

Chapter 7

Pollution, condition and genetic variability

**Gregory Maes¹, Joost Raeymaekers¹,
Christophe Pampoulie¹, Adriaan Seynaeve¹,
Geert Goemans², Claude Belpaire² and Filip
Volckaert¹**

1 - Katholieke Universiteit Leuven, Laboratory of Aquatic Ecology, Ch. de
Bériotstraat, 32, B-3000 Leuven, Belgium

2 - Institute for Forestry and Game Management, Duboislaan 14, B-1560
Groenendaal-Hoeilaart, Belgium

This chapter was published as:

Maes, G.E., Raeymaekers, J.A.M., Pampoulie, C., Seynaeve, A., Goemans, G.,
Belpaire, C., Volckaert, F.A.M., 2005.
The catadromous European eel *Anguilla anguilla* (L.) as a model for freshwater
evolutionary ecotoxicology : Relationship between heavy metal bioaccumulation,
condition and genetic variability.
Aquatic Toxicology 73, 99-114.

Summary

Understanding the effects of pollutants on the genome is of crucial importance to preserve the evolutionary potential of endangered natural populations. The highly vagile European eel (*Anguilla anguilla* L.) has suffered a dramatic decline in recruitment since two decades, urging for a better understanding of the genetic impact of pollution. Its catadromous life history constitutes a model to assess local selection of pollutants on condition and genetic variability, as juveniles recruit in European rivers without appreciable pollution load or interfering genetic background. Because of its high fat content and local benthic feeding behaviour, the feeding stage is considered extremely prone to the bioaccumulation of pollutants. We studied the relationship between heavy metal bioaccumulation, fitness (condition) and genetic variability in the European eel. The muscle tissues of 78 sub-adult eels, originating from three Belgian river basins (Scheldt, Meuse and Yser), were examined for nine heavy metal pollutants (Hg, Cd, Pb, Cu, Zn, Ni, Cr, As and Se), while in total 123 individuals were genotyped at 12 allozyme and 8 microsatellite loci. A significant negative correlation between heavy metal pollution load and condition was observed, suggesting an impact of pollution on the health of sub-adult eels. In general, we observed a reduced genetic variability in strongly polluted eels, as well as a negative correlation between level of bioaccumulation and allozymatic multi-locus heterozygosity (MLH). Microsatellite genetic variability did not show any pollution related differences, suggesting a differential response at metabolic enzymes and possibly direct overdominance of heterozygous individuals.

Introduction

Evidence from animal and plant populations indicates that allozymatic polymorphism and heterozygosity might be linked to environmental heterogeneity and stress (Nevo *et al.*, 1986; Ben-Shlomo and Nevo, 1988; Chagnon and Guttman, 1989; Gillespie and Guttman, 1989; Stanton *et al.*, 2000; Nevo, 2001). Understanding the effects of pollutants on the genome is of crucial importance to preserve the evolutionary potential of endangered natural populations, as a high genetic diversity provides a population the potential to adapt to selective forces (Gillespie and Guttman, 1989). Under natural conditions (e.g. absence of anthropogenic influences), allelic frequencies within a population fluctuate with time according to stochastic processes (drift), migration and/or environmental selection pressures (such as climate or habitat changes), while maintaining polymorphism. However, severe perturbations on a short temporal scale, such as man-induced pollution and harvesting, may lower the condition and genetic variability, reducing the viability (fitness) of natural populations. Hence the susceptibility to additional environmental stress increases, weakening the survival of the species (Thorpe *et al.*, 1981; Leary *et al.*, 1987; Stanton *et al.*, 2000). The importance of genetic variation to survive anthropogenic environmental changes relates to factors such as resistance to heat stress (mummichog, *Fundulus heteroclitus*; Powers *et al.*, 1991), oil pollution (mussels, *Mytilus edulis*; Fevolden and Garner, 1986) and radiation (central stoneroller, *Campostoma anomalum*; Gillespie and Guttman, 1989).

There are four ways in which toxicants may affect the genetic variability in a population (Van Straalen, 1999; Van Straalen and Timmermans, 2002): (1) some toxicants are mutagenic, increasing directly the mutation rate; (2) they may indirectly affect the mutation rate by affecting DNA repair mechanisms; (3) they may favour more tolerant genotypes than others and change the genetic composition of the population towards a higher mean tolerance; and (4) they may cause bottlenecks or alter migration. The first two mechanisms will increase genetic diversity, while the two latter ones will decrease it, possibly exhausting genetic variation in natural populations. This process is referred as “genetic erosion” (Van Straalen and Timmermans, 2002).

The impact of pollutants or toxicants, such as heavy metals, pesticides or industrial waste, on the genetic diversity and structure of natural populations relates to a reduced genetic variability in polluted populations, genotype-specific survivorship and subsequent shift in the distribution of tolerant genotypes without net loss of diversity, or significant correlations between pollutants and allele frequencies (Hvilsom, 1983; Fevolden and Garner, 1986; Klerks and Weis, 1987; Patarnello and Battaglia., 1992; Posthuma and Van Straalen, 1993). Heavy metal pollutants seem to strongly affect allelic selection or allele frequency shifts at polymorphic loci (Hvilsom, 1983; Ben-Shlomo and Nevo, 1988; Chagnon and Guttman, 1989; Frati *et al.*, 1992). Most of these studies focused on well-defined populations, with low dispersal capability and reproducing locally. Organisms with a catadromous life history (i.e. spawning at sea, feeding in rivers and lakes) are expected to reflect local pollutants impact faithfully, as somatic and population genetic comparisons can be made after dispersal without worrying about different genetic background, parental influence or larval pollution load. Species with a high effective population size (mostly marine) generally exhibit high levels of heterozygosity and are expected to be more resistant to pollution; multi-locus heterozygotes often show an increased fitness over homozygotes (Nevo *et al.*, 1986; David, 1998). The question remains whether the effect of pollutants can also be measured on condition and genetic variability in highly vagile species.

There are few analyses of the relationship between the bioaccumulation of contaminants and genetic diversity in natural populations (Van Straalen, 1999). An important aspect when quantifying contaminant pressure is not only the exposure concentration but also the actual uptake of the contaminant in the body, namely the level of bioaccumulation (Van der Oost *et al.*, 2003). Concentrations of environmental pollutants do not always reflect the actual level of contamination of the individuals; lab based experimental studies often use higher concentrations than present in the natural habitat (Newman and Jagoe, 1998, but see Belfiore and Anderson, 2001). Hence, a combination of experimental and field-based studies remains ideal to encompass both molecular and population-genetic influences of environmental contaminants (Bickham *et al.*, 2000).

The organism of interest in this study is the European eel (*Anguilla anguilla* L., Anguillidae, Teleostei), a marine fish spending most of its lifetime in European freshwater rivers, lagoons or lakes, but spawning in the Sargasso Sea in the central North Atlantic Ocean (Tesch, 1977). Leptocephali larvae migrate along the Gulf Stream and North Atlantic Drift to reach the European continent, enter the rivers as glass eels, feed at least for 3 (males) to 6 years (females) until their spawning migration as silver eels (Tesch, 1977). Its catadromous life history constitutes a model to assess local selection of pollutants on condition and on genetic variability, as juveniles recruit without appreciable pollution load or interfering genetic background. Despite extensive spawning migrations, the feeding stage (yellow eel) seems relatively sedentary (Tesch, 1977). In fact, because of its high fat content and local benthic feeding behaviour, the sub-adult stage is considered extremely prone to the bioaccumulation of pollutants (Linde *et al.*, 1996; Roche *et al.*, 2003).

The European eel stock is declining rapidly and is now at its minimum since 1970, probably due to climate/current changes, but compounded by anthropogenic changes like habitat destruction, migration barriers, overfishing and pollution (Dekker, 2000, Feunteun, 2002). The impact of pollutants in eel is known (see Robinet and Feunteun, 2002 for a review), but it is unclear to what extent the reproductive potential is affected and whether strong differential selection may alter the genetic composition of resident freshwater populations before the spawning migration.

Although there is firm proof that higher pollution levels decrease fitness traits, the impact of genetic variability in a catadromous organism like eel to withstand environmental stress is not known. The objectives of this study were threefold: (1) we quantified the relationship between heavy metal bioaccumulation and fitness (condition) of yellow eels from three Belgian river basins, assuming that a high environmental load is reflected in the biota. (2) We tested the hypothesis of equal post settlement allozymatic and microsatellite genetic constitution among basins and among *post-hoc* defined groups exhibiting various levels of heavy metal bioaccumulation. (3) We tested whether there is a correlation between multi-locus heterozygosity (MLH) at both genetic markers, the individual level of bioaccumulation and condition. If contaminants cause selective mortality of individuals with specific genomes, then allele and genotype frequencies should differ between populations from impacted sites (lower genetic variability) and those from not or less impacted sites (Gillespie and Guttman, 1986). A positive correlation between MLH and condition indices or negative correlation with level of bioaccumulation, would suggest a higher fitness (less contamination) for more heterozygous individuals (heterosis) (Nevo *et al.*, 1986).

