Impact of anthropogenic stressors on the population genetics of the common bioindicator *Dreissena polymorpha* (Pallas, 1771)

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Author contributions

In respect for all living things with whom we share this planet

1 SUMMARY

Toxicant inputs from agriculture, industry and human settlements have been shown to severely affect freshwater ecosystems. Pollution can lead to changes in population genetic patterns through various genetic and stochastic processes. In my thesis, I investigated the impact of anthropogenic stressors on the population genetics of the zebra mussel *Dreissena polymorpha*. In order to analyze the genetics of zebra mussel populations, I isolated five new highly polymorphic microsatellite loci. Out of those and other already existing microsatellite markers for this species, I established a robust marker set of six microsatellite loci for *D. polymorpha*.

Monitoring the biogeographical background is an important requirement when integrating population genetic measures into ecotoxicological studies. I analyzed the biogeographical background of eleven populations in a section of the River Danube (in Hungary and Croatia) and some of its tributaries, and another population in the River Rhine as genetic outgroup. Moreover, I measured abiotic water parameters at the sampling sites and analyzed if they were correlated with the genetic parameters of the populations. The genetic differentiation was basically consistent with the overall biogeographical history of the populations in the study region. However, the genetic diversity of the populations was not influenced by the geographical distance between the populations, but by the environmental factors oxygen and temperature and also by other unidentified factors. I found strong evidence that genetic adaptation of zebra mussel populations to local habitat conditions had influenced the genetic constitution of the populations. Moreover, by establishing the biogeographical baseline of molecular variance in the study area, I laid the foundation for interpreting population genetic results in ecotoxicological experiments in this region.

In a cooperation project with the Department of Zoology of the University of Zagreb, I elaborated an integrated approach in biomonitoring with *D. polymorpha* by combining the analysis techniques of microsatellite analysis, Comet assay and micronucleus test (MNT). This approach was applied in a case study on freshwater contamination by an effluent of a wastewater treatment plant (WWTP) in the River Drava (Croatia) and a complementary laboratory experiment. I assessed and compared the genetic status of two zebra mussel populations from a contaminated and a reference site. Microsatellite analysis suggested that the contaminated population had undergone a genetic bottleneck, caused by random genetic drift and selection, whereas a bottleneck was not

detected in the reference population. The Comet assay did not indicate any difference in DNA damage between the two populations, but MNT revealed that the contaminated population had an increased percentage of micronuclei in hemocytes in comparison to the reference population. The laboratory experiment with mussels exposed to municipal wastewater revealed that mussels from the contaminated site had a lower percentage of tail DNA and a higher percentage of micronuclei than the reference population. These differences between populations were probably caused by an overall decreased fitness of mussels from the contaminated site due to genetic drift and by an enhanced DNA repair mechanism due to adaptation to pollution in the source habitat. Overall, the combination of the three biomarkers provided sufficient information on the impact of both treated and non-treated municipal wastewater on the genetics of zebra mussels at different levels of biological organization.

In my thesis, I could show that the newly established marker set of six microsatellite loci provided reliable and informative data for population genetic analyses of *D. polymorpha*. The adaptation of the analyzed zebra mussel populations to the local conditions of their habitat had a strong influence on their genetic constitution. We found evidence that the different genetic constitutions of two populations had influenced the outcome of our ecotoxicological experiment. Overall, the integrated approach in biomonitoring gave comprehensive information about the impact of both treated and non-treated municipal wastewater on the genetics of zebra mussels at different levels of biological organization and was well practicable in a first case study.

2 GENERAL INTRODUCTION

Ecotoxicology is an interdisciplinary field which encompasses the impact of anthropogenic stressors on the living environment. Due to an increasing human population and activities associated with agriculture, industry and commerce, anthropogenic pollutants are widely distributed in ecosystems (Vitousek et al. 1997; Bickham et al. 2000). The majority of the toxicant inputs end up in aquatic ecosystems. They have been shown to severely affect freshwater ecosystems. Today, the biological mechanisms responsible for their toxicity are under extensive investigation (Shugart et al. 2010 and references therein).

Evolutionary toxicology investigates population genetic effects caused by environmental contamination (Shugart et al. 2010; Bickham 2011). It is likely that organisms inhabiting polluted environments are continuously exposed to mutational pressure (Crow 1997). Toxicant inputs can have impacts on wildlife populations causing somatic and heritable mutations (Bickham et al. 2000). Bearing in mind the importance of DNA in maintaining homeostasis of all organisms and for the transfer of genetic information to offspring, it is important to include genotoxicity (damage of DNA and chromosomes) in any assessment of pollution-related stress in an ecosystem (Klobučar et al. 2003). Genotoxic effects in germ cells can result in rapid alterations of gene frequencies in natural populations (Depledge 1998). However, population genetic effects are not only due to molecular toxic mechanisms. Pollution can be a selective force and consequently lead to changings in population genetic patterns. Moreover, stochastic effects leading to inbreeding in small populations, overall loss of genetic diversity, loss of heterozygosity, and genetic bottlenecks, as well as the accumulation of deleterious mutations in a gene pool (mutational load) are compounding factors that reduce fitness and accelerate the process of population extinction (Saccheri et al. 1998; Bickham et al. 2000; Theodorakis 2001).

Genetic changes in a population due to toxicants, e.g. the loss of genetic diversity, might be permanent. Genetic diversity would only be expected to recover if the population survived for a long time span and gene flow from other populations would not be interrupted (Hoffmann and Willi 2008). On the other hand, due to the mutagenic effect of chemicals, new alleles and genes could arise and increase the genetic diversity of the population. While selection and genetic drift typically reduce genetic diversity, mutation and migration are the major processes that increase genetic variability in a

population. Without any catastrophic events, such evolutionary processes act slowly, so the time scale relevant for the response of genetic diversity is years rather than months. Therefore, one advantage of monitoring population genetics is that footprints of toxicants in population genetic patterns may still be detectable after years or decades, even if the abundance of the population has already recovered. As there is often a time lag before changes in population genetic patterns become significant, an indicator based on population genetic studies is expected to be primarily useful for multigenerational exposures (Bickham et al. 2000; Bagley et al. 2002).

A bioindicator is an organism reflecting environmental conditions of its habitat by its presence or absence and its function (van Gestel and van Brummelen 1996). The zebra mussel *Dreissena polymorpha* has been applied as a bioindicator for passive as well as active biomonitoring of freshwater ecosystems (Sues et al. 1997; Roditi et al. 2000; Bervoets et al. 2005; Pain et al. 2005). It has been established as an early warning system for freshwater quality (Sluyts et al. 1996, Bocherding and Jantz 1997). Moreover, the zebra mussel has been used as a model organism for freshwater mussels in several studies dealing with anthropogenic impacts on environments (Griebeler and Seitz 2007; Hidde 2008). Individuals of different generations of zebra mussel populations can be well distinguished, as it is possible to identify the age of the individuals by counting annual rings on the shells (Jantz 1998). Therefore, zebra mussel populations are ideal for studying long-term effects in population genetics. In total, due to its aforementioned properties, we hypothesize that the zebra mussel could be a suitable indicator for demonstrating effects of pollution on freshwater habitats based on population genetic studies.

2.1 A new approach in biomonitoring freshwater ecosystems based on the genetic status of the bioindicator *Dreissena polymorpha*

Biomonitoring is the measurement of the response of living organisms to man-made changes in their environment (Hankard et al. 1993, in Ware 1998). Following the approach of biomarker-based biomonitoring (Shugart et al. 1992), we attempt to measure responses to environmental contamination at different levels of biological organization, from population down to molecular level. As defined by the National Academy of Sciences (1989), a biomarker is a xenobiotically-induced variation in

cellular or biochemical components or processes, structures, or functions that is measurable in a biological system or sample.

Here, we suggest an approach to investigate the effects of environmental contamination on the genetic status of *D. polymorpha*. In 2000, Bickham et al. proposed population genetic changes as the "ultimate" biomarker of effect. Consequently, we assess population genetic patterns of zebra mussel populations as an independent long-term indicator of environmental contamination. This "ultimate" biomarker has a slower response time than conventional biomarkers, but it has high ecological relevance. The degree of genetic diversity, e.g., reflects the genetic plasticity of the population (Shugart et al. 1992). Genetic plasticity is a precondition for the adaptation of populations to new environmental conditions. Thus a reduction in genetic diversity could cause the extinction of a population under altered environmental conditions. We assess the population genetics of zebra mussels by microsatellite analysis. Due to their high variability and codominant mode of inheritance, microsatellites are very suitable markers to detect fast changes in genetic patterns within and among populations that are caused by environmental contamination (Bickham et al. 2000; Dimsoski and Toth 2001). The biogeography of zebra mussel populations at the study site is monitored by microsatellites at the same time. This analysis is of importance since the biogeographical history of populations has a strong influence on genetic differentiation among populations. To separate this process from the effects of environmental contamination on genetic patterns, it is important to analyze biogeographical patterns of the bioindicator when conducting biomonitorings of populations from polluted and non-polluted sites (Whitehead et al. 2003). Additionally, the effects of anthropogenic stressors at the population level are assessed by measuring the population characteristics abundance and age structure. Populations exposed to pollution may have significantly reduced sizes compared to reference populations, resulting in a genetic bottleneck (Whitehead et al. 2003). If an ecological effect at the population level is due to exposure to xenobiotics, responses at lower levels of biological organization should also be or have been apparent (Shugart et al. 1992). Consequently, we also measure short-term indicators of pollution exposure at the molecular and cellular level of *D. polymorpha.* We assess two biomarkers of genotoxicity, namely Comet assay and micronucleus test, whose response is more relevant of a recent toxic influence. These are rapid techniques for the detection of genotoxic pressure and we used them for measuring DNA damage in hemocytes (blood cells). Hemocytes are easy to obtain as they are single cells in the

hemolymph and demand only very little manipulation after hemolymph withdrawal. They do not require the procedure of cell separation that could cause additional DNA damage. Moreover, hemocytes are closely exposed to environmental agents that enter the zebra mussels' bodies due to their physiological roles in the transport of toxicants and in various defense mechanisms (Mersch et al. 1996; Klobučar et al. 2003). The Comet assay and the micronucleus test are common and well established in the field of ecotoxicology. They have been successfully applied for zebra mussels and have provided reliable results with respect to freshwater pollution (Mersch and Beauvais 1997; Pavlica et al. 2001; Klobučar et al. 2003; Binelli et al. 2010). We assess the potential toxicants by water analyses. To detect pollutants in the environment, a broad range of parameters are measured, e.g. physico-chemical parameters, heavy metals and organic compounds.

In the described approach, we measure biomarkers at different levels of biological organization as well as water quality parameters. This way, we assess the short and long-term impacts of environmental contamination on the genetic status of zebra mussel populations. Possibly, our approach could become a strategy to monitor instantaneous and long-term effects of pollution on freshwater organisms, and could aid in managing anthropogenic pressures on them.

2.2 Aim of the thesis

The aim of my thesis is to integrate population genetic measures into an ecotoxicological experiment with the study species *D. polymorpha*. I therefore elaborate an integrated approach in biomonitoring and apply this approach in a first case study.
To implement the above-described approach, I designed three distinct projects. The first project is the development of five highly polymorphic microsatellite loci. This first step enables me to accurately assess the genetic patterns of populations of the study species *D. polymorpha*. In the second project, I analyze the biogeographical background of twelve populations of the zebra mussel from the rivers Danube (Hungary and Croatia), Drava (Croatia), Sio (Hungary), Vah (Slovakia), Thaya (Austria) and Rhine (Germany) by microsatellite analysis. This characterization of the baseline of molecular variance of the study species in a biogeographical context enables me to evaluate responses to contamination of a population from this region in the third project. The third project is a biomonitoring experiment carried out in cooperation with the Department of Zoology of

the University of Zagreb. We test the above-described approach in biomonitoring by assessing and comparing the genetic status of a zebra mussel population from a polluted site with a reference population in the River Drava in Croatia. Additionally, we conduct a laboratory experiment to investigate if the fitness of the two populations is altered by the different environmental conditions that the populations have been exposed to in their original source habitat. Moreover, we want to find out if the approach in biomonitoring is realizable in a first case study.

According to the three projects, my PhD thesis consists of three main chapters. Each chapter is a complete description of a project and is included in this thesis in the form of a manuscript for an international scientific journal.

Following these three chapters a conclusion is given as a synthesis of the results of all three research projects.

3 NEW POLYMORPHIC MICROSATELLITE LOCI FOR THE ZEBRA MUSSEL *DREISSENA POLYMORPHA* (PALLAS, 1771), A COMMON BIOINDICATOR

3.1 Introduction

Evolutionary toxicology investigates population genetic effects caused by environmental contamination (Bickham et al. 2000). Toxicant inputs of increasing industry, agriculture, and fast growing cities have severely altered freshwater ecosystems. These fast changing environments increase demands on the adaptability of animals. Genetic diversity enables species to adapt to changing environments. Environmental stressors are expected to reduce genetic diversity by causing mortalities, so that a recent reduction in genetic diversity is indicative of a deteriorating environmental condition. Thus, the amount of genetic diversity can be applied as a biomarker and provides essential information at two levels: First, a high genetic diversity assures the sustainability of a population and, secondly, it indicates the integrity of a whole ecosystem (Bagley et al. 2002).

Approaches employed to measure genetic diversity are selected based upon the desired level of allelic diversity (Brown et al. 2001). Microsatellites underlie high mutation rates that generate the high level of allelic diversity which is necessary for the study of processes acting on fine-scale ecological questions (Selkoe and Toonen 2006). Due to their high variability and codominant inheritance, microsatellites are adequate markers to detect fast changes in genetic diversity within and among populations that are caused by environmental contamination (Dimsoski and Toth 2001).

The zebra mussel *Dreissena polymorpha* is widely distributed in Europe and North America (Minchin et al. 2002) and has been used as a model organism for freshwater mussels in several studies (Pain et al. 2005; Griebeler and Seitz 2007; Hidde 2008). *Dreissena polymorpha* is a common bioindicator for passive as well as active biomonitoring of freshwater ecosystems (Bocherding and Jantz 1997; Sues et al. 1997; Roditi et al. 2000; Klobučar et al. 2003; Bervoets et al. 2005). Preliminary studies have shown the high genetic variability of zebra mussel populations (Müller et al. 2002; Astanei et al. 2005). It is therefore a promising approach to analyze the genetic diversity of the zebra mussel as an independent indicator of environmental conditions to assess the health of an ecosystem.

For the zebra mussel, five highly polymorphic microsatellites (Locus A6, B6, B8, B9 and C5) have been described by Naish and Boulding (2001), and two of them (Locus B8 and B9) have been modified by Astanei et al. (2005). Population genetic studies on *D. polymorpha* have shown high polymorphism of the published markers (Astanei et al. 2005) and thus, their high power to resolve the genetic structure of populations (Müller et al. 2002). However, one locus (B8) turned out to be under selective pressure (Thomas and Seitz 2008). The number of four microsatellites is critical in providing sufficient genetic variability to analyze the response of population genetic structure to anthropogenic stressors. Due to their selective neutrality, each microsatellite is an independent sample of the genome (Kalinowski 2002). Adding microsatellite loci to a study will increase the genome-wide sampling and consequently boost the power of statistical analyses (Selkoe and Toonen 2006). We therefore developed additional microsatellite loci to establish a neutral marker set that allows a robust analysis of the genetics of zebra mussel populations.

In this article, the isolation and characterization of five new microsatellite markers, developed to investigate the influence of environmental pollution on the model organism and common bioindicator *D. polymorpha*, will be depicted.

3.2 Materials and Methods

3.2.1 Microsatellite isolation and primer design

A genomic library enriched for microsatellites was developed from the pooled DNA of four individuals of *D. polymorpha*. We isolated the DNA from the posterior adductor muscle by liquid nitrogen extraction and the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH).

The Genterprise Genomics GmbH (Mainz, Germany) restricted the pooled DNA with the *HindIII* restriction enzyme (Fermentas). This company ran the products on agarose gels and excised the restricted fragments that ranged between 1.2-2.5 kb and > 2.5 kb. They then purified and cloned them into pUC 18 vector plasmids cut with the restriction enzyme *SmaI*. Afterwards, the ligated plasmids were transformed into DH10B *Escherichia coli* host cells. Colonies were screened with the following radioactively labelled oligonucleotides: (AAT)n, (GGC)n, (AAG)n, (ATG)n, (AAC)n, (GATC)n, (GATA)n, and (TAAA)n. Finally, the Genterprise Genomics GmbH (Mainz, Germany) isolated and purified the plasmid DNA from positive colonies.

