in formation of shell matrix in the limpet (*Lottia gigantea*) (Mann et al., 2012). Although potential functions were identified in this study, the field is under development and to fully understand the selection and the functional effects of sewage treatment plant effluents, functional tests of genes and their impact, including assessment of the effect of each individual SNPs within each region, are needed.

The selection regime in this system might best be explained as post-dispersal selection acting in each generation (Schmidt & Rand, 2000; Sanford & Kelly, 2011) rather than local adaptation. Local adaptation is defined as "populations that have differentiated genetically under conditions where low dispersal or active habitat choice have resulted in low gene flow" (Kawecki & Ebert, 2004), which is not true in the present case. The high nucleotide diversity ($\pi$: 0.017–0.018, Paper IV), large population size, and considerable gene flow within the studied system (Papers I, II and IV) make within-generation selection more plausible. Because this study was

conducted on sampled adult individuals and did not include sampling at different life stages or multiple cohorts, it is impossible to imply at which life stages of the blue mussels the putative selection occurs. Other studies have shown that larvae are more sensitive to pollutants compared to adults (Beiras & Bellas, 2008). To achieve a comprehensive view of this system, studies sampling early and multiple life stages and cohorts are needed.

## Species introgression

In a study of highly introgressed species, the question "*Is the observed genetic pattern an artifact of species difference?*" is highly relevant. The genetic species marker Glu-5' (ME 15/16) (Inoue et al., 1995) was used in this work to evaluate any remaining species effects on the genetic structure in relation to pollution type (Paper II) and when analyzing the occurrence of micronuclei and histopathological lesions (Paper III). No association between species identity and the pollution type-associated genetic differentiation (Paper II) or evidence of a species-specific response linked to the occurrence of micronuclei and histopathological lesions were found. Furthermore, there were no significant interactions between species identity and pollution type or location (Paper III), strengthen the presence of a strong introgression between *M. edulis* and *M. trossulus* in the Baltic Proper, which supports that the observed genetic pattern does not seem to be an artifact of species admixture. It cannot be excluded, however, that the introgression status of each individual was not fully covered by the Glu-5' marker and that other genetic markers would give a different pattern (see Kijewski et al., 2006; 2011). To fully uncover the introgression patterns in the Baltic Sea in detail, a more comprehensive whole-genome scan, including pure *M. edulis* and *M. trossulus* as reference individuals, is required.

# Concluding remarks and future directions

This thesis aimed to assess how two common point sources of pollution, sewage treatment plants and harbors, affect resident Baltic blue mussel populations. A genetic differentiation associated with sewage treatment plant effluents was found among blue mussel populations in the Baltic Proper, indicative of within-generation selection. The genetic differentiation was not associated with genotoxic effects or reduced condition. The functional analysis showed that genes identified to be under selection were putatively involved in immune functions, endocrine responses, oxidative stress and shell formation. This thesis provides information about the long-term effects of pollution on resident blue mussels in the Baltic Sea, thus adding to the understanding of the genetic effect of sewage effluents on ecosystems. In contrast, mussels resident in harbors were more influenced by genotoxic pollutants, but this was not associated with any congruent genetic differentiation. Only a few studies have investigated this issue, and our findings need to be verified by experimental studies spanning several generations. To gain further understanding of the role of the genomic regions involved in adaptive processes and the effects of sewage effluents on populations, a deeper exploration of both unique and shared genomic regions together with controlled field and genetic mapping experiments is needed. The genomic regions identified here, might be used as markers that, combined with experiments, can be used to reveal at which life stages of the blue mussels the putative selection occurs.

This thesis also contributes to the general knowledge about the blue mussel, a functionally and structurally important species in the Baltic Sea. In Paper I, the biophysical model combined with the genetic data adds information about the dispersal pattern of the blue mussel between the West Coast and the Baltic Sea and identifies an additional barrier to gene flow between the Baltic Proper and the Bothnian Sea. Paper IV provides novel genomic data that contribute both to the genomic resources within the Mytilus complex and other mollusks. This thesis has stressed the importance of heterogeneity and genetic differentiation for resilience in contaminated environments.

The results of this thesis strengthen the blue mussel's position as a keystone species in the Baltic Sea ecosystem, showing the ability to cope with stress in environments that need its filtering ability the most. The blue mussel seems to handle environmental disturbances quite well in terms of physiological adaptations and having large populations, high genetic diversity, and efficient gene flow it should not be in acute danger. However this thesis shows that sewage effluent applies a selection pressure and might, in the long run, endanger the genetic diversity of the

blue mussel. For other organisms with life history characteristics that promote local recruitment, pollution might direct evolutionary processes to an even greater degree. This might have important implications for ecosystem functioning and biodiversity conservation.

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# Södertörn Doctoral Dissertations

1. Jolanta Aidukaite, *The Emergence of the Post-Socialist Welfare State: The case of the Baltic States: Estonia, Latvia and Lithuania*, 2004
2. Xavier Fraudet, *Politique étrangère française en mer Baltique (1871–1914): de l'exclusion à l'affirmation*, 2005
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Increases in human activities releasing pollutants into aquatic habitats have created a stressful environment for many organisms. There are several examples where such activities have caused negative effects on physiological traits but there have also been evolutionary effects such as changes in the genetic structures of populations. To understand the long-term impact of human-generated pollution, it is important to investigate how these changes affect genetic structures.

The Baltic Sea is considered to be one of the world's most contaminated seas and the two main sources of pollution are harbors and sewage treatment plants. This thesis assesses the impact of harbors and sewage treatment plants on the physiological traits and genetic structure of resident populations of blue mussels at replicated sites in the Baltic Sea.

**Josefine Larsson** has a Master's Degree in biology from Uppsala University. She carries out research within the field of environmental science using genomic tools to answer ecological questions. This is her PhD thesis.

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