Materials and methods

Samples

A total of sixteen sites were sampled in three river basins (Figure 7.1). Approximately ten sub-adult freshwater eels (yellow eel stage) were collected either by electro-fishing or with fyke nets at each site during the year 2000 ($n = 123$). The sampling was part of an extensive eel pollution-monitoring network for Flanders in 2000 (Goemans *et al.*, 2003). To detect inter-basin differences and to improve the statistical power of the analyses (especially by avoiding low sampling bias in genetic variability estimates), we initially grouped samples by river basin. The first set of samples originated from the River Scheldt (S1, S2, S3, S4, S5, S6 and S7), the second set was sampled in the River Meuse (M1, M2, M3 and M4), while the third set was sampled in the River Yser (Y1, Y2, Y3, Y4 and Y5) (Figure 7.1). Eels were kept alive in oxygenated tanks for maximally three days and processed in the laboratory. Standard length (L), body weight (W_B) and liver weight (W_L) were determined for each individual. Samples from muscle and liver tissues were collected for ecotoxicological (heavy metal concentration) and genetic (allozymes and microsatellites) analyses.

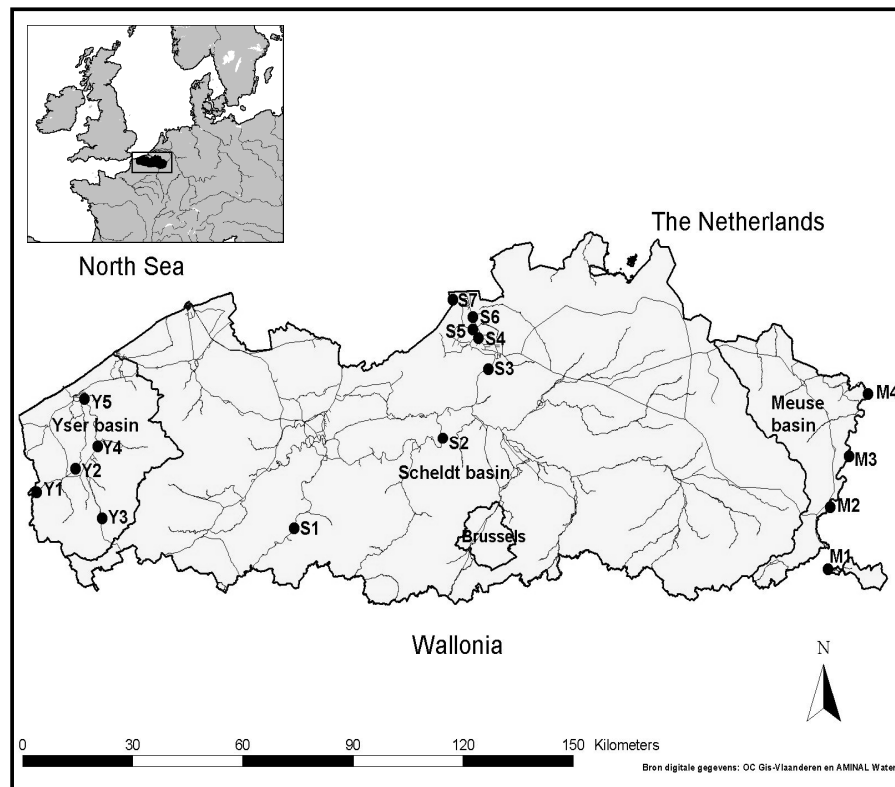


Figure 7.1. *Anguilla anguilla*: Flanders with sampling sites along three Belgian river basins; Yser: Y1, Y2, Y3, Y4, Y5; Scheldt: S1, S2, S3, S4, S5, S6, S7; Meuse: M1, M2, M3, M4.

Heavy metal load measurements and analysis

A sample of about 3-5 g of muscle tissue was removed, labelled and frozen at -20°C before analysis. A total of nine heavy metal concentrations were measured for three to six eels per sampling site ($n = 78$) by ICP-OES (Inductive Coupled Plasma-Optical Emission Spectrometry) for Cr, Ni, Cu, Zn, Cd and Pb. Quantification of As and Se was performed by GF-AAS (Graphite Furnace Atomic Absorption Spectrometry) according to standard procedures (Skoog, 1997, Goemans *et al.*, 2003). Concentrations were expressed in $\mu\text{g.kg}^{-1}$ (Hg, Cd, Pb, Ni, Cr, As and Se) or mg.kg^{-1} (Cu and Zn) wet weight.

Allozyme electrophoresis

A total of 123 individuals (including the 78 individuals characterised for pollutants) were genotyped using Cellulose Acetate Gel Electrophoresis (CAGE, Harris and Hopkinson, 1976; Richardson, Baverstock and Adams, 1986). Electrophoresis and staining procedures followed Maes and Volckaert (2002). The most common allele was called '100' and other alleles were classified according to their relative mobility to allele '100' for the locus under study. The nomenclature used for enzymes followed Shaklee *et al.* (1990). Buffers used in the electrophoretic analyses were Tris-Glycine (TG) and Tris-Maleate (TM); both liver (L) and muscle (M) tissue were used. The following nine enzyme systems (coding for 16 loci) were scored: Alcohol dehydrogenase (*ADH-1**, *ADH-2**, EC 1.1.1.1, TG, L), Aspartate aminotransferase (*AAT-1**, *AAT-2**, EC 2.6.1.1, TM, L), Glucose-6-phosphate isomerase (*GPI-1**, *GPI-2**, EC 5.3.1.9, TG, M), Isocitrate dehydrogenase (*IDH-1**, *IDH-2**, EC 1.1.1.42, TM, L), L-lactate dehydrogenase (*LDH-A**, *LDH-B**, EC 1.1.1.27, TM, M), Malate dehydrogenase (*MDH-1**, *MDH-2**, EC 1.1.1.37, TM, L), Malic enzyme (*MEP-1**, *MEP-2**, EC 1.1.1.40, TM, L), Mannose-6-phosphate isomerase (*MPI*-1*, EC 5.3.1.8, TG, L) and Phospho-glucomutase (*PGM-1**, EC 5.4.2.2, TG, M). Twelve presumed polymorphic loci were scored to examine genetic diversity and genotype distribution.

DNA extraction and microsatellite amplification

Minute sections of tissue from ethanol preserved yellow eel fins (same individuals as allozymes, $n = 123$) were digested in a lysis buffer containing 200 μl 5% Chelex 100 solution (BioRad), 7 μl of 1M DTT (Dithiothreitol) solution pH 5.2 (diluted in 0.08M NaAc) and 10 μl Protein K solution (10 mg.ml^{-1}) for at least 4 h at 56°C . After incubation at 100°C for 10 min, the samples were centrifuged at 13,000 rpm (10,000 g) for another 10 min; the supernatant was stored at -20°C for later analysis. Genotypes were examined at 8 dinucleotide repeat microsatellite loci: *AAN 01*, *AAN 02*, *AAN 05* (Daemen *et al.*, 2001); *ARO 095*, *ARO 054*, *ANG 151*, *ANG 114* and *ARO 121* (Wirth and Bernatchez, 2001). PCR reaction conditions were as follows: denaturation at 95°C for 3 min followed by a cycle of denaturation at 95°C for 35 s, annealing at 61°C (*AAN 01*, *AAN 02*) or 57°C (*AAN 05*) for 30 s and finally elongation at 72°C for 40 s. This cycle was repeated 30 (*AAN 01*, *AAN 02*) or 25 (*AAN 05*) times, after which an additional elongation of 10 min at 72°C was performed. Single PCR reactions consisted of 1 X PCR buffer (supplied with polymerase), MgCl_2 at a concentration of 1 mM (*AAN 02*, *AAN 05*) or 1.5 mM (*AAN 01*), 200 μM of dNTP, 0.4 μM of labeled forward and non-labeled reverse primer, 0.5 U of Goldstar *Taq* polymerase (Eurogentec, Seraing, Belgium) and 10-100 ng of gDNA. Double distilled water was added up to 10 μl . Loci *ARO 095*, *ARO 054*, *ANG 151*, *ANG 114* and *ARO 121* were run in a multiplex with the following PCR

conditions: denaturation at 95°C for 5 min followed by a cycle of denaturation at 95°C for 30 s, annealing at 57°C for 30 s and a final elongation at 72°C for 30 s. This cycle was repeated 25 times, after which an additional elongation of 8 min at 72°C was performed. Multiplex PCR reactions consisted of 1X PCR buffer (supplied with polymerase), MgCl₂ at a concentration of 1.5 mM, 80 μM of dNTP, on average 0.4 μM of fluorochrome labeled (IRD700 or 800, Westburg, The Netherlands) forward and non-labeled reverse primer, 0.5 U of Goldstar *Taq* polymerase and 10-100 ng of gDNA. Double dH₂O was added up to 25 μl. PCR products were run on a 5.5% acrylamide 7 M urea sequencing gel using an automated sequencer (LICOR 4200). Along with the PCR products, a molecular ladder (Westburg) was run in order to quantify the allele sizes.

Analyses of condition and heavy metal data

To assess the relative condition of individuals under pollutant stress, two condition factors were used. (1) Ricker's (1975) condition index (CI) was calculated as $1000(W_B / L^b)$, where respectively L and W relate to standard length in millimetres and body weight in milligrams (King, 1995). (2) The hepato-somatic index (HSI) was calculated as $HSI = (W_L / W_B) \cdot 100$, where W_L and W_B represent wet liver weight and wet body weight, respectively. The coefficient b in (1) was calculated as the slope from the $\text{Log } W_B - \text{Log } L$ regression analysis for all three basins, as allometric growth was detected. We then assessed the relative condition of each individual from each basin using the formula (1). ANOVA tests were performed to compare the mean relative condition between basins. Since the liver is the major detoxification and lipid storage reserve organ, changes in weight of this organ will relate to detoxification and energy storage. Weight effects on HSI were removed from (2), followed by an ANOVA on the residuals of the weight-HSI regression for group comparison.