In our laboratory, we amplified the inserts in both directions, using the standard forward and reverse pUC18 primers. Sequencing reactions were performed in a 10 µl volume containing 6 µl plasmid DNA, 2 µl 5x sequencing buffer (Genterprise Genomics GmbH), 1 µl primer (10 pmol/µl), and 1 µl Big Dye Terminator v3.1 Sequencing Standard Kit (Applied Biosystems). After purification, we ran the products on the capillary sequencer (ABI genetic analyzer 3130xl, Applied Biosystems). We checked the sequences visually for the presence of microsatellites consisting of five or more non-interrupted repeats, using the free software FinchTV 1.4.0 (Geospiza 2009). For these microsatellites, primers were designed using the free software PRIMER3PLUS (Untergasser et al. 2007). The 5'-ends of the forward primers were modified with an M13(-21) sequence tag (5´-ACGACGTTGTAAAACGAC-3´) following Schuelke (2000).

3.2.2 PCR and evaluation of the microsatellite loci

For fragment analysis, we slightly modified the single-reaction nested PCR-method described by Schuelke (2000). Thermocycling conditions consisted of a denaturation at 94 °C for 5 min, 30 further cycles of denaturation at 94 °C for 30 sec, annealing at 45 °C–65 °C for 45 sec, and extension at 72 °C for 45 sec, eight additional cycles of denaturation at 94 °C for 30 sec, annealing at 53 °C for 45 sec, and extension at 72 °C for 45 sec, and a final extension of 72 °C for 15 min. The PCR was carried out in a 25 µl-volume, using Ready-To-Go^{TM} PCR-Beads (Amersham Pharmacia Biotech Inc.). In addition to the beads, the reaction contained 50 - 200 ng DNA, 2 pmol of the forward-primer, 8 pmol of the reverse-primer and the fluorescent labelled M13-primer, 0.25-0.3 µl of $MgCl_2$ (50 mM), and sterile water up to a volume of 25 µl. In a first step, we optimized the PCR protocol, considering the annealing temperature in the range of 45 °C to 65 °C and the concentration of $MgCl_2$, using the DNA of four individuals. Secondly, the polymorphism of the successfully optimized loci was tested on 24 individuals of one population (River Danube, near Budapest, Hungary). By this standard test, it was possible to prove that the microsatellite loci were variable enough to even discriminate between individuals within a population. We estimated the observed (H_O) and expected (H_E) heterozygosity and tested for Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) with the program GENEPOP 1.2 (Raymond and Rousset 1995) for all new loci. Additionally, we tested the new loci for

pairwise LD with the already published loci A6, B6, B8, B9, and C5 (Naish and Boulding 2001; Astanei et al. 2005).

3.3 Results

After plasmid preparation, a total of 192 positive colonies were sequenced forward and reverse. Among these, we identified 28 colonies with repeat motifs that were not interrupted and had at least five repeats. The other 164 positive colonies were second copies of a specific locus or did not contain any repetitive sequence. Nine of the identified repeats were $(AAT)_n$, one was $(AAG)_n$, five were $(ATG)_n$, one was $(TAAA)_n$, two were $(TAG)_n$, one was $(GT)_n$, three were $(AT)_n$, two were $(GA)_n$, two were $(GTC)_n$, one was $(AGC)_n$, and one was $(AATAATT)_n$. Twenty-two primer pairs were designed, and PCR was carried out on those 22 loci. Eight of these loci were successfully amplified. They were tested on a single population of 24 individuals. Five of these loci displayed distinct peaks and showed allelic polymorphism among the tested individuals. Allele numbers of the five loci ranged from 3 to 14 (mean = 8.4). Observed and expected heterozygosity was between 0.55 and 0.91 (mean H_O = 0.77) as well as between 0.43 and 0.83 (mean H_E = 0.74), respectively. Table 3.1 provides the locus designations, repeat motifs, primer sequences, ranges of PCR product sizes, numbers of observed alleles, annealing temperatures, and observed and expected heterozygosities. All five loci conformed to Hardy-Weinberg expectations, suggesting no evidence for null alleles. None of these five loci was significantly linked to any other locus, including the five that had already been published.

Table 3.1 Five new microsatellite loci tested on 24 individuals of one population. Locus designations, repeat motifs of the positive clones, sequences of forward primers (**F**) with M13-tag and of the reverse primers (**R**), size ranges of detected alleles, numbers of alleles (A), annealing temperatures of the specific primers, observed heterozygosities (H_O), expected heterozygosities (H_E), and the accession numbers in NCBI GenBank are provided.

Locus	Repeat Motif	Primer Sequence (5' – 3')	Size Range (bp)	A	Tempera-ture (°C)	H_O	H_E	GenBank Accession No.
Dpol6	$(AAT)_{40}$	**F: M13(-18)** TGTTTCACCCCATTAATGACAG **R:** GTCCATTGTTGATGCCACATTA	479-509	6	64	0.74	0.82	JF318965
Dpol7	$(AAT)_{30}$	**F: M13(-18)** TCATACCGCCATTGATATGC **R:** TGCGCTCGAATAAATGACAA	205-290	14	64	0.83	0.77	JF318961
Dpol9	$(TAAA)_{12}$	**F: M13(-18)** TGGTTGATGCAGTGACCCTA **R:** TGTCGCTTGATCCATGTTTT	218-249	8	59	0.83	0.83	JF318962
Dpol15	$(TTC)_{10}(ATC)_8$ $(ATT)_{11}$	**F: M13(-18)**GATCCCCTATTACTG ACATAATGC **R:** GGAAGGTCATTTACCATACTA TTGC	450-461	3	45	0.55	0.43	JF318963
Dpol19	$(ATG)_{40}$	**F: M13(-18)**GCATTCCATCAAAAA CACAGAT **R:** GATCAACACCAAAGTTCGTTT C	293-418	11	58	0.91	0.83	JF318964

3.4 Discussion

In this study, we isolated five new microsatellite loci. With the five already published loci (Naish and Boulding 2001; Astanei et al. 2005), we now have a set of ten polymorphic loci for *D. polymorpha*. One of these loci (B8) appeared to be not neutral to selection (Thomas and Seitz 2008). We assume that the nine other markers are neutral, as they did not show any significant deviation from HWE. The relatively high expected heterozygosity (mean $H_E = 0.74$) of the new loci indicates their high degree of polymorphism. There was no significant genetic linkage disequilibrium, suggesting that all nine loci are transmitted to offspring independently.

All the nine neutral microsatellite loci are appropriate to analyze the population genetic structure of *D. polymorpha*. Due to their high polymorphism, these loci are suitable to resolve a fine-scaled population structure and to detect recent changes in populations, such as changes in genetic diversity and population bottlenecks. Microsatellites that are neutral to selection and are not significantly linked to each other are independent samples of the genome and the genetic patterns detected by microsatellite analysis

reflect patterns of the whole genome (Kalinowski 2002). It is therefore reasonable to conclude that if we detect a high genetic diversity at neutral microsatellite markers in a population, then genetic diversity is probably high at genes that encode fitness-related characters, which contributes to the sustainability of the population in the long term. Likewise, by measuring a low genetic diversity at neutral markers, we are able to detect a potentially decreased genetic diversity at fitness loci (Bagley et al. 2002), which results in a loss of adaptability and a potentially decreased fitness of the population (Murdoch and Hebert 1994). In summary, estimating the biomarker genetic diversity by using these microsatellites will contribute to elucidating the condition and sustainability of zebra mussel populations.

Due to their high polymorphism, the microsatellite loci are also applicable to detect differences in genetic structure, even among closely adjacent populations. Comparing the genetic patterns of populations in front of and behind a pollution source can resolve the relationship between stressor exposure and genetic diversity (Murdoch and Hebert 1994). As we apply the genetic diversity of *Dreissena* populations as an independent indicator of environmental condition, this can help us to understand the consequences of anthropogenic stressors for freshwater ecosystems.

This established marker system for the bioindicator *D. polymorpha* is a powerful tool to investigate the influence of anthropogenic stressors on zebra mussel populations and on freshwater ecosystems in general. The markers are currently used in several projects including studies on the population structure in the River Danube and the genetic consequences of pollution stress for zebra mussel populations.

4 BIOGEOGRAPHY AND GENETIC ADAPTATION OF POPULATIONS OF THE COMMON BIOINDICATOR *DREISSENA POLYMORPHA* IN THE RIVER DANUBE AND SOME OF ITS TRIBUTARIES

4.1 Introduction

The long-term impact of environmental stressors on natural populations and on the sustainability of ecosystems has become an important focus in the research field of ecotoxicology (Bickham et al. 2000, Bickham 2011). When assessing the state of an ecosystem, it is important to characterize the natural genetic structure of populations and the exchange of individuals between populations (Bagley et al. 2002). The genetic structure of populations can be influenced by both their biogeographical history as well as past and current environmental conditions. The biogeographical history has a strong influence on the genetic differentiation among populations. Historical factors and thereby induced population genetic processes subdivide a species' distribution range into distinct genetic subgroups and lead to present biogeographical patterns (Staton et al. 2001). Restricted gene flow caused by geographic barriers or the gradual loss or gain of alleles in the gene pools corresponding to the geographic distance of the populations both lead to genetic population differentiation. Past and present biotic and abiotic environmental changes (e.g. pollution), however, may alter these genetic patterns of populations (Bickham et al. 2000). If the genetic constitution of a population deviates from the overall biogeographical background of this species, this could indicate that local environmental factors including environmental pollution have altered its genetic constitution (Whitehead et al. 2003; Thomas et al. 2010). For this reason, it is also important to analyze the biogeographical patterns of bioindicators in biomonitoring experiments: By establishing the baseline of molecular variance in the context of biogeography of the bioindicator, it is possible to discriminate between the impact of biogeography and of contamination on the genetic constitution of populations from polluted and non-polluted sites (Whitehead et al. 2003; Fratini et al. 2008). Several authors that conducted ecotoxicological studies on exposure of populations to contamination found that biogeographical processes had mainly influenced population genetic patterns for intertidal crabs (*Pachygrapsus marmoratus*, Fratini et al. 2008), freshwater fish (*Catostomus occidentalis*, Whitehead et al. 2003), wood mice

(*Apodemus sylvaticus*, Berckmoes et al. 2005), and bank voles (*Myodes glareolus*, Meeks et al. 2009). Other studies, however, detected a significant impact of contamination on population genetic patterns, when controlling for biogeographical effects for marine mussels and barnacles (*Mytilus galloprovincialis* and *Balanus glandula*, Ma et al. 2000), perches (*Perca flavescens*, Bourret et al. 2008), and rats (*Rattus norvegicus*, Kohn et al. 2003). The purpose of our study is to establish the baseline of molecular variance of the zebra mussel *Dreissena polymorpha* in a biogeographical context to be able to interpret genetic responses to pollution in ecotoxicological studies.

The common bioindicator *D. polymorpha* has been successfully applied as bioindicator for passive as well as active biomonitoring of freshwater ecosystems (Sues et al. 1997; Bervoets et al. 2005). Its physiological and ecological characteristics are well described in literature (Ludyanskiy et al. 1993; Orlova 2002; Karatayev et al. 2006). The zebra mussel is well suitable as a bioindicator due its high filtration rate that favors the accumulation of xenobiotics in the mussel body (Bervoets et al. 2005). The zebra mussel is a bivalve native to the Ponto-Caspian Sea basin and has spread over Europe since the early 19^{th} century (Thienemann 1950). Rapid colonisation has been facilitated by the high fecundity of the mussel and its free-swimming veliger larvae stage (Minchin et al. 2002). The larvae remain in the plankton up to three weeks or even longer until they settle down and attach to the substrate with their byssal threads, and can be drifted over 300 km downstream before settlement (Sprung 1989; Stoeckel et al. 1997). The rapid upstream and downstream spreading of the mussel observed in European shipping routes has been facilitated by transporting the larvae in ship ballast water and by adult mussels attached to the hulls of watercrafts (Bossenbroek et al. 2007). The River Danube is one of the main immigration routes of the zebra mussel to Western Europe (Müller 2001; Müller et al. 2002).

In this study we analyzed eleven populations of the common bioindicator *D. polymorpha* in the River Danube and some of its tributaries. Using six microsatellite loci we first established the biogeographical baseline data on molecular variance for *D. polymorpha* for this geographic region. Neutral microsatellite markers allow accurate genetic assessment of population differentiation (Sekino et al. 2001). Due to their high variability and their rapid evolution, microsatellites can reveal present day demography and the connectivity of populations (Selkoe and Toonen 2006). Moreover, they can resolve genetic differences of contaminated and non-contaminated sites (Dimsoski and

Toth 2001) and also rapid changes in genetic variation, as provoked by the impact of environmental mutagens (Bickham et al. 2000).

Furthermore, we analyzed if the genetic constitution of the studied populations deviates from the overall biogeographical genetic background in the study area and tested if current environmental conditions have influenced their genetic constitution. This will enable us to evaluate genetic responses to pollution seen in this species in future ecotoxicological studies in this study region.

4.2 Materials and Methods

4.2.1 Study area and sampling

A section of the River Danube and some of its tributaries were chosen as study area (Fig. 4.1). To characterize the genetic structure of zebra mussel populations, we sampled populations from Budapest (Hungary) to Aljmaš (Croatia) in the River Danube (DAN1, DAN2, DAN3, and DAN5) and in its tributaries Thaya (THA), Váh (VAH), Sió (SIO) and Drava (Dc and Dp), in the nature park Kopački Rit (DAN4) and in the Lake Jarun (LJAR) in Zagreb (Croatia, Fig. 4.1). The sampling site DAN4 is a lake which is regularly flooded by the River Danube. Consequently, we allocated the DAN4 population to the Danube populations. As a genetic outgroup, we used a population from Iffezheim (RHI), at the River Rhine (Germany). We sampled population RHI in July 2006, populations THA, DAN1, DAN2, DAN3, and SIO in July 2008, and populations DAN4, DAN5, Dc, Dp, and LJAR in August 2009. Population VAH was sampled in August 2007 by members of the Joint Danube Survey II (for details see JDSII Final scientific report 2008, therein designated as population 21L).

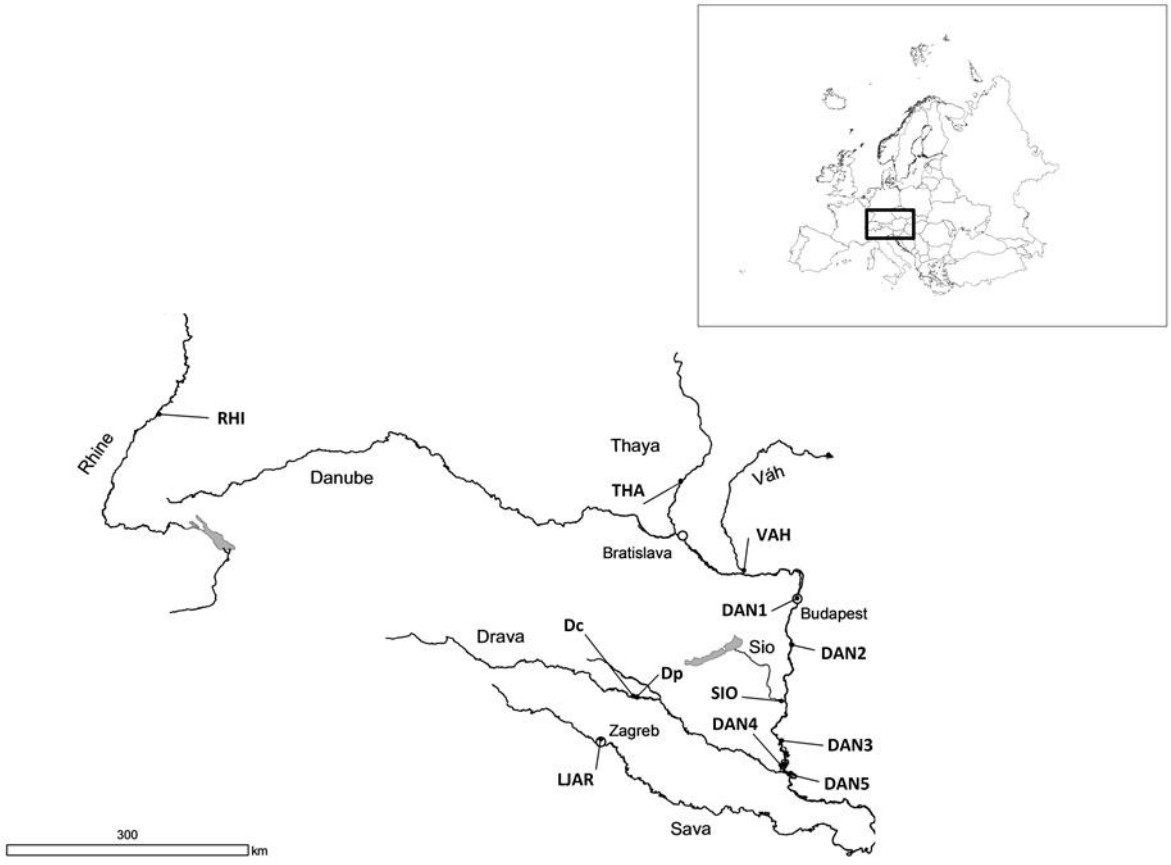

Fig. 4.1 Geographical locations of the sampling sites. River Rhine: RHI (Iffezheim, Germany); River Thaya: THA (Bernhardsthal, Austria); River Váh: VAH (800 m upstream of the estuary, Slovakia); River Danube: DAN1 (Budapest, Hungary), DAN2 (Dunaújváros, Hungary), DAN3 (Batina, Hungary), DAN4 (nature park Kopački Rit, Croatia), DAN5 (Aljmaš, Croatia); River Sió: SIO (100 m upstream of the estuary, Hungary); River Drava: Dc (behind the concrete dam of Čakovec lake, Croatia), Dp (600 m downstream from Dc, Croatia); Lake Jarun: LJAR (Zagreb, Croatia).