We used a Multivariate ANOVA on a set of seven heavy metals and univariate ANOVA's per metal followed by Tukey tests to detect the influence of basin on heavy metal load. We calculated a relative bioaccumulation index by dividing (standardizing) the individual concentration of heavy metal i (C_i) by the maximum observed concentration ($C_{i \max}$) and averaging over all metals, to relate heavy metal bioaccumulation to condition and genetic variability. Thus, the individual mean (multi-metal) bioaccumulation index (IMBI) was defined as:

$$\text{IMBI} = \left[\sum_{i=1}^n (C_i / C_{i \max}) \right] / n$$

with

n = total number of metals,

C_i = individual concentration of heavy metal i ,

$C_{i \max}$ = maximal observed concentration of heavy metal i and $0 < \text{IMBI} < 1$.

To compare heavy metal bioaccumulation among basins, an ANOVA analysis was performed on the IMBI values, followed by post-hoc analyses (Tukey tests). We calculated Pearson's correlation coefficients between individual IMBI values and condition indices (CI and HSI) to assess pollutant impact on condition. All analyses were performed in STATISTICA version 6.0 (StatSoft, 2001).

Genetic data analyses

Allozymatic and microsatellite genetic diversity was evaluated based on genotype and allele frequencies, the level of polymorphism (P), observed and expected heterozygosity (H_O and H_E), total number of alleles and mean number of alleles per locus (MNA). Multi-locus heterozygosity (MLH) was calculated as the percentage heterozygous loci per individual (corrected for non scored loci). Homogeneity of allele frequencies among samples was tested with the program GENEPOP version 3.1d (Raymond and Rousset, 1995). Departures from Hardy-Weinberg (H&W) equilibrium were calculated as $D = (H_O - H_E) / H_E$ with GENEPOP version 3.1d (Raymond and Rousset, 1995) using the Markov chain method. The standard deviation of each value was estimated by the jack-knife method over loci as implemented in GENETIX version 4.02 (Belkhir *et al.*, 1999) and the linkage disequilibrium between loci was calculated using the LINKDIS procedure implemented in GENETIX (Belkhir *et al.*, 1999). Population structure was characterised using hierarchical F-statistics (theta) and G_{ST} -values as implemented in the GENETIX 4.02 software package (Belkhir *et al.*, 1999). Due to the subtle differentiation and the high number of rare alleles, we chose to estimate the fixation index ($F_{ST(RB)}$) following Robertson and Hill (1984) after correction by Raufaste and Bonhomme (2000). Significance of multi-locus F_{ST} was assessed with permutation tests (1000 replicates). Genetic diversity indices (H_E , H_O , MNA, MLH and P) were compared between individuals, river basins and *post-hoc* defined LOW-HIGH pollution groups (LOW = IMBI < 0.22 and HIGH = IMBI > 0.25, values of 0.22 < IMBI < 0.25 were removed to avoid overlap between both groups). Because of the absence of reproductively isolated populations within each river basin (Tesch, 1977; Maes and Volckaert, 2002), the proposed division by pollution load is justified. In all cases significance levels were corrected for multiple comparisons using a sequential Bonferroni correction (Rice, 1989). Locus-by-locus heterozygosities (H_O), Allelic richness (AR) were compared using a pairwise t-test for dependant samples, while individual MLH values of both pollution groups were compared using an ANOVA. Bivariate regression analyses helped us to assess the relationship between condition and genetic estimators. Subsequent multiple regression analysis (Sokal and Rolf, 1997) was performed to test the overall contribution of MLH (allozymes and microsatellites) and condition (CI and HSI) on the level of bioaccumulation (IMBI). Analyses were performed in STATISTICA version 6.0 (StatSoft, 2001).

Results

Heavy metal bioaccumulation

Comparisons of heavy metal pollution between river basins pointed to a strong heterogeneity in pollution load between sites (MANOVA, $F_{14,138} = 5.044$, $p < 0.0001$, $n = 78$, (see Appendix 1 and Table 7.1). The metals As and Se were not included in the statistical treatment because of the low number of analyses performed (1-5 individuals per river basin). The Meuse basin exhibited the highest concentrations for six out of nine heavy metal measurements (when including As and Se), but only two out of seven heavy metal (Hg, Cd) concentrations differed significantly between river basins (ANOVA, Table 7.1). The Scheldt showed the lowest Hg concentration, while the Meuse had the highest level of Cd. The distribution of heavy metal concentrations was heterogeneous between sites within river basin (Appendix 1), as shown in Figure 7.2a. The distribution of the IMBI values (based on seven metals) ranged from 0.113 to 0.479 and showed a roughly bimodal pattern of lowly and highly polluted individuals (Figure 7.2b). Later on this separation was used in the genetic analyses to define the "HIGH" and "LOW" pollution groups. An ANOVA of the IMBI values followed by a Tukey test indicated the Meuse basin as being significantly stronger polluted than the other two rivers ($F_{2,75} = 6.834$, $p < 0.01$, $n = 78$, Figure 7.3). Considering the possible relationship between size and pollution load (length is only weakly correlated with age in yellow eels), we found no significant correlation between length and pollutant concentration for any heavy metal (data not shown).

Table 7.1. Average heavy metal concentration per river basin of *Anguilla anguilla* L. Multivariate and univariate ANOVA's for equal heavy metal bioaccumulation in eel tissue originating from the Yser, Meuse and Scheldt basin. Values for Hg, Cd, Pb, Ni, Cr, As and Se are expressed in $\mu\text{g.kg}^{-1}$. Values for Cu and Zn are expressed in mg.kg^{-1} . The highest values are listed in bold.

Basin	n	Hg	Cd	Pb	Cu	Zn	Ni	Cr	As	Se	All metals
Yser	25	150.32	2.448	41.68	0.518	23.88	46.52	295.68	135	329	W-value = 0.438
Meuse	20	173.6	19.485	37.6	0.493	26.31	65.7	361.5	371.25	663.5	F = 5.044
Scheldt	33	93.6	2.993	52.78	0.643	25.14	46.54	174.36	308.67	1022.8	df = 14
p-value		0.0006	0.0000	0.4600	0.5222	0.5152	0.1482	0.3818	/	/	0.0000

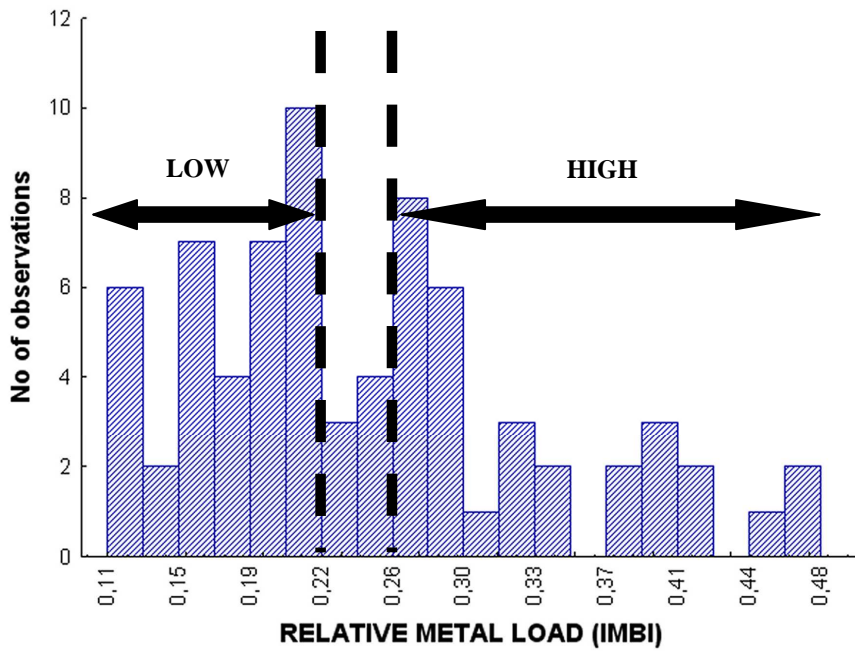
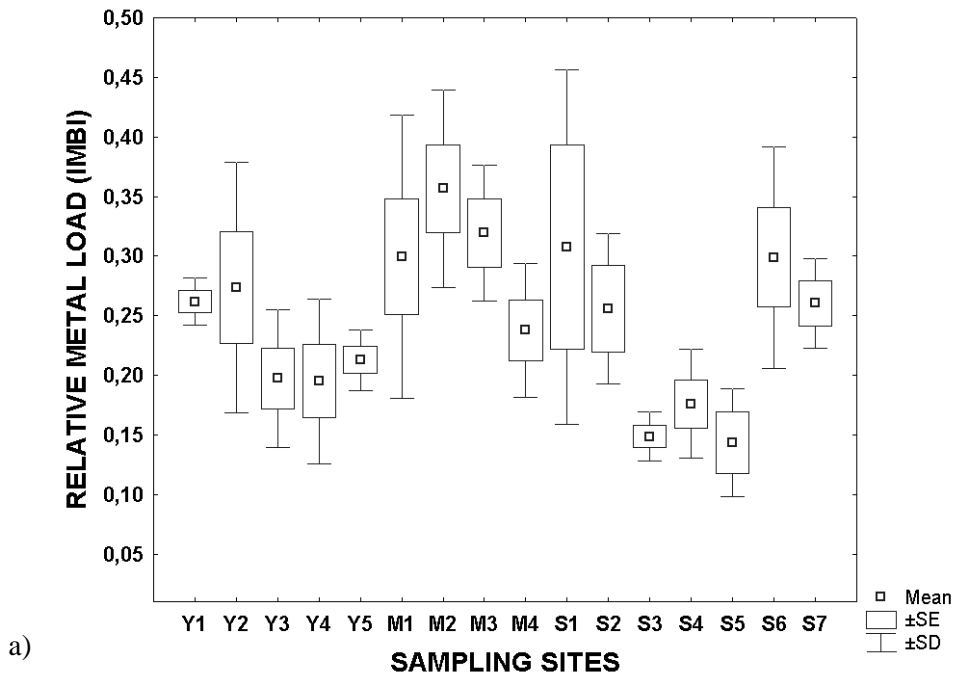


Figure 7.2. Level of heavy metal pollution (IMBI) in *Anguilla anguilla*. a) Per sampling site within river basin; b) Histogram: the "HIGH" and "LOW" group are defined from the bimodal distribution of IMBI values.

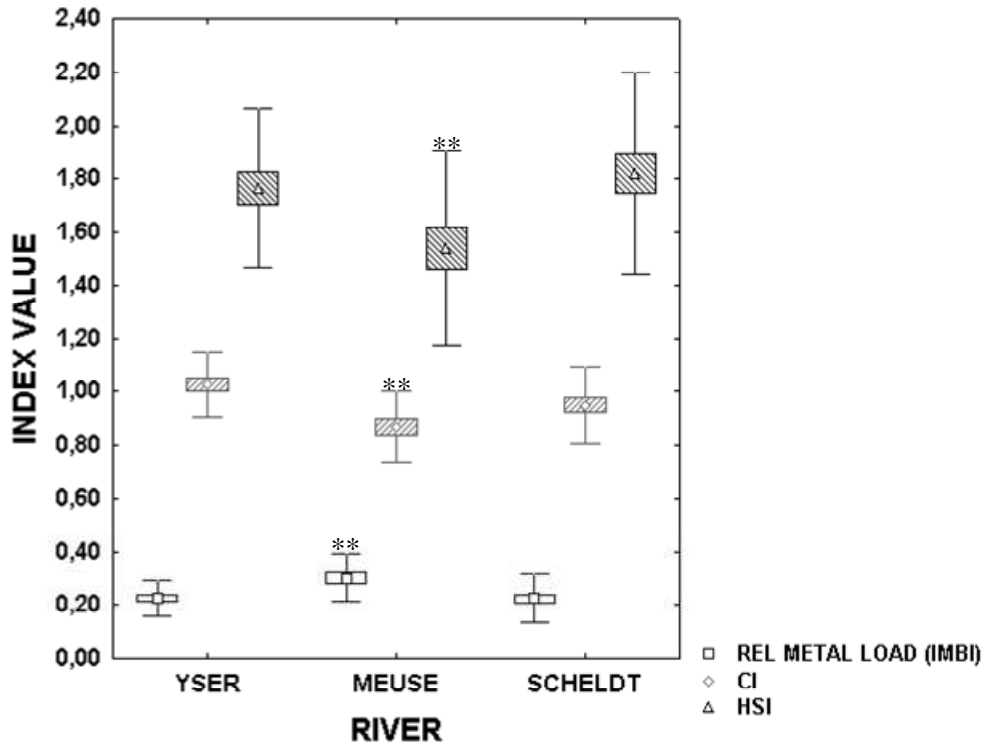
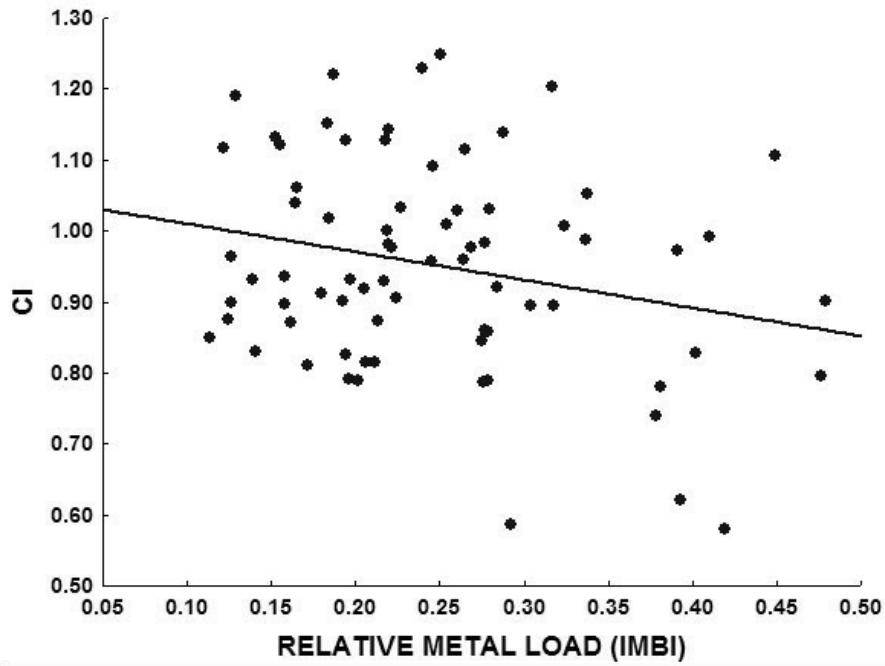


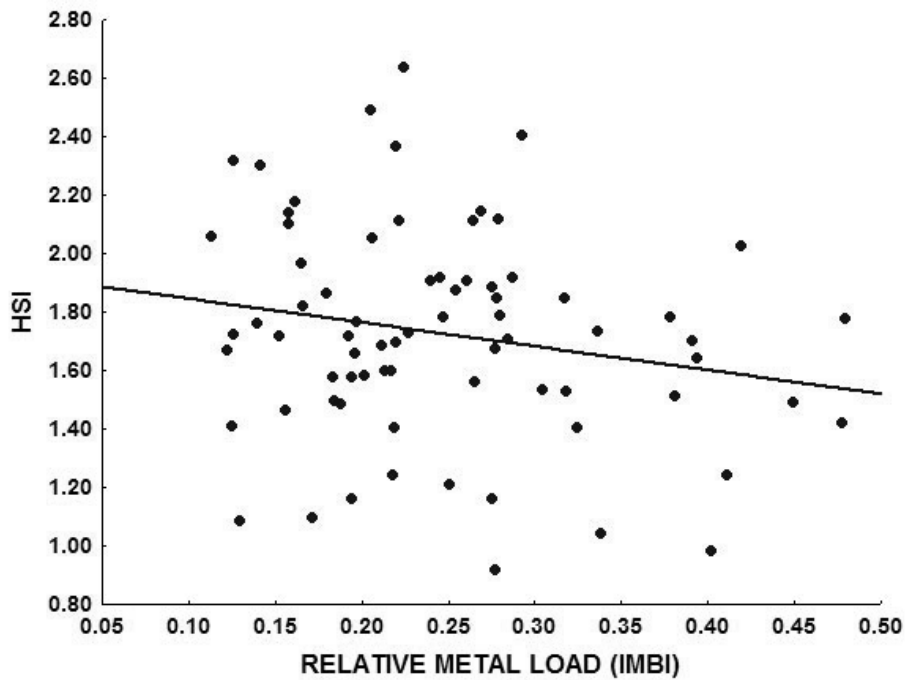
Figure 7.3. Boxplots representing relative condition index (CI \pm SD), hepatosomatic index (HSI \pm SD) and level of heavy metal pollution (IMBI \pm SD) of *Anguilla anguilla* for each river basin. ** = $p < 0.01$

Condition (CI) and Hepatosomatic (HSI) indices

The regression equation between length and weight was $\log(W) = 3.155 \log(L) - 3.032$ ($r = 0.9746$, $n = 123$, $p < 0.001$). The relative condition index (CI) was calculated as $1000(W/L^{3.155})$ and varied significantly among basins (ANOVA, $F_{2, 120} = 10.565$, $p < 0.001$), with the Meuse showing the lowest condition (Figure 7.3). HSI values varied from 0.917 to 2.639 among basins. The correlation between Weight and HSI was $r = -0.27$; $p < 0.01$. The relative hepato-somatic index, measured as the residuals of the former regression, differed significantly among basins (ANOVA, $F_{2, 120} = 5.897$, $p < 0.01$), pointing to the Meuse river as exhibiting the lowest values (Tukey test). Finally, there was a significant negative correlation between heavy metal bioaccumulation (IMBI) and condition ($r = -0.24$; $p < 0.05$, Figure 7.4a) and a negative relationship between IMBI and HSI ($r = -0.20$; $p = 0.09$) (Figure 7.4b). No correlation was observed between CI and HSI (data not shown).



a)



b)

Figure 7.4. Correlation between heavy metal bioaccumulation (IMBI) of *A. anguilla* and a) condition index (CI) with $R = -0.24$; $p = 0.039$; b) hepatosomatic index (HSI) with $R = -0.20$; $p = 0.09$ for all individuals ($n = 73$).