Individuals of *D. polymorpha* were sampled at the river shore in 1-50 cm water depth for genetic analyses. We estimated the abundance of *D. polymorpha* (a) for each population from the number of captured individuals (i) per time (t) and the number of collecting agents (c) as

$$a = \frac{i}{t \cdot c} \quad [\min^{-1}].$$

The sampling time varied between one and 120 minutes, depending on the abundance of the mussels at the sampling site. The abundance of population VAH is unknown as it was not sampled by the authors of this study.

4.2.2 Assessment of water parameters

We estimated four major general water quality indicators (JDSII Final scientific report 2008), i.e. temperature, dissolved oxygen, pH-value, and conductivity, as well as current velocity of the sampling sites during sampling. Temperature, dissolved oxygen, pH-value and conductivity were measured with a multiparameter instrument (Multi 340i, WTW GmbH). The current velocity was measured with a flow measuring device (Messtechnik Reinhardt GmbH). For population VAH, none of the abiotic parameters could be measured, because the population was not sampled by the authors. For population RHI (genetic outgroup), which was sampled during another study (unpublished data), water temperature and current velocity are known, but pH, dissolved oxygen and conductivity are unknown.

4.2.3 DNA extraction and microsatellite analysis

In general, we used 48 individuals per population for microsatellite analyses. For population VAH and SIO, however, we could only genotype 29 and 37 individuals, respectively, because of the low numbers of sampled individuals at the respective sampling sites. Tissue used for DNA extraction was taken from the posterior adductor muscle of ethanol-preserved samples. Genomic DNA was extracted using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH). The six primers used for microsatellite analysis and the respective PCR protocols are based on Naish and Boulding (2001), Astanei et al. (2005), and Thomas et al. (2011). Four of the primers (A6, B6, B9, and C5) are from Naish and Boulding (2001) and Astanei et al. (2005), but they were partially modified in our institute (Table 5.1) using the program PRIMER3PLUS (Untergasser et al. 2007), or by PIG-tailing (Brownstein et al. 1996). The two other primers (Dpol9 and Dpol19) are published in Thomas et al. (2011). Genotypes were generated on a capillary sequencer (ABI genetic analyzer 3130xl, Applied Biosystems). Genotype scoring was conducted with the program GENEMAPPER 4.0 (Applied Biosystems). The allele size calls were reviewed twice and if necessary corrected manually. Individuals that did not show a result for a specific locus or did not show distinct peaks were repeated in amplification and fragment analysis.

4.2.4 Statistical analyses

Quality of microsatellite data. Only nine out of a total of 546 individuals studied (1.65%) produced missing data for more than a third of the analyzed microsatellite loci. As this was most probably caused by low DNA quality, we removed these individuals from the total genetic data set. The jackknife procedure as described by Morin et al. (2009) and executed by the program "R" 2.9.1 (R Development Core Team 2009) was used to identify further individuals that were influential on deviations from Hardy-Weinberg-Equilibrium (HWE) of single loci. This procedure identified another 28 individuals. Six of these individuals were directly removed from the data set as they were homozygous in at least four loci, which was possibly caused by low DNA quality. For all other 22 individuals, we repeated the PCR. As this procedure confirmed their genotypes, the respective individuals were not excluded from the total data set.
After this procedure, we checked the microsatellite data set of the remaining 531 individuals for possible null alleles, scoring errors due to stuttering and large allele dropouts with the program Microchecker (van Oosterhout et al. 2004). We also tested for linkage disequilibria between pairs of loci for each of the populations using the program GENEPOP 4.0.10 (Raymond and Rousset 1995).

Genetic diversity and Hardy-Weinberg-Equilibrium. Random genetic drift and selection alter frequencies of genotypes within populations and can cause loss of genetic diversity and deviations from Hardy-Weinberg-Equilibrium (HWE). We therefore estimated genetic diversity and assessed potential deviations from HWE with the program GENEPOP 4.0.10 (Raymond and Rousset 1995). We calculated the observed and expected heterozygosity (genetic diversity) for each of the populations across all six microsatellite loci (Raymond and Rousset 1995). For testing HWE in each of the populations across loci we applied the exact "HW test" (Haldane 1954; Guo and Thompson 1992) and the statistically more powerful score test (U test) of heterozygote deficiency and heterozygote excess (Rousset and Raymond 1995). To detect if each of the loci conformed to HWE in the populations we applied the score test of deficiency and excess of heterozygotes to each of the loci. Markov chain parameters were 10,000 for dememorization, with 500 batches and 5000 iterations per batch in all score tests conducted.

Bottlenecks. To find out if any of the populations had recently experienced a severe reduction in population size and had undergone a recent genetic bottleneck, e.g. due to mortalities resulting from water contamination, we applied the program BOTTLENECK 1.2.02 (Cornuet and Luikart 1997) to all populations. In this program, the genetic data are tested for an excess of heterozygosity in comparison to the heterozygosity expected under mutation-drift equilibrium ($H_E > H_{EQ}$). In a bottlenecked population, the number of alleles is reduced faster than the allele frequencies by a strong reduction in population density. As H_E is calculated from allele frequencies and H_{EQ} is calculated from allele numbers, a significant excess of heterozygosity ($H_E > H_{EQ}$) indicates a recent bottleneck in a population (Piry et al. 1999).
In this analysis, we chose the two-phase model (TPM), as it is the most appropriate mutation model for microsatellite loci (Di Rienzo et al. 1994; Piry et al. 1999). We used the Wilcoxon test to test for statistical significance of bottlenecks in the populations. This test is the most powerful one provided by the program BOTTLENECK if less than 20 loci have been analyzed (Piry et al. 1999). We adopted the default parameters for the TPM given in this program (30% variance and 70% probability).

Population differentiation. To assess overall differences in gene pools of populations caused by historic and recent processes we calculated the genetic differentiation between the populations (F_{ST}) using the F-statistics by Weir and Cockerham (1984) as implemented in the program FSTAT (Goudet 2001). Weir and Cockerham (1984) weight allele frequencies according to sample sizes, and therefore the estimates of these statistics are not influenced by differences in sample sizes of populations.
The genetic differentiation between the populations based on pairwise F_{ST}-values was represented in a cladogram by the program TreeView 1.6.6 (Page 1996). We used the UPGMA method of clustering and applied pairwise F_{ST}-values (1,000 bootstraps), calculated by the program POPULATIONS 1.2.30 (Langella 1999), to establish the cladogram.

Isolation by distance. To identify Danube populations deviating from the overall biogeographical background we conducted an isolation-by-distance analysis, as we assumed that populations originating from one single stream would reflect the correlation of increasing genetic differentiation of populations with geographical distance. We therefore calculated a regression of pairwise estimates of genetic

distances, as defined by $F_{ST}/(F_{ST}-1)$, against the corresponding logarithms of the pairwise geographical distances (along the rivers) between populations. A Mantel test with 1000 random permutations was conducted to test for significance of isolation by distance. All analyses were carried out with the program GENEPOP 4.0.10 (Raymond and Rousset 1995).

Spatial analysis of molecular variance. To derive which populations belong together geographically and genetically, we conducted a Spatial Analysis of Molecular Variance (SAMOVA) with the program SAMOVA 1.0 (Dupanloup et al. 2002). The algorithm implemented in this software is based on a simulated annealing procedure that maximizes the proportion of total genetic variance due to differences between the groups of populations and minimizes genetic variance within groups. The estimated F_{CT}-value indicates the variance between groups and therefore the genetic differentiation between the groups of populations (Dupanloup et al. 2002). The program is also able to identify genetic barriers between the groups of populations and to detect outlier populations.

Within-population structure. To elaborate the genetic structure within the populations, we created clustering profiles for each of the populations using the program STRUCTURE 2.3.3 (Pritchard et al. 2000). We chose the admixture model with correlated allele frequencies (Falush et al. 2003) and used the LOCPRIOR model option (Hubisz et al. 2009) with twelve predefined populations (K = 12), according to the twelve sampling locations. The value of *r*, calculated by the program, parameterizes the amount of information carried by the twelve predefined populations. A value larger than one indicates that either there is no population structure, or that the structure is independent of the sampling locations. If *r* is close to 1 or smaller than 1, the defined locations are informative of the structure of the populations.

Correlation of genetic estimates with abiotic parameters and geographical distances. We finally tested whether the genetic constitution of populations is influenced by current environmental conditions. We therefore first examined whether the pairwise F_{ST}-values of the ten populations THA, DAN1, DAN2, DAN3, DAN4, DAN5, SIO, Dc, Dp, and LJAR (without population VAH and the genetic outgroup RHI) correlated with differences in abiotic parameters pH, dissolved oxygen, temperature, current or

conductivity by conducting Mantel tests with the program SsS 1.1k (Rubisoft Software GmbH).

Second, by applying multiple regression models, we assessed the impact of all abiotic parameters and of geographical distance (along the rivers) to the sampling site of population DAN5 (which was the most downstream location of our stream network) on the abundance of the populations, the overall and locus-wise genetic diversity of populations (H_E), and the overall and locus-wise number of alleles of populations. The aforementioned ten populations were included in these analyses. The multivariate regression analyses were conducted with the program Statistica 7.0 (Statsoft Inc.).

4.3 Results

4.3.1 Population abundance and abiotic water parameters

The abundance of *D. polymorpha* was very high in the populations Dc, LJAR and DAN4 with an abundance index of 100 for Dc, and 50 for LJAR and DAN4, respectively. The populations RHI, THA, DAN5, SIO, and Dp had low abundances (0.50-0.79) while those of DAN1, DAN2 and DAN3 were intermediate (2.00-6.69, Table 1).

Table 4.1 Number of alleles (A), genetic diversity (H_E), population abundance and the abiotic parameters pH, concentration of dissolved oxygen (O_2, mg/l), temperature (T, °C), conductivity (µS/cm), and current (m/s) at the sampling sites. For abbreviations of populations see Fig. 4.1.

Pop.	A	H_E	Abundance	pH	O_2 (mg/l)	T (°C)	Cond. (µS/cm)	Current (m/s)
RHI	75	0.82	0.61	-	-	24.7	-	0.8
THA	61	0.79	0.69	7.9	3.51	23.1	615	< 0.1
VAH	65	0.81	-	-	-	-	-	-
DAN1	85	0.83	2.00	7.9	3.00	22.6	348	0.3
DAN2	91	0.85	6.69	8.6	4.32	25.0	336	0.1
DAN3	87	0.84	4.40	7.9	2.98	23.0	350	0.1
DAN4	85	0.84	50.00	7.4	3.20	26.0	400	< 0.1
DAN5	86	0.85	0.50	8.0	7.00	23.0	306	0.3
SIO	50	0.69	0.79	8.5	0.00	24.2	1084	< 0.1
Dc	67	0.82	100.00	7.9	6.40	21.7	255	0.1
Dp	59	0.81	0.83	7.8	6.40	20.5	284	0.5
LJAR	62	0.82	50.00	7.9	6.90	26.4	533	< 0.1

Abiotic conditions varied between sampling sites (Table 4.1). The pH-values were between 7.8 and 7.9 for most of the sampling sites, whereas DAN4 had the lowest pH-value (7.4) and DAN2 (8.6) and SIO (8.5) had the highest values. The concentration of dissolved oxygen was beyond the lower detection limit for SIO, and it was highest for DAN5 (7.0 mg/l), LJAR (6.9 mg/l), Dc (6.4 mg/l), and Dp (6.4 mg/l), while all other sites (THA, DAN1, DAN2, DAN3, and DAN4) had intermediate concentrations. The water temperatures ranged between 20.5 °C (Dp) and 26.4 °C (LJAR) for all sampling sites. At the sampling sites THA, DAN4, SIO, and LJAR the current velocity was lower than 0.1 m/s, whereas for all other sites it was higher. The current velocity was the highest for RHI with 0.8 m/s. The conductivity ranged between 306 µS/cm (DAN5) and 400 µS/cm (DAN4) for the five sampling sites at the River Danube. The lowest conductivity of all study sites was measured for Dc with 255 µS/cm and it was by far the highest for SIO with 1084 µS/cm.

4.3.2 Data review

The analysis of the total genetic data set with the program Microchecker (van Oosterhout et al. 2004) did not detect any scoring errors due to stuttering or large allele drop outs. It also did not indicate any evidence for existing null alleles, except for locus C5 in population Dp, and locus A6 in the populations DAN3 and SIO. These loci had a general excess of homozygotes for most allele size classes in the respective populations which could indicate the presence of null alleles. For population Dp and locus C5, the jackknife analysis (Morin et al. 2009) identified one individual to be influential on the deviation from HWE. This individual, however, was not suspected to have a null allele by the software Microchecker. Consequently, we did not remove it from our data set, and we assumed that its homozygote genotype was real and did not contribute to the observed deviation from HWE because of a null allele. For population SIO and locus A6, one single individual was considered to be influential on HWE by the jackknife procedure. This individual, however, did not carry a null allele according to the software Microchecker and was therefore left in the data set as well. For population DAN3, no specific individual was responsible for the deviation from HWE at locus A6, according to the jackknife procedure. Therefore, no further samples of DAN3 were removed from the data set. The estimated rate of potential null alleles was 0.07 for C5 in Dp, 0.08 for A6 in DAN3, and 0.08 for A6 in SIO. Such rare frequencies of null alleles are reported to have a negligible impact even on parentage analyses, whereupon

parentage analyses are expected to have a higher bias from null alleles than analyses of population structure (Dakin and Avise 2004).

After sequential Bonferroni correction, we detected no significant linkage disequilibrium between all pairs of the six loci that were applied in this study. For population LJAR, however, the loci A6 and B9 showed significant linkage disequilibria. But since there was no significant linkage disequilibrium in all other populations between these loci, it is obvious that it was not caused by a real physical genetic linkage of the two loci. As the current velocity in the Lake Jarun in Zagreb is low (< 0.1 m/s), it is very likely that the larvae are not drifted away and that they settle down close to their relatives. Consequently, alleles from the same lineages tend to cluster together in the same individual and linkage disequilibria are observed (Hartl and Clark 1989).

4.3.3 HWE, bottlenecks and genetic diversity

Allelic diversity assessed by the total number of alleles across loci varied between the studied populations (Table 1). The Danube populations (DAN1, DAN2, DAN3, DAN4, DAN5) had the highest numbers of alleles. Allele numbers observed for these populations ranged from 85 (DAN1, DAN4) to 91 (DAN2). Population SIO had the lowest number of alleles (50) of all studied populations.

The test of HWE across loci showed that all populations conformed to HWE, after sequential Bonferroni correction. The score tests of heterozygote excess and of heterozygote deficiency across loci corroborated HWE for all populations, after sequential Bonferroni correction.

The six analyzed microsatellite loci were polymorphic in all populations. The number of alleles per locus over all populations was 27 for locus A6, 26 for B6, 22 for B9, 11 for C5, 15 for Dpol9 and 31 for Dpol19. The locus-specific tests of heterozygote deficiency and excess across populations revealed that all loci conformed to HWE after sequential Bonferroni correction, except of locus A6 that showed a significant heterozygote deficiency. To detect which of the populations generated the heterozygote deficiency at locus A6, score tests of heterozygote deficiency were carried out for each of the populations for A6. These tests revealed that the populations SIO, DAN3 and DAN5 had a significant deficit of heterozygotes (SIO: $p = 0.005$, DAN3: $p = 0.007$, DAN5: $p = 0.041$) and that the deficit was marginally significant for DAN4 ($p = 0.068$).

Genetic diversity estimated as expected heterozygosity (H_E) varied between populations (Table 4.1). H_E was highest in the Danube populations (DAN1, DAN2, DAN3, DAN4,

DAN5) with values ranging between 0.83 and 0.85. Population SIO from the River Sio had by far the lowest genetic diversity of all populations studied ($H_E = 0.69$). The values of the other six populations were slightly lower than those of the Danube populations and ranged between 0.79 and 0.82.