Intra- and inter-basin genetic variability

A total of 12 enzymatic loci were scored. The total number of alleles per locus ranged from 1 to 6 and from 2.3 to 2.6 per sample over all loci. Observed (H_O) and expected (H_E) heterozygosities per sample ranged from 0.103 to 0.119 and from 0.122 to 0.129 respectively (Table 7.2; Appendix 2). An overall probability test of H&W equilibrium based on 1000 iterations was highly significant ($p < 0.001$), pointing to the non-random distribution of alleles within some samples. A global test based on 1000 iterations with H_1 = heterozygote deficiency was only highly significant for the Meuse river (F_{IS} : 0.21; $p < 0.001$, Table 7.2), mainly due to loci *GPI-1**, *GPI-2**, *MDH-2** and *ADH-1**. A more detailed analysis of heterozygosities within river basin showed that the Meuse exhibited the highest expected heterozygosity ($H_E = 0.129$), the highest mean number of alleles (MNA = 2.58) and the highest level of polymorphism ($P_{(0.95)} = 0.50$). In contrast, this population exhibited the lowest observed heterozygosity ($H_O = 0.107$) (Table 7.2). No linkage disequilibrium was observed in the three populations.

The microsatellite loci revealed higher levels of variability than the allozymes as the total number of alleles per locus ranged from 12 (*AAN 05*) to 40 (*ANG 114*) and heterozygosity values (H_E) per locus ranged from 0.735 to 0.939 (Appendix 2). The mean number of alleles per locus by population varied between 14.4 (Scheldt) and 16.3 (Meuse). Observed and expected heterozygosity (H_O and H_E) per population were highly variable, ranging from 0.792 to 0.822 and from 0.850 to 0.869, respectively (Table 7.2). Exact tests assuming H_1 = heterozygote deficiency, revealed significant departures from the null hypothesis of H&W equilibrium in all samples (Table 7.2). The deficits could be attributed to a particular locus, namely *AAN 02* which exhibited the strongest inbreeding coefficients ($F_{IS} = 0.22$, $p < 0.001$), most likely due to null alleles. Detailed analysis of population specific genetic variability defines the Meuse population as the most variable, with the highest mean number of alleles (MNA = 16.3), expected heterozygosity ($H_E = 0.869$) and observed heterozygosity ($H_O = 0.822$) (Table 7.2). No linkage disequilibrium was observed in the three populations.

Table 7.2. Allozymatic and microsatellite genetic variability of *Anguilla anguilla* L. in the three river basins and in the LOW and HIGH pollution group. Expected (H_E) and observed (H_O) heterozygosity per sample/*post-hoc* group over all loci, level of polymorphism (P) and mean number of alleles (MNA) per sample/*post-hoc* group over all loci. N : number of individuals; S.E.: standard error; $P_{(0.95)}$ or $P_{(0.99)}$: 95% or 99% polymorphism criterion respectively. ** = $p < 0.01$.

	SAMPLE	N	$H_E \pm S.E.$	$H_O \pm S.E.$	$P_{(0.95)}$	$P_{(0.99)}$	M.N.A.	F_{IS}
ALLOZYMES	Yser	41	0.122 ± 0.154	0.111 ± 0.119	0.417	0.750	2.333	0.104
	Meuse	41	0.129 ± 0.147	0.103 ± 0.107	0.500	0.750	2.583	0.212**
	Scheldt	41	0.125 ± 0.165	0.119 ± 0.171	0.417	0.583	2.417	0.063

	LOW	35	0.140 ± 0.159	0.131 ± 0.151	0.500	0.750	2.833	0.075
	HIGH	32	0.109 ± 0.140	0.088 ± 0.094	0.417	0.667	2.333	0.212**
MICROSATELLITES	Yser	41	0.850 ± 0.073	0.792 ± 0.089	1.0	1.0	14.875	0.082**
	Meuse	41	0.869 ± 0.068	0.822 ± 0.087	1.0	1.0	16.250	0.069**
	Scheldt	41	0.851 ± 0.078	0.802 ± 0.054	1.0	1.0	14.375	0.072**

	LOW	35	0.863 ± 0.073	0.803 ± 0.077	1.0	1.0	15.875	0.087**
	HIGH	32	0.856 ± 0.080	0.817 ± 0.049	1.0	1.0	15.250	0.062**

Micro-scale genetic structure

Overall genetic differentiation was significant ($p < 0.05$), but the multi-locus unbiased differentiation estimators were very low for allozymes ($F_{ST(RB)} = 0.007$, $G_{ST} = 0.001$) and for microsatellites ($F_{ST(RB)} = 0.018$, $G_{ST} = 0.003$). Pairwise genetic differentiation shows discrepancies between both markers. The microsatellite genotypes of the Meuse basin are most distinct from the Yser ($F_{ST(RB)} = 0.025$, $p < 0.01$), while the allozyme genotypes differentiate Yser and Scheldt the most ($F_{ST(RB)} = 0.017$, $p < 0.05$).

Genetic composition of the “HIGH” and “LOW” pollution group

The bimodal distribution of the IMBI values allowed us to define two groups ranked by their magnitude of relative metal load (Figure 7.2b). A total of 67 individuals were ultimately selected, with 35 and 32 individuals in “HIGH” and “LOW” polluted condition respectively. To exclude redundancy, we tested for the independence between basin and HIGH-LOW pollution groups (Chi-square = 7.33; $df = 5$; $p > 0.05$). The “HIGH” pollution group clearly exhibited a lower allozymatic genetic variability (H_E , H_O , level of polymorphism and MNA) than the “LOW” pollution group (Table 7.2, Appendix 2). The proportional difference between both groups ($H_{LOW} - H_{HIGH} / H_{LOW}$) amounted to 21.5% H_E and 34.6% H_O between both pollution groups. Locus-by-locus heterozygosity (H_O) and allelic richness (AR) analysis points to a significantly lower AR (t-test, $p = 0.01$, Figure 7.5a) and lower H_O (t-test, $p = 0.03$, Figure 7.5b) for polluted individuals. Finally, the number of multi-locus genotypes (28 vs. 22) was higher in the “LOW” pollution group. In contrast, microsatellite variability (multi- and locus-by-locus analyses) showed no appreciable difference in expected or observed heterozygosity, allelic richness or number of alleles between both groups (data not shown). When individual MLH were compared for allozymes and microsatellites, we observed marginally significant lower allozyme MLH values for HIGH polluted individuals (ANOVA, $F_{1, 65} = 3.898$, $p = 0.05$), while for microsatellites no differences could be detected ($p > 0.05$) (Figure 7.5c)

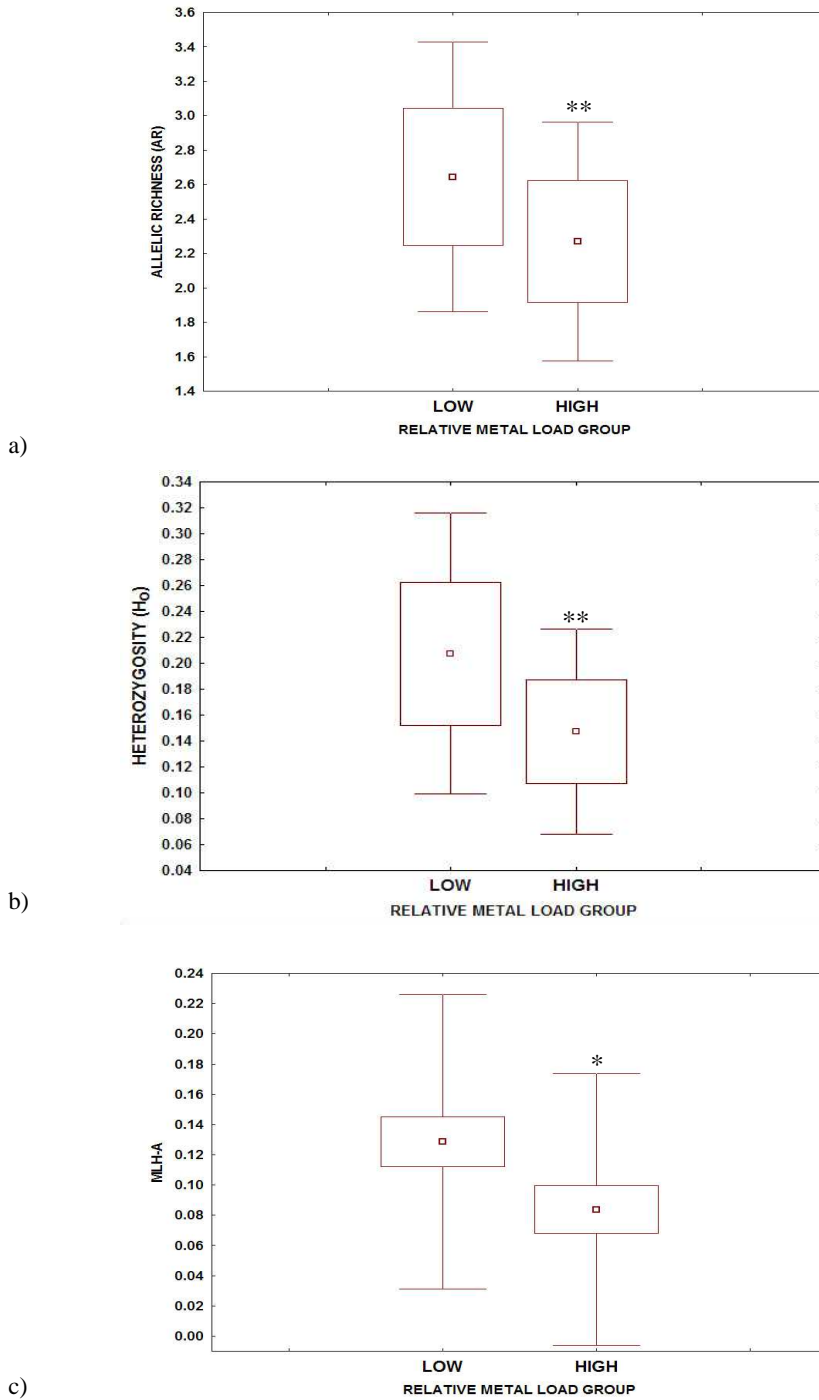


Figure 7.5. Allozymatic genetic variability of *A. anguilla*. Boxplots representing mean (\pm SE) a) allelic richness (AR, t-test; $p = 0.01$), b) observed heterozygosity (H_o , t-test; $p = 0.03$) locus-by- locus and c) Multi-locus Heterozygosity (MLH, ANOVA, $p = 0.05$) comparisons between “HIGH” and “LOW” heavy metal pollution groups. * = $p = 0.05$, ** = $p < 0.05$

MLH, condition and heavy metal load regression

Regression analysis between MLH (allozymes and microsatellites) and condition indices (CI and HSI) yielded a negative trend, but no significant correlation (n = 123, data not shown). Regression between individual allozyme MLH and IMBI values yielded a significant negative correlation (R = -0.28; p = 0.016), while microsatellite MLH was not correlated with IMBI (R = 0.07, p > 0.05) (Figure 7.6). Similarly, when performing a regression analysis using the mean IMBI and MLH values per sampling site (n = 16), a marginally significant negative correlation (R = -0.48, p = 0.05) is observed (data not shown), indicating that similar results can be obtained if comparing individual sampling sites. Multiple regression analysis of IMBI as dependent variable versus MLH allozymes, MLH microsatellites, CI and HSI resulted in a better model to explain variation in bioaccumulation ($F_{4, 68} = 5.776$, $p < 0.001$; adjusted $R^2 = 0.21$) than each variable alone (see Table 7.3, Figure 7.6). All variables, except the MLH of microsatellites, were correlated with IMBI values. MLH of allozymes contributed the most (β -weights) to the variation in IMBI, followed by CI, HSI and MLH of microsatellites (Table 7.3).

Table 7.3. Multiple regression analysis relating individual heavy metal bioaccumulation (IMBI) to condition (CI and HSI) and multi-locus heterozygosity (allozymes; MLH-A and microsatellites; MLH-M). The relative contribution of each variable is reported as beta-weight (standardized regression coefficient). Standard errors are given in parentheses.

	Parameter (s.e.)	DF	t	p-value	Beta weights (s.e.)
Intercept	0.535 (0.100)	1	5.352	0.000	/
CI	-0.210 (0.066)	1	-3.185	0.002	-0.343 (0.108)
HSI	-0.070 (0.026)	1	-2.653	0.010	-0.285 (0.107)
MLH- A	-0.350 (0.100)	1	-3.486	0.001	-0.376 (0.108)
MLH- M	0.086 (0.071)	1	1.209	0.231	0.129 (0.107)
Error	/	68	/	/	/

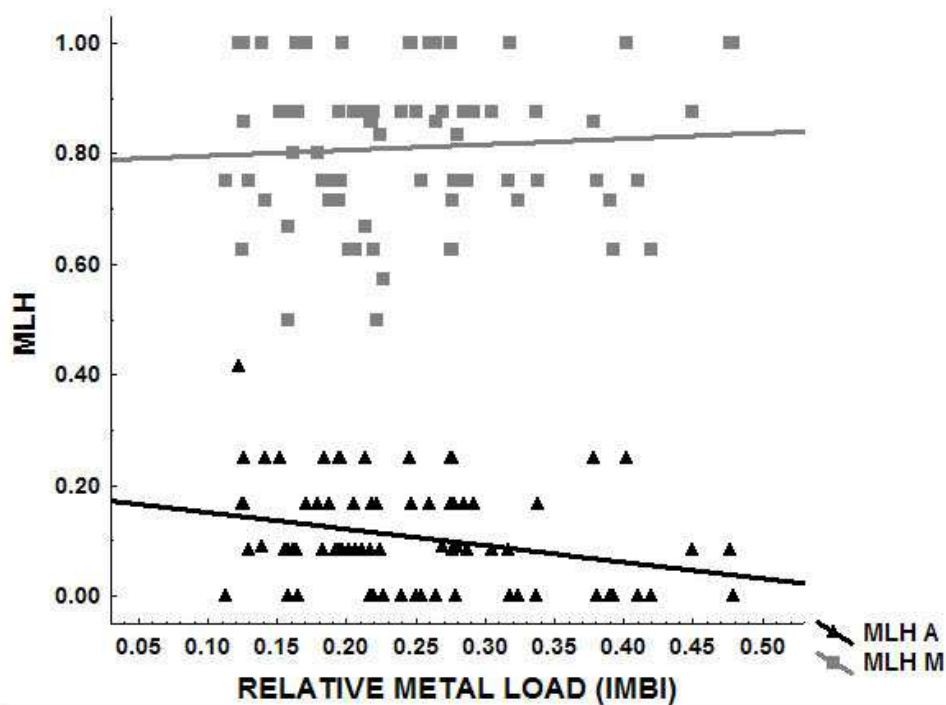


Figure 7.6. Correlation between relative heavy metal bioaccumulation (IMBI) and multi-locus heterozygosity (MLH) in *A. anguilla* for allozymes (MLH A) with $R = -0.28$; $p = 0.016$ and microsatellites (MLH M) with $R = 0.07$; $p = 0.56$.

Discussion

Although the impact of pollution on genetic variability has been assessed previously (Bickham *et al.*, 2000; Belfiore and Anderson, 2001 for a review), this study is novel in several aspects. First, our study focused on the level of bioaccumulation in a species extremely prone to pollution due to its high fat content, reflecting the actual pollution stress in the organism (Collings *et al.*, 1996). Its catadromous life history enables the detection of local pollutant influences on somatic and genetic characteristics, as juveniles enter rivers with much less pollution load or differential genetic background than locally reproducing and genetically distinct freshwater species. Their level of bioaccumulation after a few years spent in the rivers can be considered as indicative of their fitness, because strongly polluted eels detoxify less efficiently, have a lower condition and might be less successful spawners (Feunteun, 2002). Secondly, it has been suggested that several genetic markers should be used to discriminate between the influence of selection and other factors that might be marker specific (Belfiore and Anderson, 2001). In this study we compared patterns from strictly neutral genetic markers (microsatellite DNA) with enzymatic markers (allozymes), for which the assumption of selective neutrality has often been challenged (Eanes, 1999). The significance of assessing biometric

(weight, condition, growth) responses has also been underlined as a measure of pollutant impact on the organism (Van Straalen and Timmermans, 2002). Finally, the study of highly vagile organisms with a catadromous life-history like eel remains underrepresented, due to the difficulty of defining biologically relevant populations. Earlier studies used reproductively isolated populations, enabling straightforward population comparisons in the light of the “genetic erosion” hypothesis (Van Straalen and Timmermans, 2002). Here, we explain this issue in two ways, namely (1) by assessing the impact of pollutants on genetic variability (“Genetic Erosion” hypothesis) and (2) by considering individual genetic variability as an advantage to cope with pollution (“Heterosis” or “overdominance” hypothesis). Nevertheless, due to the catadromous life-history of eel and its failure to breed in captivity, no strong conclusions about evolutionary consequences can be drawn from our observations.

Spatial heterogeneity in pollution and condition

Although the European eel is a highly vagile fish species (Tesch, 1977), the feeding stage inhabiting the freshwater environment is remarkably sedentary and pollutants are expected to have a local influence. Our results confirm this knowledge; the accumulation of heavy metals is strongly heterogeneous between and within basins (Table 7.1, Figure 7.2). We found significant differences between individuals originating from other river basins, pointing to locally highly and less polluted sites. Despite intra-riverine variability, the Meuse basin was the strongest polluted river (Table 7.1), in line with current perception (Maeckelberghe, 2003; Cellule Etat de l'Environnement Wallon, 2003). The bioaccumulation of heavy metals, defined as a relative index (IMBI), confirmed single metal predictions, namely pointing to the Meuse eels as significantly stronger polluted than the eels of other basins. Earlier studies on European eel have confirmed the heterogeneous distribution of pollutants in rivers and lakes (Linde *et al.*, 1996; Belpaire *et al.*, 2002; Goemans *et al.*, 2003). The individual level of bioaccumulation might provide both an estimate of the environmental quality of the sediment (eels are benthic feeders) and a measure of health condition (fitness) of the organism (Bervoets and Blust, 2003). As no correlation was found between eel length and pollutants, the capacity of detoxification of individuals seemed unrelated to their size.