The Wilcoxon test of the program BOTTLENECK (Cornuet and Luikart 1997) revealed a significant excess of heterozygosity (H_E) in comparison to the heterozygosity expected at mutation-drift equilibrium ($H_E > H_{EQ}$) for the populations Dp ($p = 0.023$) and LJAR ($p = 0.016$). This result suggests that the populations Dp and LJAR had undergone a recent genetic bottleneck (Piry et al. 1999).

4.3.4 Population structure

F_{ST}-statistics conducted for all pairs of populations revealed that all populations were significantly differentiated from each other, despite of the populations Dc, Dp and LJAR, and the pairs of the Danube populations DAN5-DAN1, DAN5-DAN2, DAN5-DAN3, DAN1-DAN2, DAN1-DAN3, DAN1-DAN4, DAN2-DAN3, DAN2-DAN4 (Table 4.2).

Table 4.2 Genetic differentiation between pairs of populations. Pairwise F_{ST}-values (above diagonal) and significance values (≠0, below diagonal). For abbreviations of populations see Fig. 4.1. Significant values are in bold.

Pop.	RHI	THA	VAH	DAN1	DAN2	DAN3	DAN4	DAN5	SIO	Dc	Dp	LJAR
RHI	-	**0.091**	**0.030**	**0.013**	**0.011**	**0.022**	**0.006**	**0.016**	**0.134**	**0.069**	**0.072**	**0.068**
THA	**< 0.001**	-	**0.049**	**0.054**	**0.046**	**0.039**	**0.058**	**0.044**	**0.079**	**0.033**	**0.033**	**0.041**
VAH	**< 0.001**	**< 0.001**	-	**0.005**	**0.015**	0.005	**0.019**	0.006	**0.091**	**0.049**	**0.049**	**0.050**
DAN1	**< 0.001**	**< 0.001**	**< 0.001**	-	0.002	-0.001	0.002	-0.001	**0.090**	**0.039**	**0.039**	**0.041**
DAN2	**< 0.001**	**< 0.001**	**< 0.001**	0.302	-	0.004	-0.003	-0.001	**0.088**	**0.038**	**0.037**	**0.034**
DAN3	**< 0.001**	**< 0.001**	0.033	0.322	0.182	-	0.004	-0.002	**0.075**	**0.032**	**0.031**	**0.033**
DAN4	**< 0.001**	**< 0.001**	**< 0.001**	0.208	0.427	0.005	-	0.000	**0.094**	**0.042**	**0.042**	**0.040**
DAN5	**< 0.001**	**< 0.001**	0.002	0.091	0.426	0.966	0.023	-	**0.072**	**0.030**	**0.030**	**0.029**
SIO	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	-	**0.067**	**0.065**	**0.064**
Dc	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	-	-0.004	0.002
Dp	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	0.520	-	0.005
LJAR	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	0.477	0.139	-

In the cladogram (Fig. 4.2) the five Danube populations (DAN1, DAN5, DAN3, DAN2 and DAN4) comprised one group, as well as the populations LJAR, Dc and Dp.

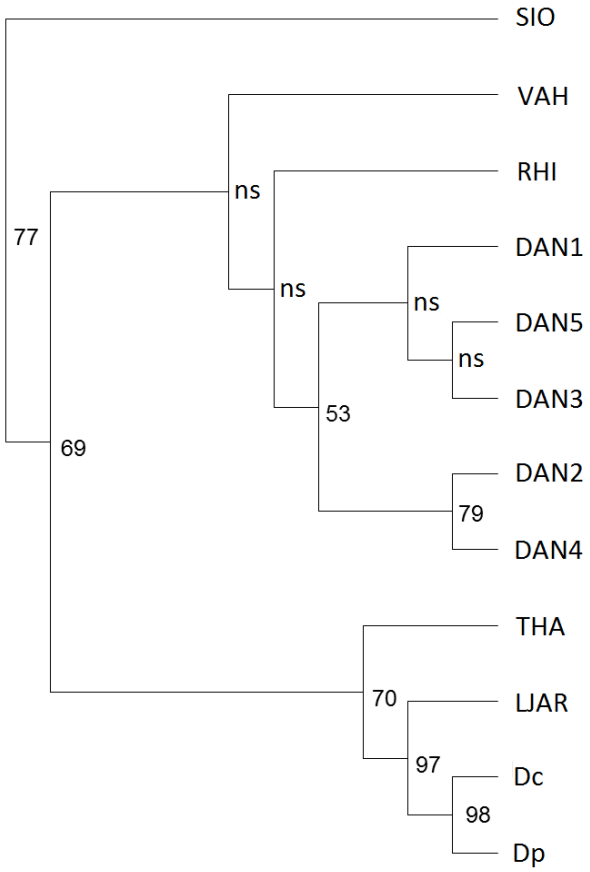

Fig. 4.2 Cladogram of genetic differentiation between populations studied. For abbreviations of populations see Fig. 4.1. To construct the cladogram we used the UPGMA method of clustering, based on F_{ST}-values (Latter 1972). Bootstrap-values are given as percentages over 1000 replications. Non-significant bootstrap-values are indicated by ns (due to clade support < 50%).

Population THA clustered together with the population from the Lake Jarun (LJAR) and the two Drava populations (Dc and Dp). The three other populations SIO, VAH, and RHI formed distinct branches, whereupon the sample from the River Sio (SIO) was most differentiated from all other populations.

The isolation-by-distance analysis did not reveal a significant correlation between genetic and geographic distances of the populations of the River Danube ($p = 0.744$).

The SAMOVA carried out for the twelve studied populations revealed eight groups ($F_{CT} = 0.035$, $p = 0.000$) for which genetic differentiation between groups was maximized and within groups minimized. One group comprised five populations

originating from the River Vah (VAH) and the River Danube (DAN1, DAN2, DAN4, and DAN5). All other groups consisted of one population.

The analysis of the genetic structure within populations conducted with the program STRUCTURE revealed two groups of populations with similar clustering profiles: one group consisted of the Danube populations DAN5, DAN1, DAN2, and DAN3, and the second comprised populations from the River Drava (Dc and Dp) and the Lake Jarun (LJAR). The remaining five populations (VAH, THA, SIO, RHI, and DAN4) showed a unique clustering profile (Fig. 4.3). The value of r = 0.468 (< 1) indicated that the twelve predefined populations were informative for this grouping.

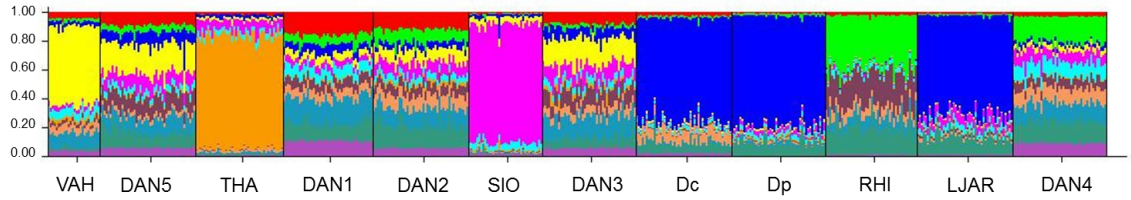

Fig. 4.3 Clustering profile of populations studied. The profile was obtained from the software STRUCTURE 2.3.3 (Pritchard et al. 2000) for K = 12 predefined populations under the LOCPRIOR model and correlated allele frequencies (see methods for details). Each individual is represented on the graph by a vertical line divided into colored segments corresponding to different genetic clusters. The length of each colored segment is equal to the estimated proportion of the individuals' membership in the cluster of corresponding color (indicated on the left-hand scale). For abbreviations of populations see Fig. 4.1.

4.3.5 Correlation of population genetic parameters with abiotic parameters

To test whether differences in environmental parameters between sampling sites had influenced the genetic differentiation of the populations, we correlated the differences seen in each of the abiotic parameters at two sampling sites with the pairwise F_{ST}-values of the respective populations. There was no significant correlation between the F_{ST}-values and the differences in abiotic parameters of the respective populations. This suggests that differences in environmental parameters had not affected the amount of genetic differentiation between populations.

4.3.6 Multiple regression analyses

In contrast, multivariate analysis of abiotic parameters measured at sampling sites and geographical distance versus population genetic parameters revealed eight significant models: overall genetic diversity (H_E), and H_E of the loci B6, C5 and Dpol9, overall number of alleles and number of alleles of the loci B6, C5 and Dpol9 were related to

abiotic parameters (Table 4.3). In all eight models, population genetic parameters significantly decreased with increasing conductivity. In addition, overall H_E, H_E of locus Dpol9 and number of alleles of locus Dpol9 significantly increased with increasing temperature. Overall number of alleles and number of alleles of the loci B6 and Dpol9 significantly decreased with increasing concentrations of dissolved oxygen. The coefficients of determination of all eight regression models were high (R^2: 0.62 – 0.92, Table 4.3), suggesting a good model performance. The abiotic parameters pH and current as well as geographical distance did not correlate with any of the population genetic parameters. Further, the abundance of the populations was not related to any of the abiotic parameters.

Table 4.3 Results of forward, stepwise multiple regression models assessing the influence of abiotic factors and the geographical distance (along the rivers) to population DAN5 on the populations' abundance, expected heterozygosity (H_E, overall and per locus), and number of alleles (overall and per locus). Ten populations (N = 10; THA, DAN1, DAN2, DAN3, DAN4, DAN5, SIO, Dc, Dp, and LJAR) were included in all regression analyses. For abbreviations of populations see Fig. 4.1. Listed are partial regression coefficients (β), coefficients of determination (corrected for degrees of freedom, corr. R^2), and results of the F-test (F, p) for significance of the model.

Model/ parameter	β_{pH}	β_{O2}	$\beta_{°C}$	$\beta_{current}$	$\beta_{conductivity}$	$\beta_{geo.\ dist.}$	corr. R^2	F	p
Abundance	-	0.62^{+}	-	-0.60^{+}	-	-	0.28	2.74	0.132
H_E (overall)	-	-	0.40**	-	-1.03***	-	0.92	54.81	< 0.001
H_E (A6)	-	0.92*	-	-	-	-	0.58	4.15	0.075
H_E (B6)	-	-	-	-	-1.02***	-	0.78	16.54	0.002
H_E (B9)	-	-	-	-	-	-	0.29	2.22	0.187
H_E (C5)	-	-	-	-	-1.00***	-	0.90	41.77	< 0.001
H_E (Dpol9)	-	-0.36^{+}	0.79**	-	-1.09**	-	0.87	15.61	0.005
H_E (Dpol19)	-	-	-	-	-1.11*	-	0.55	4.61	0.053
Alleles (overall)	-	-0.76*	0.39^{+}	-	-1.28**	-	0.74	7.31	0.026
Alleles (A6)	-	-	0.98*	0.66^{+}	-0.84*	-	0.70	5.16	0.068
Alleles (B6)	-	-0.89**	-	-	-1.40**	-0.37^{+}	0.83	11.80	0.009
Alleles (B9)	-	-0.85*	-	-	-1.19*	-	0.58	4.16	0.075
Alleles (C5)	-	-	-	-	-0.97**	-	0.62	8.31	0.014
Alleles (Dpol9)	0.32^{+}	-0.81**	0.47*	-	-1.38**	-	0.83	11.80	0.009
Alleles (Dpol19)	-	-	-	-	-1.05*	-	0.35	2.59	0.148

$^{+}$ p < 0.1, * p < 0.05, ** p < 0.01, *** p < 0.001

4.4 Discussion

4.4.1 Biogeography and local population differentiation

The F-statistics indicated that all populations were significantly genetically differentiated from each other, except for the Danube populations and a group consisting of the two Drava populations (Dc and Dp) and the population from the Lake Jarun (LJAR). This pattern of genetic population differentiation is basically consistent with the overall biogeographical history of the populations in our study region. The fact that the Danube populations were not significantly differentiated from each other is concordant with the observation that the larvae of *D. polymorpha* can be drifted several hundred kilometers until they settle down (Stoeckel et al. 1997). There is a very high migration of larvae from upstream to downstream populations, leading to high gene flow across larger distances. The lack of genetic differentiation between the two Drava populations (Dc and Dp) conforms to the close proximity of the two sampling sites, although other genetic measures revealed genetic differences between the two populations (see below). The isolated population from the Lake Jarun (LJAR) was unexpectedly not genetically differentiated from the two Drava populations (Dc and Dp). This could indicate that the Lake Jarun was colonized by mussels originating from the River Drava. One of the most important vectors concerning overland transport of zebra mussels is recreational boating, although other vectors such as birds also exist (Johnson and Carlton 1996; Minchin et al. 2002; Bossenbroek et al. 2007). The additionally detected significant genetic bottleneck corroborates that population LJAR had undergone a strong founder process. Thus, a possible scenario for the introduction of the zebra mussel into the Lake Jarun could be that boats with mussels attached to their hull and which had been used in the River Drava were transferred to the Lake Jarun.

However, a graphic representation of genetic differentiation between the populations detected deviations from the overall biogeographical pattern elucidated by the F-statistics. Contrary to its geographical location the population from the River Thaya (THA) clustered together with the two Drava populations and the Lake Jarun population (Dc, Dp and LJAR) in the cladogram based on F_{ST}-values. By contrast, based on genotype frequencies, population THA differed from the Drava and Jarun populations (results of the STRUCTURE analyses). This means that, while allele frequencies were numerically similar in the populations THA, Dc, Dp and LJAR, genotypes of population

THA differed from those of the other three populations. This finding questions the idea that the Thaya population was genetically similar to the Drava and Lake Jarun populations, as indicated by the cladogram. The UPGMA analysis underlying this cladogram further revealed that SIO is the population which was most genetically differentiated from all other populations. In accordance with this observation, the clustering profile of this population created by the software STRUCTURE suggested that this population differed from all other populations. Population SIO owned one dominant genotype (Fig. 4.3) that was barely existent in any of the other populations. On the one hand, this strong genetic distinctness of population SIO could be explained by its isolated geographic location. SIO was the only population studied that was located in the River Sio (Hungary), and therefore it could share a different gene pool than all other populations. On the other hand, we measured a concentration of dissolved oxygen close to zero at the SIO site (Table 4.1). Such extreme environmental conditions could have caused strong selection or genetic drift (due to significant mortality) in this population leading to the observed dominance of one genotype and also to a low abundance (Table 4.1). The fact that environmental factors can strongly affect the genetic differentiation of populations could also be valid for the two Drava populations Dc and Dp: Despite a very low geographic distance of 600 m between these two populations, they formed two distinct branches in the cladogram (with 98% bootstrap support, Fig. 4.2) and the SAMOVA analysis yielded a separate group for each of the two populations. We additionally found a significant genetic bottleneck in population Dp, but not in Dc, and a highly reduced abundance in Dp in comparison to Dc. Thus, a high mortality rate in population Dp may have led to a loss of alleles. We did not find any differences between abiotic water parameters measured at the two sampling sites that could explain a higher mortality in population Dp than in Dc. An output of a wastewater treatment plant (WWTP) effluent into the River Drava, however, located behind the Dc but in front of the Dp sampling site, could eventually have provoked differences in water quality. The fact that wastewater pollution can result in a high mortality in a population has been described for other mussels (e.g. fingernail clams, Wilson et al. 1995). Further analyses of the water chemistry and additional ecotoxicological analyses at this study site are needed to clarify whether the wastewater has really influenced the genetics of population Dp (see chapters 5, 6).

That the SAMOVA analysis merged the populations VAH, DAN1, DAN2, DAN4 DAN5 into one group, whereas population DAN3 from the Danube was not included, is

inconsistent with a biogeographical interpretation of overall population differentiation. The SAMOVA algorithm calculates the genetic variances of populations according to allele frequencies. This calculation is based on nucleotide distances, whereas the F-statistics utilizes allelic distances (Slatkin 1995; Michalakis and Excoffier 1996). As locus A6 showed a significant or at least a marginally significant deviation from HWE for the geographically neighboring populations SIO, DAN3, DAN4, and DAN5, we carried out a further SAMOVA analysis for all populations using all microsatellite loci, except for locus A6. This analysis now yielded the same grouping of the five Danube populations (DAN1, DAN2, DAN3, DAN4, and DAN5) that was derived from the F-statistics. Locus A6, deviating from HWE, had obviously strongly impacted the outcome of the SAMOVA analysis. As the four populations SIO, DAN3, DAN4, and DAN5 are geographically close-by, local processes, e.g. selection to ambient water conditions (see below), could have caused the Hardy-Weinberg disequilibrium observed at locus A6.