Life-history traits, such as condition, growth and fecundity, reflect the environmental quality and the organism's historic experience (Meffe, 1991; Ridley, 1996). We expected an impact of the level of bioaccumulation on the condition of European eel, due to the excess energy required for detoxification. We clearly showed a relationship between an increased heavy metal content and a lower condition in eel. The Meuse population exhibited a significantly lower condition than the other two river basins. Regression analysis revealed a strong negative correlation between individual bioaccumulation and condition indices (Figure 7.4a and b), which confirms the literature. For example, in the Sydney rock oyster (*Saccostrea commercialis*), bioaccumulation strongly correlated with condition (Avery *et al.*, 1996). Hence, we have strong indications that the bioaccumulation of heavy metals is a predictor of the condition in European eel and that pollution might significantly affect individual fitness. Due to the mobilisation of fat reserves during the spawning migration, it is expected that highly polluted individuals will have a lower reproductive success during spawning (van Ginneken & van den Thillart, 2001; Robinet and Feunteun, 2002).

Bioaccumulation vs. intra river and post-hoc genetic variability

Because of the absence of reproductively isolated groups in Belgium and Europe (Wirth and Bernatchez, 2001; Maes and Volckaert, 2002), it remains difficult to sustain the concept of "populations" in a river basin. Analyses performed at the population level are mostly testing for Hardy-Weinberg equilibrium, which can also be interpreted as randomness in genotypic distribution within rivers instead of random mating amongst individuals. Our proposal to analyse on the one hand natural populations (river basin) and on the other hand phenotypic traits (pollution charge), aims first at analysing the influence of a geographically divergent pollution level (local pattern of genetic variability) and subsequently mainly at comparing the genetic variability based on pollutant concentration in "general", where an individual's heterozygosity determines its response to pollutants. The level of bioaccumulation does not necessarily reflect the environmental pollution but also the individual capacity for detoxification. Hence, we argue that individual bioaccumulation is also determined by the genetic make-up.

Considering genetic variability within a river basin, we showed that the Meuse, despite exhibiting the highest expected variability, was in strong H&W disequilibrium, pointing to a non-random distribution of genotypes and possibly differential selection. Such results may have several causes, like population substructure, null alleles, inbreeding and selection (Hartl and Clarck, 1997). Because of the absence of reproductively active populations and the lack of similar results on microsatellites (excluding locus AAN 02), selection seems the most plausible explanation for the genotypic shift. The most strongly polluted population was the least heterozygous (H_o) at allozymes, possibly attributing weaker detoxification ability to more homozygous individuals and/or shifts towards certain homozygote classes.

Due to the heterogeneous distribution of metals and the absence of "biological" populations in rivers, we ranked individuals in *post-hoc* groups according to their level of bioaccumulation. The pattern exhibited here was much more unambiguous, namely a lower overall genetic allozymatic variability in strongly polluted individuals and again a strong H&W disequilibrium. A similar decrease in genetic variability has been demonstrated in various other freshwater, marine and terrestrial organisms under natural and laboratory conditions (Hvilsom, 1983; Fevolden and Garner, 1986; Klerks and Weis, 1987; Patarnello *et al.*, 1989; Posthuma and Van Straalen, 1993). Changes in diversity were mostly attributed to the selective advantage of certain genotypes or a reduction in population size (N_e). Remarkably, in most studies either only a few enzymatic loci were screened or an impact was observed at few loci (Chagnon and Guttman, 1989; Gillespie and Guttman, 1989; Paternello and Battaglia, 1992; Newman and Jagoe, 1998). In the present study, we observed a multi-locus response on pollution, namely at seven out of nine enzymatic loci. Only locus *GPI-1** remained constant, while *MPI-1** even exhibited a higher variability in strongly polluted individuals, possibly pointing to a heavy metal tolerant allele. We observed a lower number of genotypes in the highly polluted group, which fits the expectations (Ben-Shlomo and Nevo, 1988; Chagnon and Guttman, 1989; Diamond *et al.*, 1991), and suggests differential mortality or genotype shifts. Interestingly, 74% of Meuse individuals belong to the HIGH pollution group compared to 40% and 35% for the Yser and Scheldt respectively. This confirms the lower observed variability (H_o) in the Meuse, while the remaining low polluted individuals from this river may have raised the MNA and hence the expected heterozygosity by carrying rare alleles. The genetic variability at strictly neutral markers did not show any pollution related differences, despite the high number of alleles and the higher resolution expected from this marker (Hedrick, 1999). Nevertheless, other studies using similar markers have found a strong correlation between a decrease in neutral genetic variation and the level of pollution in natural

aquatic (Nadig *et al.*, 1998; Krane *et al.*, 1999; Ma *et al.*, 2000; Matson *et al.*, 2000) and terrestrial (Theodorakis *et al.*, 2001) habitats. This result was somewhat expected as the only selection possibly influencing the genetic pattern of eel is direct selection on metabolically important enzymes, as microsatellites evolve strictly neutrally, mainly enabling the detection of post-reproductive selection (Bickham *et al.*, 2000; Belfiore and Anderson, 2001).

Bioaccumulation vs. individual genetic variability

A comparison between individual-based pollution characteristics and population summary statistics (H_E , P, MNA, H&W equilibrium) holds several difficulties for the interpretation, due to the assumption of "population" in genetic estimators. Therefore, we chose to analyse the relationships between all variables using individual based regression analyses. A negative correlation was observed between IMBI and condition indices, as well as between IMBI and allozymatic MLH. This suggests that strongly polluted individuals need more energy for detoxification and are on average in a worse condition, while more heterozygous individuals may accumulate less (Van Straalen and Kammenga, 1998). Increased fitness with heterozygosity has been empirically demonstrated in a large number of plants and animal species (see David, 1998 for a review), as heterozygotes are better buffered against environmental fluctuations, are superior due to their multimeric enzymes (Nevo *et al.*, 1986) and have a lower energetic demand, favouring such individuals in strongly polluted conditions (heterosis). Due to the multi-locus response or cause of the correlation, an overall metabolic gain in efficiency may be proposed as cause for the correlation (Eanes, 1999); most allozymes studied belong to the glycolysis or citric acid cycle. No Heterozygosity Fitness Correlation (HFC) was found at microsatellites, results concordant with recent findings in farmed eel where growth rate was correlated to allozymatic but not to microsatellite MLH (Pujolar *et al.*, 2005). The relative importance of condition and genetic variability to explain differences in heavy metal bioaccumulation as assessed by multiple regression analysis, pointed to allozymatic MLH, followed by the condition index as the main factors influencing bioaccumulation. Hence, an individual's enzymatic heterozygosity (and not necessarily its genome-wide heterozygosity) seems to play an important role in the potential to counteract pollutant bioaccumulation.

Conclusions

We clearly showed a strong correlation between the level of bioaccumulation and a reduced condition within resident eel populations. We also found an obvious link between pollution and a lower allozymatic genetic variability at the individual level and in two *post-hoc* defined groups of different pollution levels. Microsatellite variability did not reflect any pollution or condition related trend, and no individual HFC pattern. We hypothesize that enzymatic genetic variability (MLH) is a key issue to explain differences in the bioaccumulation of toxicants (or detoxification success), in other words to retain fitness. Hence, direct overdominance seems the most likely explanation for the observed pattern in eel and thus not associative overdominance or genetic erosion (only detectable after reproduction). Complementary sampling and experimental studies should increase our confidence about the strength of ecological consequences in catadromous organisms, as well as about the heterosis effect (HFC) detected in this study. Conditional is the optimisation of artificial breeding before evolutionary inferences can be made experimentally. Our results also underline the complexity of evolutionary toxicology research in diadromous species, which switch

between habitats. The knowledge of the genetic make-up is crucial to infer evolutionary consequences of pollutants in such species, which is only possible when assessing the interaction between ecology and genetics.

Acknowledgements

This research was funded by The Ministry of the Flemish Community (AMINAL). Many thanks go to K. Peirsman for organizing the fishing and processing of the samples. We thank J.P. Nash, J.K.J. Van Houdt and two anonymous referees who helped improve the quality of this manuscript. G.E. Maes received a PhD scholarship from the IWT (Institute for the Promotion of Innovation by Science and Technology in Flanders). J.A.M. Raeymaekers received a PhD fellowship of the Fund for Scientific Research (F.W.O.-Vlaanderen). C. Pampoulie received a post-doctoral fellowship from FWO-Vlaanderen and the KULeuven.