Based on our results of the population genetic analyses we hypothesize that, in addition to geographical effects, the genetics of the analyzed zebra mussel populations was strongly influenced by environmental factors at the level of single loci and across all loci.

4.4.2 Local population differentiation and environmental factors

We found a significant influence of the concentrations of dissolved oxygen, temperature and conductivity on the genetic variation (defined as genetic diversity and/or number of alleles according to DiBattista (2008)) of the populations (Table 4.3). The genetic variation in the populations at the loci B6 and Dpol9 significantly decreased with increasing concentration of dissolved oxygen. The genetic variation at locus Dpol9 significantly increased with increasing temperature. The genetic variation at the loci B6, C5 and Dpol9 significantly decreased with increasing conductivity. The differences found in abiotic water parameters between sampling sites did not correlate with the pairwise F_{ST}-values (identity of alleles, Weir and Cockerham 1984) of the populations. An explanation for this is that pairwise F_{ST}-values were calculated based on all six microsatellite loci, whereas environmental factors sometimes affected only single loci (although an effect was still visible across all loci) and additionally, the effects were heterogeneous among loci. We are aware that we have measured only snapshots of the environmental conditions at the sampling sites. A long-term monitoring of the

conditions would be necessary to really demonstrate a significant influence of environmental parameters on the genetics of zebra mussel populations. Still we hope that the relations found between genetic variation and measured environmental parameters give us some hints for factors that could have influenced the genetics of the populations at the sampling sites.

Genetic diversity observed at microsatellite loci could be indirectly influenced by genetic hitchhiking of specific individual loci with one or several coding genes that are under selection (Smith and Haigh 1974). Moreover, if mortality is high in a population, genetic drift is affecting the whole gene pool of the population and therefore also the neutral microsatellite loci (Bagley et al. 2002). Both genetic hitchhiking and genetic drift can result in a loss of alleles and hence in a decrease in the genetic variation of the analyzed genetic markers (Ma et al. 2000; Athrey et al. 2007; Bourret et al. 2008).

Dissolved oxygen. We unexpectedly found that the number of alleles of our studied zebra mussel populations decreased with an increasing concentration of dissolved oxygen suggesting ongoing processes that cause mortalities and thus a loss of alleles in the populations. For *D. polymorpha* the optimum water oxygen saturation is above 80%-85% and the mussel is considered to be tolerant to low levels of oxygen, with a critical value of 25%-26% at 20 °C (Orlova 2002). The concentrations of dissolved oxygen that we measured at our sampling sites correspond to levels of oxygen saturation ranging between 34% and 86%. They are all well within the tolerance range of *D. polymorpha*, except for population SIO, where oxygen saturation was close to zero. Thus, no high mortality would be expected from the oxygen levels recorded in all other populations, even though our measurements only reflect a snapshot of the environmental conditions at the sampling sites. Consistent with the broad oxygen saturation tolerance of the zebra mussel, Garton and Haag (1991) observed that multiple locus heterozygosity of seven allozyme loci was not correlated to oxygen consumption of individuals. As we detected the decrease in the number of alleles with increasing oxygen concentration only for two out of six analyzed loci, it could be a random pattern. Genetic hitchhiking of the respective microsatellite loci with coding genes is a further possible explanation.

Temperature. We detected an increase in genetic diversity with increasing temperature for pooled loci. Water temperatures measured by us (20.5 °C–26.4 °C) during sampling

in summer were within the tolerance range of *D. polymorpha* and thus no high mortalities were expected from these values (Orlova 2002; Karatayev et al. 2006). The optimal average water temperature in summer ranges between 17 °C and 23 °C for this mussel (Ludyanskiy et al. 1993). As water temperatures recorded at sampling sites coincide more or less with optimum temperature conditions for the mussel, the increase in genetic variation with increasing temperatures is most probably not a direct effect of temperature. Buschini et al. (2003), however, showed that cells of *D. polymorpha* have probably more efficient detoxifying and repair mechanisms at 18 °C than at lower (4 °C), but also than at higher temperatures (28 °C). They further suggest that the mussel's sensitivity towards environmental pollutants could be temperature dependent. Based on the observation of Buschini et al. (2003) we argue that zebra mussels living in water bodies with moderately high temperatures (below 28 °C) could have a higher resistance to xenobiotics and therefore a lower mortality than at lower temperatures. Thus other environmental factors including xenobiotics could have caused the increasing genetic variation with increasing temperatures found in our study area.

Conductivity. Genetic variation of populations decreased with increasing conductivity across loci but also for several single loci. This observation could indicate mortalities due to ongoing random genetic drift or selection. The level of conductivity can reflect the degree of water contamination due to wastewater, agricultural leachate, or the presence of inorganic dissolved solids of natural origin, for example (US EPA 2012). Thus, the decrease in genetic variation with increasing conductivity could reflect different levels of pollution at sampling sites. Contrary to this hypothesis the sampling site DAN4 (nature park Kopački Rit) had a higher conductivity (400 µS/cm) than the site Dp at the River Drava behind a WWTP effluent (284 µS/cm). In accordance, the highest conductivity was observed for the polluted River Sio (pers. observation, 1084 µS/cm) where the concentration of dissolved oxygen was close to zero. Detailed chemical analyses of the water and the sediment are needed for sampling sites to definitely proof our hypothesis that conductivity reflects the pollution at the sampling sites in our study.

Plasticity of the zebra mussel. *Dreissena polymorpha* has an unprecedented success in colonizing European and North American waters under strongly differing environmental regimes. Marsden et al. (1995) found that only little genetic variation

was lost in zebra mussel populations that had invaded North America. They concluded that *D. polymorpha* was able to adapt to a wide variety of environmental parameters without a loss of genetic diversity. A similar result at a smaller geographic scale was observed in this study for the population of the Lake Jarun that was presumably colonized by zebra mussels from the River Drava. Although environmental conditions in the Lake Jarun differ from those of the River Drava (Table 4.1) and a founder effect was still detectable, there was no substantial loss of genetic variation in population LJAR (genetic diversity H_E, number of alleles, Table 4.1). Minchin et al. (2002) stated that zebra mussels were sufficiently plastic to colonize a wide range of habitats. In accordance, Fetisov et al. (1992) reported on zebra mussel populations that had been exposed to temperatures of up to 40 °C and even higher, which are considered to be above the upper limit of the temperature tolerance range of *D. polymorpha* (Buschini et al. 2003). The authors of this study found heterozygote deficiencies due to selection in these populations which is again a hint of adaption of zebra mussels to extreme environmental conditions. The observations of these authors and of our study suggest a rapid adaptation of mussel populations to local ambient environmental conditions.

4.4.3 Conclusion

Our study demonstrated a genetic population differentiation that is consistent with the overall biogeographical history of the populations, but also indicated that zebra mussels are genetically adapted to local habitat conditions. The latter effect seemed to have a strong influence on the genetic constitution of the populations, so that the genetic patterns of some populations deviated strongly from the baseline of geographic population differentiation. This finding is important when conducting biomonitoring experiments with zebra mussels as bioindicator. The different abiotic and biotic parameters at the source habitat of the test organisms and the resulting different genetic constitution of its populations could provoke different responses in experiments. We think it is therefore useful to monitor abiotic and biotic water parameters and the genetic background and thus the local adaptation of these bioindicators to their source habitat to facilitate a comparison of results obtained in biomonitoring experiments. Finally, it must be stated that our measurements of abiotic factors of the sampling sites were only snapshots of the momentary environmental conditions of the populations. A long-term monitoring of abiotic factors at sampling sites would be necessary to corroborate our

hypotheses on the relationships between genetic variation and environmental parameters found in our study.

5 EFFECTS OF FRESHWATER POLLUTION ON THE GENETICS OF ZEBRA MUSSELS (*DREISSENA POLYMORPHA*) AT THE MOLECULAR AND POPULATION LEVEL

5.1 Introduction

The fast growing human population has altered freshwater ecosystems profoundly (Vitousek et al. 1997). Hazardous substances present in the wastewater from agriculture, industry, and human settlements end up in aquatic ecosystems (Jha 2008). Effluents of wastewater treatment plants (WWTP) are one of the major sources of genotoxicants in surface waters (Ohe et al. 2004; Lacaze et al. 2011). Evolutionary toxicology investigates the effects of chemical pollutants on the genetics of natural populations (Matson et al. 2006; Shugart et al. 2010; Bickham 2011). Toxicants may induce DNA damage along with long-term DNA changes in freshwater organisms. It has been suggested that the resulting genomic instability plays an important role in decreasing fitness of populations and hence may have significant consequences for short- and long-term survival of populations (Jha 2008). Moreover, there are stochastic effects on population genetics that result from pollution causing a decline in population size and consequently inbreeding and the overall loss of genetic diversity. These genetic processes can reduce overall population fitness and accelerate population extinction (Bickham et al. 2000; Theodorakis 2001; Reed and Frankham 2003).

To obtain an integrated assessment of the impact of pollution on populations, the use of multiple biomarkers at different levels of biological organization has been strongly suggested (Shugart et al. 1992; Jha 2008; Bickham 2011). If chemical contamination is responsible for an emergent effect at the population level, responses at lower levels of biological organization should also be apparent (Shugart et al. 1992). The establishment of this causal relationship is essential to support the conclusion that an emerging population effect is due to contamination exposure (Bickham 2011).

The zebra mussel *Dreissena polymorpha* has been applied as bioindicator for passive as well as active biomonitoring (Sues et al. 1997; Bervoets et al. 2005; Pain et al. 2005), e.g. for genotoxicity monitoring in freshwater ecosystems (Klobučar et al. 2003). To our knowledge, our study is the first to combine microsatellite analysis, Comet assay and micronucleus test (MNT) for the use in biomonitoring, and furthermore, the first study

on the impact of pollution on the population genetics of the common bioindicator *D. polymorpha* applying microsatellite analysis.

In particular, we present a case study of two mussel populations from the River Drava (Croatia). These two populations are considered to have the same genetic background, but are exposed to different environmental conditions. The first population (Dp) was collected at a polluted site where mussels were constantly exposed to the effluent of the municipal WWTP of the city of Varaždin, whereas the second population (Dc) was collected at a reference site.

We hypothesized to detect a genetic bottleneck in population Dp, caused by increased mortality resulting from the exposure of the population to the WWTP effluent. This bottleneck could be a result of selection or of random genetic drift due to a decrease in population size. At the molecular and cellular level, we expected increased genotoxic effects in Dp compared to Dc.

In a laboratory experiment, we exposed randomly selected individuals from both populations to polluted municipal wastewater from the city of Zagreb. To mimic a real polluted environment as much as possible, we used the municipal wastewater containing a mixture of pollutants rather than a solution with a single pollutant. For this experiment, our hypothesis was that the two populations would react differently to these altered environmental conditions. Under the assumption that selective processes had changed the genetic constitution of population Dp in the source habitat, Dp could be more resistant to the wastewater and show less DNA damage than Dc. If, alternatively, Dp had a decreased genetic diversity due to random genetic drift leading to a decreased fitness under altered environmental conditions (Reed and Frankham 2003; Nowak et al. 2012), we expected that Dp would be more severely affected by the wastewater and exhibit a higher level of genotoxic effects than the control population Dc.

We believe that this research is a case study reinforcing the need to integrate population genetic measures into ecotoxicological investigations (Shugart et al. 2010; Bickham 2011; Nowak et al. 2012).

5.2 Materials and Methods

5.2.1 Sampling sites

The zebra mussels were sampled in July and August 2009 in northern Croatia (Fig. 5.1). The reference site (Dc: 46.306274° N 16.471809° E) was situated just behind the dam

of the Čakovec lake. The contaminated sampling site (Dp: 46.303452° N 16.478352° E) was about 600 m downstream from Dc. Ca. 10 m upstream from the Dp site, the WWTP effluent of the city of Varaždin flows into the River Drava. Varaždin is a city with 47,000 inhabitants and 17,000 households. Its wastewater treatment plant collects wastewater from the public sewage system and industrial wastewater. Due to the high water level at the Dc site at the time of the sampling of zebra mussels, the Comet assay and the MNT of the control population had to be carried out on individuals from the Čakovec lake (CL: 46.3143678° N 16.4154166° E), which is situated directly upstream from the Dc site (150 m). For the sake of simplicity, we refer to the sampling location CL (Fig. 5.1) as Dc in the remaining text.

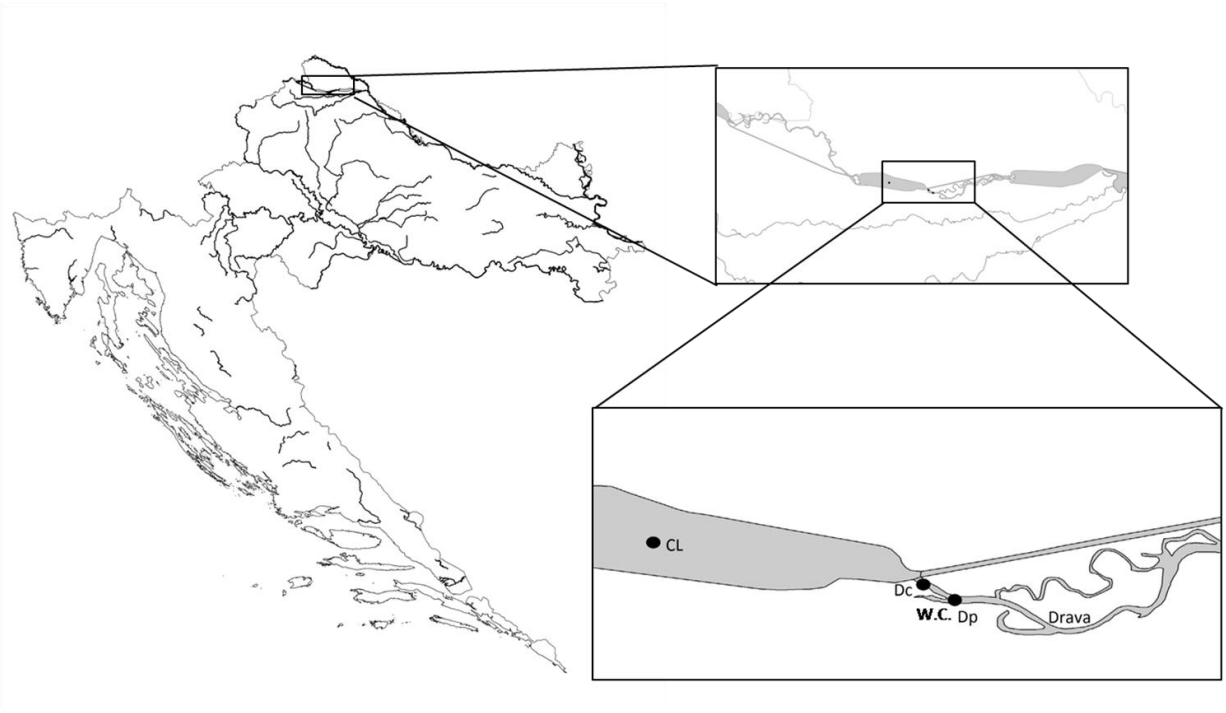

Fig. 5.1 Geographical locations of the sampling sites in Croatia. CL: Čakovec lake, Dc: control site, Dp: contaminated site downstream from the mouth of the wastewater treatment plant effluent channel (W.C.).

5.2.2 Water and sediment analyses

Chemical analyses of water and sediment were performed by the Institute of Public Health, Dr. Andrija Štampar (Zagreb, Croatia), except for pH and oxygen, which were directly measured in the field. Water samples were collected at the two investigated sites (Dc, Dp), and at the WWTP effluent channel, approximately 20 m upstream from the outlet into the River Drava. Additionally, we sampled water in a wastewater channel leading to the main city collector of municipal wastewater in Zagreb for the laboratory

experiment. In the water samples, pH and the concentrations of dissolved oxygen, chromium, copper, nickel, lead, iron, cadmium, zinc, manganese, twelve polycyclic aromatic hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs) were measured. The pH was measured with a pH-meter (pH 526, WTW GmbH). Dissolved oxygen was measured with an oximeter (OXI 730, WTW GmbH). The concentrations of metals (Cr, Cu, Ni, Pb, Fe, Cd, Zn, Mn) in the water were determined using inductively coupled plasma-optical emission spectrometry (ICP-OES, IRIS Intrepid II XSP, Thermo) according to the HRN EN ISO 11885 (1998) standard method. PAHs were determined by a high performance liquid chromatography (HPLC system Agilent 1100 Series – thermostatted autosampler G1329A, binary pump G1312A, Agilent Technologies) with fluorimetric detection (fluorescence detector G1321A, Agilent Technologies) (HPLC-FLD). The twelve PAHs (naphthalene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, benzo(g,h,i)perylene, indeno(1,2,3-cd)pyrene) were analyzed according to the EPA 550 standard method. The total PAH concentration corresponded to the sum of the concentrations of the twelve PAHs analyzed. For PCBs, water samples were extracted by dichloromethane. The extracts were evaporated on a rotary evaporator (Rotavapor R-210 with Heating Bath B-491, Vacuum Pump V-700, and Vacuum Controller V-850, Büchi), purged and then concentrated in a stream of nitrogen gas. PCBs were quantified by a gas chromatography with a 63Ni electron capture detector (GC-17A-ECD, Shimadzu) according to the HRN EN ISO 6468 (2002) standard method.

Only one sediment sample could be collected in the WWTP effluent channel, since the bottom at Dc and Dp consisted of small pebbles. To evaluate the degree of pollution of this sediment sample we measured the concentrations of heavy metals (chromium, copper, nickel, lead, cadmium, zinc, cobalt, and mercury), arsenic, molybdenum, mineral-oil hydrocarbons, PAHs and PCBs.

The methods used for determining heavy metal concentrations (Cr, Cu, Ni, Pb, Cd, Zn, Co, Hg), arsenic, mineral oil hydrocarbons, and PAHs have been previously described in detail (Klobučar et al. 2011). The concentration of molybdenum was determined in the same way as the heavy metals. For PCB detection, the oven-dried samples (5 g) were transferred to a Soxhlet apparatus and extracted with 100 ml of n-hexane. The extracts were evaporated on a rotary evaporator (Rotavapor R-210 with Heating Bath B-491, Vacuum Pump V-700, and Vacuum Controller V-850, Büchi) and concentrated in

a stream of nitrogen gas. They were then transferred into a centrifuge tube and cleaned-up with concentrated sulphuric acid (min. 96%). If the samples were highly contaminated repeated acid clean-up was employed. PCBs were quantified by a gas chromatography with a 63Ni electron capture detector (GC-17A-ECD, Shimadzu) according to EPA 8082 standard method.

5.2.3 Estimation of population abundance

To estimate the abundance of the two field populations Dc and Dp, individuals of *D. polymorpha* were sampled at the riverbank in 1 - 50 cm water depth. The population abundance (a) was estimated from the number of captured individuals (i) per time (t) and the number of collecting agents (c) as

$$a = \frac{i}{t \cdot c} \quad [\min^{-1}].$$

The sampling time t was one minute at Dc and 60 minutes at Dp.

5.2.4 Microsatellite analysis

For population genetic analyses, the sample size was 48 individuals for each of the populations. Genomic DNA was extracted from the posterior adductor muscle of ethanol-preserved samples using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH). The six primers used for microsatellite analysis and the respective PCR protocols are based on Naish and Boulding (2001), Astanei et al. (2005), and Thomas et al. (2011). The primers A6, B6, B9, and C5 from Naish and Boulding (2001) and Astanei et al. (2005) were partially modified in our institute (Table 1) using the program PRIMER3PLUS (Untergasser et al. 2007), or by PIG-tailing (Brownstein et al. 1996). The primers Dpol9 and Dpol19 were those from Thomas et al. (2011).

Table 5.1 Primer set used for microsatellite analysis. The primers are based on Naish and Boulding (2001), Astanei et al. (2005), and Thomas et al. (2011). We slightly modified the primer pairs of the loci A6, B6, B9, and C5 (Naish and Boulding 2001; Astanei et al. 2005).

Locus	Primer sequence (5' – 3')
A6	**F:** GTT TCT TTG CCG GTC TAA TAA TAG AGT TAA C
	R: GTG ATT GTG TAT CTG CTA TAA ACC
B6	**F:** GTT TCT TCG TGT GCT CAT GTT TCC TCC
	R: CGT TGT TCA AGC AAT AAG AAA GAC
B9	**F:** GTT TCT TTT GAC AAT ATC CTG TCT AAT G
	R: GCG TGT GTT TTT GAA ACG TG
C5	**F:** GTT TCT TGC ACT GTC AAC GTC ACA CTT TTG
	R: CCT TGC TAA CAG CTC GGT TGT ATC
Dpol9	**F: M13(-18)**TGG TTG ATG CAG TGA CCC TA
	R: TGT CGC TTG ATC CAT GTT TT
Dpol19	**F: M13(-18)**GCA TTC CAT CAA AAA CAC AGA T
	R: GAT CAA CAC CAA AGT TCG TTT C

The genotype data were generated on a capillary sequencer (ABI genetic analyzer 3130xl, Applied Biosystems). The allele fragment size calls were made using the GENEMAPPER 4.0 software (Applied Biosystems). One individual of population Dp provided a low DNA quality and was therefore removed from the genetic data set. The microsatellite data set was checked for scoring errors due to stuttering, large allele dropouts, and null alleles with the program Microchecker (van Oosterhout et al. 2004). We tested the potential influence of scoring errors on Hardy-Weinberg-Equilibrium (HWE) with the jackknife procedure described by Morin et al. (2009) and executed by the program "R" 2.9.1 (R Development Core Team 2009). Linkage disequilibria between pairs of loci were tested using the program GENEPOP 4.0.10 (Raymond and Rousset 1995).

5.2.5 Comet assay

Mussels collected from the two sites Dc and Dp were transported within 90 min to the Department of Zoology, Faculty of Science in Zagreb. Hemolymph was withdrawn from the posterior adductor muscle sinus with a hypodermic syringe for subsequent Comet assay and MNT. The Comet assay was conducted as described in detail by Štambuk et al. (2008). For each individual, one slide with 50 cells was examined, and the extent of DNA migration was determined as a percentage of the tail DNA using the image analysis system Komet 5 (Kinetic Ltd).

5.2.6 Micronucleus test

Aliquots of 0.1 ml hemolymph mixed with 0.1 ml phosphate-buffered saline (PBS) in 10 mM ethylenediaminetetraacetic acid (EDTA) were placed on slides and left for 15 min in a humidified chamber at room temperature allowing hemocytes to settle down. The subsequent procedure was conducted as described previously (Štambuk et al. 2008). Micronuclei were identified according to the criteria given in Majone et al. (1987) and Kirsch-Volders et al. (2003).

5.2.7 Statistical analyses

Bottlenecks. We checked both field populations for possible bottlenecks using the program BOTTLENECK, version 1.2.02 (Cornuet and Luikart 1997). With this program, genetic data are tested for an excess of heterozygosity in comparison to the heterozygosity expected at mutation-drift equilibrium ($H_E > H_{EQ}$). In a bottlenecked population, the number of alleles is reduced faster than the allele frequencies by a strong reduction in population density. As H_E is calculated from allele frequencies and H_{EQ} is calculated from allele numbers, a significant excess of heterozygosity ($H_E > H_{EQ}$) is evidence of a recent bottleneck in a population (Piry et al. 1999). We chose the two-phase mutation model (TPM) in this analysis, since it has been described as the most appropriate model for microsatellite loci (Di Rienzo et al. 1994; Piry et al. 1999). The Wilcoxon test was used to test for statistical significance of bottlenecks.

Genetic diversity and HWE. The overall observed and expected heterozygosity (genetic diversity) of the two populations and respective heterozygosities for each of the loci were estimated with the program GENEPOP 4.0.10 (Raymond and Rousset 1995).

The same program was applied for testing overall HWE by the exact "HW test" for each population, and by the statistically more powerful score test (U test) differentiating between heterozygote deficiency ($H_O < H_E$) and excess ($H_O > H_E$) (Rousset and Raymond 1995). In case of overall deficiency of heterozygotes the score test of heterozygote deficiency was also applied to each of the six loci, to detect which of the loci had caused a deficiency of heterozygotes in the populations Dc and Dp. In all score tests conducted, Markov chain parameters were 10,000 for dememorization, with 500 batches and 5,000 iterations per batch.

Population differentiation and inbreeding. To assess overall differences in gene pools of the populations Dc and Dp, we calculated the differentiation between the two populations (F_{ST}) and the inbreeding coefficients (F_{IS}) using the F-statistics by Weir and Cockerham (1984) as implemented in the program FSTAT (Goudet 2001). Weir and Cockerham (1984) weight allele frequencies according to sample sizes, and therefore the estimates of these statistics are not influenced by differences in sample sizes of populations. The F_{ST}- and F_{IS}-values were calculated over all loci, as well as separately for each of the loci.

AMOVA. To rate changes in the genetic composition of the field populations between generations, the individuals of both populations were divided into three distinct age classes. We determined the age of individuals by counting the annual rings on the surface of their shells (Jantz 1998). The first age class consisted of one- and two-year-old zebra mussels, the second age class contained three- and four-year-old mussels, and the third age class comprised five-year-old mussels. Mussels older than five years were excluded from these analyses because the sample size for this age class for Dp (one individual) was too small in comparison to the sample size for Dc (nine individuals). In total, the analysis of the age classes was carried out with 39 individuals from population Dc, and 46 from population Dp. We initially conducted a pairwise comparison of the age classes using the program Arlequin 3.5.1.2 (Excoffier and Lischer 2010), to ensure that the unequal sample sizes of age classes did not affect the following overall analyses of molecular variance (AMOVA). In the overall AMOVA analyses, all age classes that belonged to one population comprised one group.

The F-statistics of the age classes (over all six loci) were estimated and tested for significant deviation from zero, using a non-parametric permutation approach described

by Excoffier et al. (1992). Furthermore, the age class-specific inbreeding coefficients (F_{IS}) were estimated and also tested for significance.

The distance method applied in the locus-by-locus AMOVA was the number of different alleles. With the help of this method, the locus-specific inbreeding coefficients (F_{IS}), variances among age classes within populations (F_{SC}), variances among age classes among populations (F_{CT}), and the overall fixation indices (F_{IT}) were calculated and tested for significant deviation from zero. All AMOVA analyses were computed with the program Arlequin 3.5.1.2 (Excoffier and Lischer 2010).

Comet assay and Micronucleus test. For each group, mean values of DNA damage were calculated based on the mean of each individual within a group. The data are presented as mean ± SEM (standard error), for both Comet assay and MNT. To assess differences between groups, we performed the Mann-Whitney U-test.

5.2.8 Laboratory exposure

Approximately 200 mussels per site were acclimatized to laboratory conditions for two weeks in glass aquaria containing de-chlorinated, well-aerated water, which was renewed every other day, at ambient room temperature. Every other day the mussels were fed with algae (*Chlorella* sp). After this acclimatization period, 50 mussels of each population were exposed to the polluted municipal wastewater of Zagreb in aquaria for three days at ambient room temperature. The ten liters of wastewater in the aquaria were aerated and renewed every day. At the same time, samples of 50 mussels of each population were exposed to ten liters of aerated de-chlorinated tap water at ambient room temperature and were used as control. At the end of the experiment, seven to twelve individuals were taken from each treatment to assess genotoxicity by the Comet assay and MNT.

5.3 Results

5.3.1 Water and sediment analysis

Results of the water and sediment chemical analysis are presented in Table 5.2. In the water analyses, the sampling site in the WWTP effluent channel and Dp had lower concentrations of O_2 and Fe compared to Dc, but increased concentration of Mn. As

there was no other inflow between the two sites Dc and Dp, the observed differences in the water parameters between the Dc site and the Dp site can be considered as a result of the WWTP effluent inflow.

Table 5.2 Results of water and sediment chemical analyses. Concentrations of abiotic parameters and contaminants of four water samples and one sediment sample are depicted. Dc = reference site in River Drava, Dp = contaminated site in River Drava, WcW = water sample of the wastewater treatment plant effluent, ZaW = water sample of municipal wastewater in Zagreb used in the laboratory experiment, WcS = sediment sample of wastewater treatment plant effluent channel.

Water parameters	Dc	Dp	WcW	ZaW	Sediment parameters	WcS
pH	8.07	7.74	7.87	7.20	Co (mg/kg)	10.4
O_2 (mg/l)	8.68	4.62	4.27	NA	As (mg/kg)	< 0.05
Cr (µg/l)	< 2	< 2	< 2	19	Cr (mg/kg)	804
Cu (µg/l)	< 20	< 20	< 20	36	Cu (mg/kg)	242
Zn (µg/l)	< 20	< 20	< 20	88	Zn (mg/kg)	1300
Ni (µg/l)	< 5	< 5	< 5	17	Ni (mg/kg)	44.8
Fe (µg/l)	314	126	99.2	1110	Hg (mg/kg)	1.36
Pb (µg/l)	< 5	< 5	< 5	7	Pb (mg/kg)	218
Cd (µg/l)	< 1	< 1	< 1	< 1	Cd (mg/kg)	< 3
Mn (µg/l)	24.9	38.8	58.5	84	Mo (mg/kg)	42.4
PCB (µg/l)	< 0.02	< 0.02	< 0.02	< 2.0	PCB (mg/kg)	< 5
Total PAH (µg/l)	< 0.005	< 0.005	< 0.005	0.255	Total PAH (mg/kg)	27.8
					Mineral-oil hydrocarbons (mg/kg)	176.6

The sediment analysis of the WWTP effluent channel clearly showed that the water running through this channel had been heavily polluted, at least from time to time.

5.3.2 Population abundance

The estimated abundance of population Dc (a_{Dc} = 100.00) was approximately 120 times higher than the abundance of Dp (a_{Dp} = 0.83).

5.3.3 Review of microsatellite data

The analysis of the genetic data set with the program Microchecker (van Oosterhout et al. 2004) did not detect any scoring errors due to stuttering or large allele dropouts. It also did not reveal any hint for existing null alleles, except for locus C5 in Dp. This

locus had a significant excess of homozygotes, which could indicate the presence of null alleles. The estimated rate of possible null alleles of locus C5 in Dp was low (0.07). The six individuals that were suspected to have null alleles by the software Microchecker were not considered to be influential on HWE estimates for the population based on the jackknife procedure (Morin et al. 2009). Therefore, we did not remove these individuals from the data set. Moreover, low null allele frequencies have a negligible impact even in parentage analysis, whereupon parentage analysis is expected to have a higher bias from null alleles than analysis of population structure (Dakin and Avise 2004). We detected no significant linkage disequilibrium between all pairs of the six loci studied applying sequential Bonferroni correction.

5.3.4 Genetic bottlenecks

The Wilcoxon test revealed a significant excess of heterozygosity ($H_E > H_{EQ}$) in Dp ($p = 0.023$), but not in Dc ($p = 0.219$). This result suggests that the Dp population has undergone a recent genetic bottleneck (Piry et al. 1999). Furthermore, there was a significantly higher loss of rare alleles in Dp than in Dc (chi^2-test: $chi^2 = 4.17$, $df = 1$, $p = 0.041$). This loss of rare alleles in population Dp was observed at four out of six of the analyzed loci.

5.3.5 Population specific genetic diversity and HWE

The genetic diversity computed over all loci by GENEPOP was similar for both populations with $H_E = 0.82$ in Dc, and $H_E = 0.81$ in Dp. Based on all loci, the observed heterozygosity H_O was 0.83 in Dc and 0.76 in Dp. H_O only deviated significantly from H_E for Dp, but not for Dc (Table 5.3). While for locus Dpol19 H_O was slightly higher in Dp than in Dc, H_O was lower in Dp than in Dc for the other five loci. For Dp, locus C5 showed the strongest difference between observed and expected heterozygosity ($H_O = 0.64$, $H_E = 0.77$) while no difference was observed for this locus in Dc ($H_O = 0.79$, $H_E = 0.78$) (Table 5.3).

Table 5.3 Locus-specific and overall genetic characteristics of populations. Inbreeding coefficients (F_{IS}), observed and expected heterozygosity (H_O/H_E) of population Dc and Dp, genetic differentiation (F_{ST}) between Dc and Dp, and number of alleles (A) for Dc and Dp. None of the F_{ST}-values was significantly different from zero. Significant deficits of heterozygotes from Hardy-Weinberg equilibrium are in bold.

Locus	F_{IS} (Dc)	F_{IS} (Dp)	H_O/H_E (Dc)	H_O/H_E (Dp)	F_{ST} (Dc/Dp)	A (Dc)	A (Dp)
A6	-0.057	0.014	0.94 / 0.89	0.90 / 0.91	0.0003	13	15
B6	-0.004	0.064	0.92 / 0.91	0.83 / 0.89	-0.003	18	13
B9	-0.036	0.016	0.90 / 0.87	0.81 / 0.82	-0.005	13	9
C5	-0.010	0.167	0.79 / 0.78	**0.64 / 0.77**	-0.008	7	6
Dpol9	-0.061	0.092	0.71 / 0.67	0.60 / 0.66	-0.008	8	7
Dpol19	0.042	0.004	0.75 / 0.78	0.79 / 0.79	-0.004	8	9
Over all loci	-0.021	0.057	0.83 / 0.82	**0.76 / 0.81**	-0.004	67	59

The exact tests implemented in GENEPOP did not show any significant deviation from HWE for both populations (p_{Dc} = 0.417, p_{Dp} = 0.237). The score tests of heterozygote excess also corroborated HWE (p_{Dc} = 0.438, p_{Dp} = 0.967), whereas the score tests of heterozygote deficiency revealed a significant deficit of heterozygotes in Dp (p = 0.033), but not in Dc (p = 0.562). Score tests for heterozygote deficiency individually carried out for each of the six loci and each of the two populations revealed that all loci conformed to HWE, except for locus C5 in Dp, which had a significant deficit of heterozygotes (p = 0.013, Table 5.3).

5.3.6 Population differentiation

Population Dc had a negative inbreeding coefficient (F_{IS} = -0.021), whereas for Dp the inbreeding coefficient was positive (F_{IS} = 0.057, Table 5.3). Population Dp had a marginally significantly increased percentage of homozygotes in comparison to Dc (chi^2-test: chi^2 = 3.69, df = 1, p = 0.055). This observation corroborated the score test, which indicated a significant deficit of heterozygotes in Dp. The largest F_{IS}-value was observed for locus C5 in population Dp (F_{IS} = 0.167, Table 5.3). The fixation index F_{ST} of the two populations did not differ significantly from zero (F_{ST} = -0.004, p = 0.521) and thus indicated no genetic differentiation between the two populations (Table 5.3).

5.3.7 AMOVA of the different age classes in Dc and Dp and locus-by-locus AMOVA

The AMOVA indicated that the major proportion of genetic variation was due to genetic differences within individuals (97.58%). The overall inbreeding coefficient (F_{IS} = 0.027, p = 0.075) and the overall fixation index (F_{IT} = 0.024, p = 0.085) both deviated marginally significantly from zero. In contrast, the variances among age classes within the populations (F_{SC} = 0.001, p = 0.364) and among the populations (F_{CT} = -0.005, p = 0.913) were not significant (Table 5.4). The AMOVA analysis for the age classes of each of the two populations revealed that the second and third age class of population Dp had significant F_{IS}-values above zero, whereas all other age classes had insignificant F_{IS}-values close to zero. This observation across loci demonstrated that the second age class (F_{IS} = 0.073, p = 0.037) and third age class (F_{IS} = 0.111, p = 0.024) of Dp had an increased proportion of homozygotes in comparison to the first age class of Dp and to all three age classes of Dc.

Table 5.4 Analysis of molecular variance (AMOVA) across all six microsatellite loci. The results are weighted averages across all six loci for 85 individuals, comprising the two populations Dp and Dc, and divided into three age classes. For the group level, the individuals of the three age classes belonging to the same population were pooled. Listed are the source of variation, sum of squared deviations, the variance component estimates, the percentage of total variance, the fixation indices, and the significance of the variance components and of the fixation indices estimated by performing 1,023 permutations for the analyses.

Source of variation	Sum of squares	Variance component	% of total variation	Fixation index
Among groups	1.68	-0.011	-0.45	F_{CT} = -0.005
Among age classes within groups	10.37	0.003	0.14	F_{SC} = 0.001
Among individuals within age classes	196.22	0.066	2.72	F_{IS} = 0.027
Within individuals	200.00	2.361	97.58	F_{IT} = 0.024
Total	408.27	2.419		

The locus-by-locus AMOVA carried out over all age classes showed that only locus C5 was significantly different from zero in the F-statistics. The inbreeding coefficient was significantly larger than zero (F_{IS} = 0.100, p = 0.042) and the overall fixation index was marginally significant (F_{IT} = 0.095, p = 0.061) for locus C5 (Table 5.5).

Table 5.5 Locus-by-locus analysis of molecular variance (AMOVA) across two populations (Dc, Dp). Estimates and p-values of F_{IS} (locus-specific inbreeding coefficients), F_{SC} (variances among age classes within populations), F_{CT} (variances among age classes among populations), and F_{IT} (overall fixation indices) for each of the six microsatellite loci. Significant ($p < 0.05$) and marginally significant ($p < 0.1$) p-values are in bold.

Locus	F_{IS}	p-value	F_{SC}	p-value	F_{CT}	p-value	F_{IT}	p-value
A6	-0.020	0.754	0.006	0.217	0.002	0.482	-0.012	0.684
B6	0.018	0.391	0.012	0.115	-0.008	1.000	0.022	0.289
B9	0.006	0.509	-0.018	0.976	0.003	0.408	-0.009	0.644
C5	0.100	**0.042**	0.004	0.348	-0.001	1.000	0.095	**0.061**
Dpol9	0.030	0.368	-0.009	0.721	-0.005	0.820	0.017	0.433
Dpol19	0.040	0.259	0.011	0.198	-0.010	0.692	0.041	0.241

5.3.8 Comet assay - field populations

Results of the Comet assay carried out for field populations are shown in Fig. 5.2. The percentages of tail DNA did not differ significantly between the Dc and Dp site. The frequency of cells exceeding 50% of tail DNA was 0.29% for the control population Dc and 0.30% for population Dp.

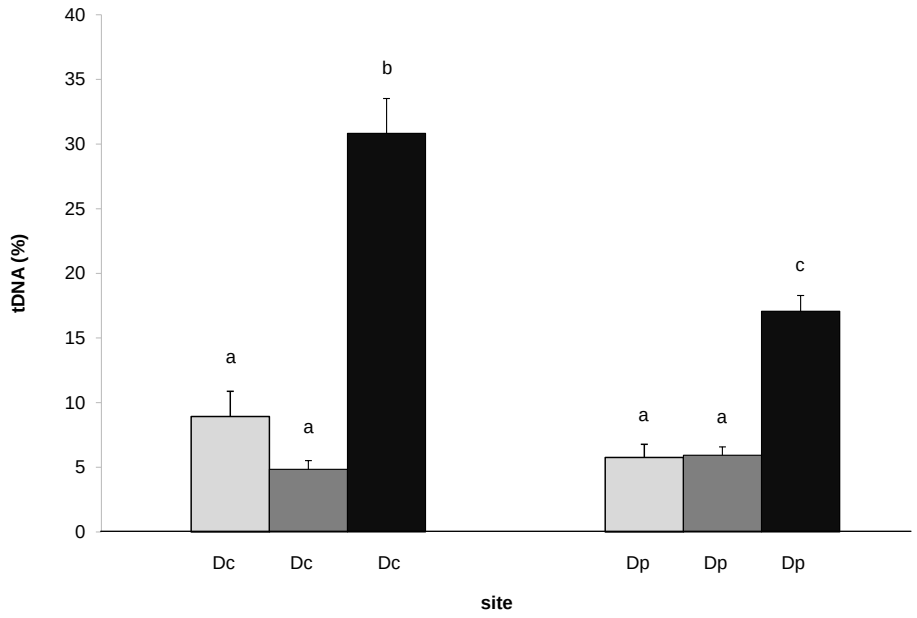

Fig. 5.2 DNA damage measured by the Comet assay in the hemocytes of zebra mussels from the Dc and Dp site, River Drava. Light grey bars correspond to field populations, dark grey bars to population samples exposed to dechlorinated tap water in the laboratory experiment, and black bars to population samples exposed to wastewater in the laboratory experiment. Different letters indicate statistically different DNA damage ($p < 0.05$).

5.3.9 Micronucleus test - field populations

DNA damage as assessed by the MNT showed a significantly increased number of micronuclei in hemocytes for field mussels collected at the Dp site in comparison to the mussels originating from the Dc site ($p = 0.008$, Fig. 5.3).

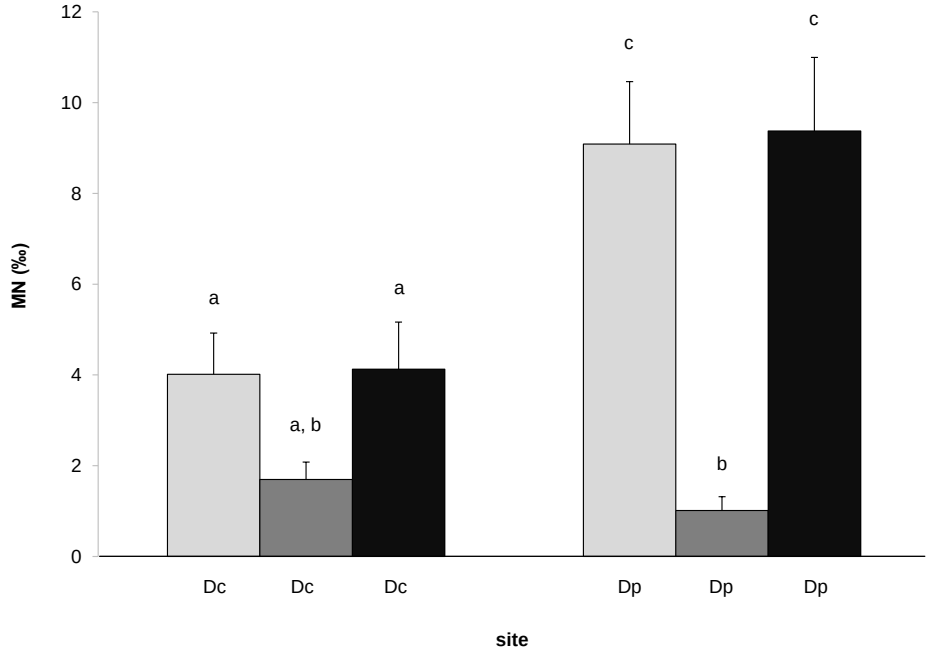

Fig. 5.3 Number (per mill) of micronuclei (MN) in hemocytes of zebra mussels from the Dc and Dp site, River Drava. Light grey bars indicate field populations, dark grey bars population samples exposed to dechlorinated tap water in the laboratory experiment, and black bars population samples exposed to wastewater in the laboratory experiment. Different letters indicate statistically different numbers of micronuclei ($p < 0.05$).

5.3.10 Laboratory exposure

After two weeks of acclimatization and three days of exposure to aerated dechlorinated tap water DNA damage reached basal levels that were very similar between the two populations, in both Comet assay and MNT. After the exposure to municipal wastewater, the Comet assay revealed a significantly higher DNA damage in hemocytes of mussels originating from Dc and Dp sites than in the mussels of the control populations exposed to the dechlorinated tap water (Dc: $p = 0.0002$, Dp: $p = 0.0002$). The genotoxic response measured by the Comet assay in the individuals from the Dc site exposed to the wastewater was significantly higher in comparison to the response of mussels from the Dp site ($p = 0.012$, Fig. 5.2). The frequency of cells exceeding 50% of tail DNA for the Dc population was 0% for the mussels in dechlorinated tap water and 10.99% for the mussels exposed to the wastewater. For the Dp population it was 0% for the mussels in dechlorinated tap water and 1.77% for the mussels exposed to the wastewater.

A significant increase in the number of micronuclei in hemocytes was detected in the mussels originating from the Dp site after the exposure to wastewater in comparison to

the mussels in dechlorinated tap water ($p = 0.0002$, Fig. 5.3), and also in comparison to the mussels originating from the Dc site and exposed to the wastewater ($p = 0.023$). The number of micronuclei in hemocytes from mussels from the Dc site exposed to the wastewater was not significantly different from the Dc mussels in tap water ($p = 0.089$).
There was no mortality of the mussels exposed to dechlorinated tap water during the two weeks of acclimatization or during the three days of exposure to the tap water. After the third day of exposure to municipal wastewater, the mortality rate was 77% for population Dc and 84% for population Dp, but these rates are not significantly different from each other (chi^2-test: $chi^2 = 0.66$, $df = 1$, $p = 0.416$).
DNA damage measured by the Comet assay and MNT did not differ significantly between the control laboratory population Dc (in tap water) and the control field population Dc (Comet assay: $p = 0.118$, MNT: $p = 0.076$, Figs. 5.2, 5.3). Thus, it was correct to consider the results from the field mussels of the CL/Dc site as from an unpolluted control site.

5.4 Discussion

5.4.1 Abundance and genetic bottleneck

The results of this study indicated a severely lower abundance of the field population Dp in comparison to population Dc. Moreover, Dp had undergone a significant genetic bottleneck which reflects a strong reduction in the number of individuals in the past. While allele numbers were decreased in Dp in comparison to Dc, the allele frequencies did not significantly differ between the two populations. The genetic bottleneck in Dp was accompanied by a loss of rare alleles in four out of six of the analyzed microsatellite loci. This suggests that non-selective random genetic drift had affected population Dp. Based on the results obtained from our water and sediment analyses the bottleneck in population Dp had probably resulted from WWTP effluent contamination.

5.4.2 Genetic diversity and population differentiation

Populations Dc and Dp had nearly identically high levels of genetic diversity (Dc: 0.82, Dp: 0.81). Similarly high levels of genetic diversity (0.79 – 0.94) assessed by microsatellite analysis have been reported for other populations of *D. polymorpha* in the

literature (Müller et al. 2002; Astanei et al. 2005). The fact that the genetic diversity of the contaminated population was not altered by the WWTP effluent is coherent with the conclusions derived from a metaanalysis across different taxa (plants, invertebrates, vertebrates) by DiBattista (2008). He found that pollution could both decrease and increase genetic variation in populations (defined as genetic diversity or mean number of alleles per locus) through genetic drift and directional selection on the one hand, and increased mutation rate and selection for heterozygotes on the other hand. Thus, the high genetic diversity seen in population Dp could have resulted from various selective and stochastic genetic processes. If the high genetic diversity of Dp was mainly due to increased mutational load, this would not imply a high adaptability of the population under altered environmental conditions and a constant high population fitness. If so, we would expect that Dp would show a decreased fitness in comparison to the unimpacted Dc population in our laboratory experiment. The lower fitness of Dp would result from increased mutational load, the directional selection of Dp to the specific environmental conditions in its habitat (as reflected by the microsatellite locus C5) and the experienced genetic drift (shown by the microsatellite analysis across all loci) (Nowak et al. 2009). Moreover, decreases in genetic diversity can be diluted by recent migration events, as it was suggested for populations of the arctic marine amphipod *Orchomenella pinguis* exposed to contamination (Bach and Dahllöf 2012).

In accordance with their similar allele frequencies, there was no significant genetic differentiation between the two populations Dc and Dp. A high gene flow between the two populations is consistent with their high genetic similarity. As there was no boat traffic in the old River Drava behind the Čakovec lake, gene flow of the *D. polymorpha* mussels could only result from the downstream swamping of free-swimming larvae in high densities (Haag and Garton 1992). Larvae remain in the plankton up to three weeks or even longer, and consequently can be drifted over 300 km before settlement (Sprung 1989; Stoeckel et al. 1997). This passive dispersal mode of larvae strongly prevents genetic population differentiation (Müller et al. 2001). As the distance between the two sampling sites Dc and Dp is only about 600 m it is very likely that the mussels of the Dc and Dp site are offspring of the same mussel populations that inhabited the Čakovec lake or even a location more upstream the River Drava. High levels of gene flow preventing population differentiation are also consistent with the similar high levels of genetic diversity observed in the two populations.

5.4.3 Selection and genetic drift

We found a significant deviation from HWE for the Dp population, whereas population Dc showed HWE. This deviation observed in Dp was caused only by locus C5, which deviated significantly from HWE and showed a deficit of heterozygotes. While Astanei et al. (2005) also reported a deficit of heterozygotes in *D. polymorpha* populations based on microsatellite loci and concluded that it was most likely caused by null alleles, other studies did not find deviations from HWE (Müller et al. 2002; Hidde 2008). Our analyses did not indicate a potential influence of null alleles and suggest that the deviations from HWE at locus C5 must have been caused by another genetic process. Alternatively, it is possible that a locus-specific analysis of population genetic structure reveals novel candidate loci that are the object of selection due to genetic hitchhiking (Smith and Haigh 1974; Kohn et al. 2003). A linkage of the neutral microsatellite locus C5 to a gene undergoing selection could explain the observed deviation from HWE. We hypothesize that locus C5 is located near a coding gene that is under selection only in the contaminated Dp population, but not in the non-contaminated Dc population. A possible candidate gene could be a gene that is involved in DNA repair mechanisms, and/or in antioxidative or detoxification processes (see molecular and cellular level). Our interpretation is corroborated by another *D. polymorpha* population studied by the authors which is located at the heavily polluted site in the River Sio, Hungary. This population also showed a deviation from HWE at locus C5 (unpublished data). Other population genetic studies also found a selective effect on a single microsatellite locus that was explained by the genetic hitchhiking model (Kohn et al. 2003; Bourret et al. 2008). A genetic linkage mapping of the microsatellite loci for *D. polymorpha* could clarify which coding gene is linked to C5.

The results obtained for age classes also indicated an indirect selection process affecting locus C5. For population Dp, the inbreeding coefficients (F_{IS}) increased with the age of the mussels and significantly differed from zero for the age classes 2 and 3. This suggests that the proportion of homozygotes increased with the age of the mussels, indicating ongoing selection. The increase in homozygosity is reflected by the overall deficit of heterozygotes observed in Dp.

In total, our results support our first hypothesis that a genetic bottleneck had affected the Dp population, but not the Dc population. This bottleneck was caused by random genetic drift. Moreover, we also detected a strong indirect selective effect on locus C5 in the Dp population. The selective process causing this indirect effect on C5 has

obviously been acting on the zebra mussels for years, which we demonstrated by the age class analysis.

5.4.4 Molecular and cellular level

The second part of our first hypothesis, where we assumed that the field population Dp should have a higher level of genotoxic effects than the field population Dc, was confirmed only by MNT. We observed a significantly higher percentage of micronuclei for Dp than for Dc. This result indicates pollution at the Dp site. The levels of micronuclei in hemocytes found for zebra mussels from the Dp site is comparable to the levels reported for other polluted sites in the literature (Mersch and Beauvais 1997; Klobučar et al. 2003; Binelli et al. 2010). The MNT result is contrary to the Comet assay, in which the Dc and Dp population did not show significantly different responses. Several studies have also described that a high response seen in MNT is not necessarily reflected at the same intensity in the Comet assay (Bombail et al. 2001; Klobučar et al. 2008). The following reasons are given for this inconsistency: first, MNT and Comet assay assess different aspects of DNA damage. MNT detects clastogenic and aneugenic effects while Comet assay measures single- and double-strand breaks, alkali labile sites, DNA-DNA and DNA-protein cross-links (Cotelle and Férard 1999; Klobučar et al. 2008). Second, the level of DNA damage measured by the Comet assay can be lower than the initial level, as it is possible that some of the damage has already been repaired (Black et al. 1996). The MNT only assesses non-repairable DNA damage and chromosome loss that will persist until apoptosis of the damaged cells. Third (and in connection to the second), an adaptation of mussels inhabiting a contaminated site through activation of the DNA repair mechanism is possible. This adaptation could be facilitated through genetic selection or differential regulation of genes coding for DNA repair (Rodríguez-Ariza et al. 1992; Black et al. 1996; Hoffmann and Willi 2008). For example, Black et al. (1996) carried out a Comet assay for the freshwater bivalve *Anodonta grandis* inhabiting a site contaminated with lead, cadmium, and zinc. They did not find any increased DNA damage in the mussels originating from this site. Mussels of the same species from a non-contaminated site kept in laboratory showed DNA breakage even at low concentrations of lead. The authors concluded that this discrepancy in response in the Comet assay was due to an enhanced DNA repair mechanism of the mussels from the contaminated area.

As the mussels from the Dp site had a low DNA damage in the Comet assay in combination with an increased rate of micronuclei, we hypothesize that they had adapted to the polluted environment by an enhanced DNA repair mechanism and/or antioxidative defense. Inducement of any of these mechanisms is expected to affect the results of the Comet assay in greater manner than the results of the MNT.

5.4.5 Laboratory exposure

After exposure to municipal wastewater, the mussels from the Dc site showed a significantly higher level of DNA damage in the Comet assay than the mussels from the Dp site. In contrast, we observed a significantly higher number of micronuclei in hemocytes in the mussels belonging to the Dp population. These results coincide with our second hypothesis that the two populations Dc and Dp would react differently under altered environmental conditions due to their different genetic constitution.

Based on the results of the population genetic analysis, the Comet assay and the MNT carried out for the field populations, we have hypothesized that the mussels of the contaminated Dp site had been adapted to the local WWTP effluent discharge by an enhanced DNA repair mechanism and/or enhanced antioxidative mechanism. This possible adaptation was apparently reflected only in the successful repair of DNA damage revealed by the Comet assay and not in MNT as clastogenic or aneugenic damage is very difficult to repair. A more efficient DNA repair system and/or antioxidative mechanisms in the Dp population resulting from adaptation processes could also explain the differences in responses of the two populations seen in the pollution treatment in the Comet assay. An adaptation of the DNA repair system and/or antioxidative mechanisms could be concordant with the low level of tail DNA found in the field population Dp, and the significantly lower percentage of tail DNA found in the laboratory Dp population in comparison to the laboratory Dc population in the pollution treatment. This explanation is consistent with the first part of our hypothesis for the laboratory experiment, where we assumed that population Dp was more resistant to wastewater due to previously acting selection processes in the source habitat. It is also possible that the DNA repair mechanism and/or antioxidative defense were facilitated by differential gene expression, but the relation between these processes is not clear. So far, the role of specific DNA sequences in the repair of DNA damage has not yet been studied in ecotoxicology and should be the focus of future studies (Jha 2008).

The second part of our second hypothesis was that if the impacted population (Dp) had a decreased genetic diversity due to random genetic drift, it would be affected by wastewater more severely than population Dc. The microsatellite analysis, however, detected no decreased genetic diversity in Dp in comparison to Dc, but we could show that random genetic drift had affected the Dp population. Possibly, genetic drift and a resulting decreased population fitness were responsible for the high rate of micronuclei of the Dp population in the laboratory experiment.

5.4.6 Conclusion

The mussels from the Dc and Dp site reacted differently to the exposure to wastewater in our laboratory experiment, which was probably due to their differing genetic constitution. This differing constitution was apparently a consequence of the different environmental conditions, to which the mussels had been exposed in their natural habitat. The combination of the responses of the three biomarkers gave comprehensive information about the impact of both treated and non-treated wastewater on the genetics of zebra mussels at different levels of biological organization. A limitation of our approach is that it is not possible to directly prove the selection of one or several specific genes by the analysis of neutral microsatellite markers. Therefore, future research should answer which genetic process (e.g. genetic selection or differential regulation of the DNA repair mechanism) enabled the adaptation to contamination. A challenging perspective could be the widening of our approach to gene expression studies or genetic linkage mapping of the microsatellite loci to coding genes of the model organism *D. polymorpha* (Hoffmann and Willi 2008). The main advantage of our approach is the possibility to measure effects on genetics at different levels of biological organization (molecular, cellular, and population level) with a time- and cost-effective system owing to the three well-established techniques.

6 GENERAL CONCLUSIONS

Integrating population genetic measures into ecotoxicological studies has become an important challenge in the research field of ecotoxicology (Bickham 2000; Bickham 2011). In my thesis, I elaborated an integrated approach in biomonitoring with the common bioindicator *Dreissena polymorpha* in cooperation with the Department of Zoology of the University of Zagreb (Thomas et al. 2010). We successfully applied this approach by measuring population genetic and genotoxic responses to wastewater contamination in a field study and a complementary ecotoxicological experiment.

Microsatellite isolation. As a first project, I developed five new polymorphic microsatellite loci for *D. polymorpha* (Thomas et al. 2011) for the population genetic analyses. Out of those and other already existing microsatellite markers for this species (Naish and Boulding 2001; Astanei et al. 2005), I arranged a set of six highly polymorphic markers. The set proved a high variability and precision in the results of my further two population genetic studies. There was no linkage disequilibrium between the markers and a very low rate of possible null alleles. Therefore, I was able to rely on a very robust genetic data set when analyzing and interpreting population genetic patterns.

Establishment of biogeographical baseline. Monitoring the biogeographical background is important when integrating population genetic measures into ecotoxicological studies (Whitehead et al. 2003). Thus, in a second project, I analyzed the biogeographical baseline of molecular variance of zebra mussel populations of a section of the River Danube (in Hungary and Croatia) and some of its tributaries. In this way, it was possible to distinguish between ecotoxicological responses to pollution and biogeographical patterns of the populations. The populations of the River Danube were not genetically differentiated from each other and therefore reflected a high connectivity and gene flow in this study. Nevertheless, some of the populations deviated from the biogeographical baseline of all other populations. I investigated if abiotic water parameters influenced the genetics of the analyzed populations. The genetic diversity of the populations was related to the environmental factors oxygen and temperature and also to other unidentified factors. My study indicated that zebra mussels were genetically adapted to the special conditions in their habitat.

Application of the approach. In a third project, I applied the elaborated approach in biomonitoring in collaboration with the Department of Zoology of the University of

Zagreb. We analyzed the genetics of a population (Dp) in the River Drava that was under the influence of an effluent of a wastewater treatment plant (WWTP) and that had shown a genetic bottleneck in the second project, as well as the genetics of a reference population (Dc). We applied the ecotoxicological tests Comet assay and micronucleus test on the two populations and analyzed water and sediment samples. To assess the impact of the WWTP effluent on the contaminated population Dp, we carried out measurements in July, August and September during the sampling period (unpublished data). The measurements of temperature, dissolved oxygen and pH at the Dp and Dc site did not show any differences between sites. In contrast, conductivity at the Dp site was always enhanced in comparison to the Dc site, obviously as a consequence of the inflow from the WWTP effluent. Thus, we assume that at the Dp sampling site, the conductivity most probably reflected freshwater pollution. This interpretation is also supported by the sediment analysis in the WWTP effluent channel which also documented a high pollution. Taken together, both results indicate a permanent pollution at the Dp site, but probably of fluctuating intensity as we did not observe strong pollution when measuring water chemistry (Table 5.2). By combining the results of microsatellite analysis, Comet assay and micronucleus test, I found that the genetic constitution of population Dp was different from the genetic constitution of the reference population Dc. It had apparently been altered by environmental parameters by adaptation and genetic drift. In addition to the field study, we carried out a laboratory experiment in which we exposed samples of both populations to municipal wastewater. The mussels from the Dc and Dp site reacted differently to the exposure to wastewater in this experiment. This was probably due to their differing genetic constitution. The differing constitution was apparently a consequence of the different environmental conditions, to which the mussels had been exposed in their natural habitat. The results of the third project indicate that different genetic constitutions of test organisms can bias the results of ecotoxicological experiments.

Synthesis. In my thesis, I isolated five new microsatellite loci for *D. polymorpha*. Out of those and other already existing microsatellite markers for this species, I established a robust marker set of six microsatellite loci. In ecotoxicological studies, this marker set facilitates precise and powerful statistical analyses of recent changes in genetic patterns of populations of this common bioindicator in response to pollution, as shown in this study. Moreover, this marker set will be useful for studies of demographic patterns of the zebra mussel, a successful invader (Astanei et al. 2005, Naish et al. 2001). Also,

ecological questions that refer to a fine geographical scale can be addressed by these markers (Selkoe and Toonen 2006). Due to the codominant mode of inheritance and the high variability of microsatellites, this marker set is adequate to detect recent changes in the level of genetic diversity within and among populations (Dimsoski and Toth 2001). Interestingly, the genetic diversity (measured as H_E) of population Dp was not affected by the obviously strong impact of the WWTP effluent in the field study, although a genetic bottleneck and a strongly decreased abundance were detected in this population. Additionally, this is surprising as I found in the second study that the genetic diversity of the populations was related to the measured factors temperature, conductivity, and the concentration of dissolved oxygen at two loci. In the third study, I showed that the measured conductivity was always enhanced at the Dp site in comparison to the reference site (Dc) and that it most probably reflected the pollution by the WWTP effluent. As DiBattista described in 2008, pollution can have increasing and decreasing effects on genetic diversity through the evolutionary forces mutation and selection for heterozygotes on the one hand and selection and genetic drift on the other hand. He also reported on several studies that did not show a significant decrease in genetic variation (measured as H_E or mean number of alleles per locus) in response to the exposure to pollution (e.g. Berckmoes et al. 2005) or that even may have found an increase in genetic variation (e.g. Baker et al. 2001). Increasing and decreasing effects of freshwater pollution at the Dp site may have both impacted the genetic diversity of the Dp population. Moreover, the aforementioned high gene flow of *D. polymorpha* found in our study had most probably blurred the possibly decreased genetic diversity at the Dp site and therewith diluted a population genetic effect as described before in Müller et al. (2002). To conclude, genetic diversity is an important population genetic parameter when investigating the influence of pollution on populations as it can give a hint for exposure to pollution (Bagley et al. 2002). However, a high genetic diversity of a population under the influence of pollution does not necessarily guarantee a high fitness (DiBattista 2008). From this point of view, and bearing in mind the high gene flow between zebra mussel populations found in this study, genetic diversity is not a reliable biomarker for the health status of zebra mussel populations if it is measured exclusively. It is important to simultaneously conduct further statistical tests, e.g. for genetic bottlenecks, analyses of molecular variances between age classes and locus-specific analyses to distinguish between effects of selection and genetic drift. Assessing the abundance of the populations also provides important indications for recent

bottlenecks. Moreover, it is important to conduct biogeographical analyses to compare the genetic diversity and other population genetic estimates to those of neighboring populations.

In the third project, I found the indication that different constitutions of test organisms can bias the results of ecotoxicological experiments. Similar effects were also assumed in previous ecotoxicological studies on mussels (Black et al. 1996; Burgeot et al. 1996; Buschini et al. 2003). It is therefore important to assure similar environmental parameters at the source habitats of the test organisms (Bickham 2000). It is also advisable to monitor the population genetics of a test organism in a biomonitoring experiment to see if molecular variances are consistent with biogeography or deviate from the patterns of neighboring populations and therefore point to potential impacts of pollution.

To summarize, the analysis of the molecular variance of zebra mussel populations in a biogeographical context and its correlation with abiotic water parameters indicate that the mussels are genetically adapted to the conditions in their habitat. A field study at a polluted site and an associated ecotoxicological experiment suggested that the different genetic constitutions of mussel populations due to variable source habitats can bias the outcome of ecotoxicological experiments. Moreover, in addition to genetic diversity, it is advisable to measure further genetic parameters of zebra mussel populations, when assessing the condition of these populations. The elaborated approach in biomonitoring allowed to measure effects on genetics at different levels of biological organization owing to the three well-established techniques microsatellite analysis, Comet assay and micronucleus test.

Outlook. In the field study and the complementary laboratory experiment of the third project, I found the indication that the zebra mussels exposed to the WWTP effluent had adapted to the polluted environment by an enhanced DNA repair mechanism and/or antioxidative defense. However, it was not possible to directly prove or neglect the selection of one or several specific genes by the analysis of neutral microsatellite markers. A further explanation for the adaptation to pollution could be differential gene expression. So far, the genetic processes that are involved in DNA repair mechanisms have not been sufficiently investigated in ecotoxicology (Jha 2008). Future research should answer which genetic processes enable the adaptation to contamination. Therefore, a future perspective would be to conduct genome scans and/or gene expression studies on *D. polymorpha*. When locating the analyzed microsatellite loci in

the genome by a genome scan, it would be possible to link the observed hitchhiking effects to neighboring coding genes. This way we could possibly detect candidates for adaptive shifts in response to environmental change (candidate genes). Moreover, gene expression studies can identify candidate genes for DNA repair mechanisms, antioxidative defense or other processes that are affected by freshwater pollution (Hoffmann and Willi 2008). For example, microarrays could detect the coding genes of *D. polymorpha* that are affected by the exposure to the WWTP effluent by measuring expression changes and contribute to elucidating the function of these genes.

A future challenge would be the widening of our approach with additional analysis techniques. Assessing the physiological condition of zebra mussels could show how severely individuals and populations that are exposed to pollution are affected in comparison to reference populations. A conditioning index would provide important information as the condition of the mussels reflects key functional processes within organisms and populations (Smolders et al. 2004).

A future perspective could be to establish our elaborated approach in biomonitoring for other mussel species that inhabit different ecosystems, e.g. to the marine mussels *Mytilus edulis* and *Perna viridis*. The Comet assay and the MNT have already been successfully applied to these species (Izquierdo et al. 2003; Rank and Jensen 2003; Siu et al. 2004) and microsatellite markers are also available for both of them (e.g. Ong et al. 2005; Lin et al. 2007; Lallias et al. 2009). Therefore, our approach should be well applicable on these species.

In my thesis, I could show that the elaborated approach in biomonitoring was well practicable in a first case study. Our approach here revealed to be sensitive enough to monitor freshwater contamination caused by a WWTP effluent. A future challenge will be to test this approach more elaborately on different populations under the influence of freshwater pollution. This is necessary to ascertain that this approach is capable of delivering comparable results on the health of zebra mussel populations from different habitats. If so, it could possibly be used for biomonitorings of pollution in freshwater ecosystems in the future.

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8 ACKNOWLEDGEMENTS

9 CURRICULUM VITAE

10 ERKLÄRUNG

Ich versichere, dass ich meine Dissertation „Impact of anthropogenic stressors on the population genetics of the common bioindicator *Dreissena polymorpha* (Pallas, 1771)" selbstständig und ohne unerlaubte Hilfe angefertigt habe und mich keiner anderen Quellen und Hilfen als der von mir ausdrücklich bezeichneten bedient habe.

Mainz, im November 2012 Emilia Godila Thomas