References

- Avery, E.L., Dunstan, R.H., Nell, J.A., 1996. The detection of pollutant impact in marine environments: condition index, oxidative DNA damage, and their associations with metal bioaccumulation in the sydney rock oyster *Saccostrea commercialis*. *Archives of Environmental Contamination and Toxicology* 31, 192-198.
- Belfiore, N.M., Anderson, S.L., 2001. Effects of contaminants on genetic patterns in aquatic organisms: a review. *Mutation Research – Reviews in Mutation Research* 489, 97-122.
- Belkhir, K., Borsa, P., Goudet, J., Bonhomme, F., 1999. Genetix: logiciel sous Windows pour la genétique des populations. version 3.0, Laboratoire Genome & Population, CNRS-UPR, Université de Montpellier II, Montpellier (France).
- Belpaire C., Derwich A., Goemans G., Van Thuyne G., Cooreman K, Guns M., Ollevier F., 2002. Intra-lake spatial variations in pollution patterns of eel *A. anguilla*. In: Aida, K., *et al.* (eds.), Proceedings of the International Symposium 'Advances in eel biology'. University of Tokyo, 28-30 September 2003, p.170-174.
- Benshlomo, R., Nevo, E., 1988. Isozyme polymorphism as monitoring of marine environments - the interactive effect of cadmium and mercury pollution on the shrimp, *Palaemon elegans*. *Marine Pollution Bulletin* 19, 314-317.
- Bervoets, L., Blust, R., 2003. Metal concentrations in water, sediment and gudgeon (*Gobio gobio*) from a pollution gradient: relationship with fish condition factor. *Environmental Pollution* 126 (1), 9-19.
- Bickham, J.W. , Sandhu, S., Hebert, P.D.N., Chikhi, L., Athwal, R., 2000. Effects of chemical contaminants and genetic diversity in natural populations: implications for biomonitoring and ecotoxicology. *Mutation Research – Reviews in Mutation* 463, 33-51.
- Cellule Etat de l'Environnement Wallon, 2003. Tableau de bord de l'environnement wallon 2003. Ed. MRW & DGRNE, 144p.
- Chagnon, N.L., Guttman, S.I., 1989. Differential survivorship of allozyme genotypes in mosquitofish populations exposed to copper or cadmium. *Environmental Toxicology and Chemistry* 8, 319-326.

- Collings, S.E., Johnson, M.S., Leah, R.T., 1996. Metal contamination of angler-caught fish from the Mersey estuary. *Marine Environmental Research* 41, 281-297.
- Daemen, E., Cross, T., Ollevier, F., Volckaert, F.A.M., 2001. Analysis of the genetic structure of European eel (*Anguilla anguilla*) using microsatellite DNA and mtDNA markers. *Marine Biology* 139, 755-764.
- David, P., 1998. Heterozygosity-fitness correlations: new perspectives on old problems. *Heredity* 80, 531-537.
- Dekker, W., 2000. A procrustean assessment of the European eel stock. *ICES Journal of Marine Science* 57, 938-947.
- Diamond, S.A., Newman, M.C., Mulvey, M., Guttman, S.I., 1991. Allozyme genotype and time-to-death of mosquitofish, *Gambusia holbrooki*, during acute inorganic mercury exposure - a comparison of populations. *Aquatic Toxicology* 21, 119-134.
- Eanes, W.F., 1999. Analysis of selection on enzyme polymorphisms. *Annual Review of Ecology and Systematics* 30, 301-326.
- Feunteun, E., 2002. Management and restoration of European eel population (*Anguilla anguilla*): an impossible bargain. *Ecological Engineering* 18, 575-591.
- Fevolden, S.E., Garner, S.P., 1986. Population-genetics of *Mytilus edulis* from Oslofjorden, Norway, in oil-polluted and non oil-polluted water. *Sarsia* 71, 247-257.
- Fрати, F., Fanciulli, P.P., Posthuma, L., 1992. Allozyme variation in reference and metal-exposed natural populations of *Orchesella cincta* (insecta, collembola). *Biochemical Systematics and Ecology* 20, 297-310.
- Gillespie, R.B., Guttman, S.I., 1989. Effects of contaminants on the frequencies of allozymes in populations of the central stoneroller. *Environmental Toxicology and Chemistry* 8, 309-317.
- Goemans, G., Belpaire, C., Raemaekers, M., Guns M., 2003. Het Vlaamse palingpolluentenmeetnet, 1994-2001: gehalten aan polychloorbifenylen, organochloorpesticiden en zware metalen in paling (report nr: IBW.Wb.V.R.2003.99). 169p.
- Harris, H., Hopkinson, D.A., 1976. Handbook of enzyme electrophoresis in human genetics. North Holland Publishing Co., Oxford.
- Hartl, D.L., Clark, A.G., 1997. Principles of population genetics. Sinauer Associates, Sunderland (USA), third edition, 542p.
- Hedrick, P.W., 1999. Perspective: highly variable loci and their interpretation in evolution and conservation. *Evolution*. 53, 313-318.
- Hvilsom, M.M., 1983. Copper-induced differential mortality in the mussel *Mytilus edulis*. *Marine Biology* 76, 291-295.
- King, M., 1995. Fisheries Biology, Assessment and Management. Fishing news books, Blackwell Science Ltd., Oxford, England. 341p.
- Klerks, P.L., Weiss, J.S., 1987. Genetic adaptation to heavy metals in aquatic organisms - a review. *Environmental Pollution* 45, 173-205.
- Krane, D.E., Sternberg, D.C., Burton, G.A., 1999. Randomly amplified polymorphic DNA profile-based measures of genetic diversity in crayfish correlated with environmental impacts. *Environmental Toxicology and Chemistry* 18, 504-508.
- Leary, R.F., Allendorf, F.W., Knudsen, K.L., 1987. Differences in inbreeding coefficients do not explain the association between heterozygosity at allozyme loci and developmental stability in rainbow-trout. *Evolution* 41, 1413-1415.
- Lewis, S.S., Klerks, P.L., Leberg, P.L., 2001. Relationship between allozyme genotype and sensitivity to stressors in the western mosquitofish *Gambusia*

- affinis* detected for elevated temperature but not mercury. *Aquatic Toxicology* 52 , 205-216.
- Linde, A.R., Arribas, P., Sanchez Galan, S., and Garcia Vazquez, E., 1996. Eel (*Anguilla anguilla*) and brown trout (*Salmo trutta*) target species to assess the biological impact of trace metal pollution in freshwater ecosystems. *Archives of Environmental Contamination and Toxicology* 31(3), 297-302.
- Ma, X.L., Cowles, D.L., Carter, R.L., 2000. Effect of pollution on genetic diversity in the bay mussel *Mytilus galloprovincialis* and the acorn barnacle *Balanus glandula*. *Marine Environmental Research* 50, 559-563.
- Maeckelberghe, H., 2003. De kwaliteit van het Vlaamse oppervlaktewater en de afvalwaterlozingen in 2002. *Water* 3, 1-6.
- Maes G.E., Volckaert F.A.M., 2002. Clinal variation and isolation by distance within the European eel (*Anguilla anguilla* L.). *Biological Journal of the Linnean Society* 77, 509-521.
- Matson, C.W., Rodgers, B.E., Chesser, R.K., Baker, R.J., 2000. Genetic diversity of *Clethrionomys glareolus* populations from highly contaminated sites in the Chernobyl region, Ukraine. *Environmental Toxicology and Chemistry* 19, 2130-2135.
- Meffe, G.K., 1991. Life-history changes in Eastern mosquitofish (*Gambusia holbrooki*) induced by thermal elevation. *Canadian Journal of Fisheries and Aquatic Science* 48, 60-66.
- Nadig, S.G., Lee, K.L., Adams, S.M., 1998. Evaluating alterations of genetic diversity in sunfish populations exposed to contaminants using RAPD assay. *Aquatic Toxicology* 43, 163-178.
- Nevo, E., 2001. Evolution of genome-phenome diversity under environmental stress. *PNAS* 98, 6233-6240.
- Nevo, E., Noy, R., Lavie, B., Beiles, A., Muchtar, S., 1986. Genetic diversity and resistance to marine pollution. *Biological Journal of the Linnean Society* 29, 139-144.
- Newman, M.C., Jagoe, R.H., 1998. Allozymes reflect the population-level effect of mercury: simulations of the mosquitofish (*Gambusia holbrooki girard*) GPI-2 response. *Ecotoxicology* 7, 141-150.
- Patarnello, T., Battaglia, B., 1992. Glucosephosphate isomerase and fitness - effects of temperature on genotype dependent mortality and enzyme-activity in 2 species of the genus *Gammarus* (Crustacea, Amphipoda). *Evolution* 46, 1568-1573.
- Posthuma, L., Vanstraelen, N.M., 1993. Heavy-metal adaptation in terrestrial invertebrates - a review of occurrence, genetics, physiology and ecological consequences. *Comparative Biochemistry and Physiology C - Pharmacology, Toxicology and Endocrinology* 106 , 11-38.
- Powers, D.A., Lauerman, T., Crawford, D., Dimichele, L., 1991. Genetic mechanisms for adapting to a changing environment. *Annual Review of Genetics* 25, 629-659.
- Pujolar, J.M., Maes, G.E. & Volckaert, F.A.M. 2005. Growth Rate Correlates To Individual Heterozygosity in European eel, *Anguilla anguilla* L. *Evolution* 59 (1), 189-199.
- Raufaste, N., Bonhomme, F., 2000. Properties of bias and variance of two multiallelic estimators of F_{ST} . *Theoretical Population Biology* 57, 285-296.
- Raymond, M., Rousset, F., 1995. Genepop (version 1.2): a population genetics software for exact tests and ecumenicism. *Journal of Heredity* 86, 248-249.
- Rice, W.R., 1989. Analyzing tables of statistical tests. *Evolution* 43, 223-225.
- Richardson, B.J., Baverstock, P.R., Adams, M., 1986. Allozyme electrophoresis: A handbook for animal systematics and population studies. Academic Press, Inc., California, San Diego. 410p.

- Ricker, W.E., 1975. Computation and interpretation of the biological statistics of fish populations. *Bulletin of the Fisheries Research Board of Canada* 191, 1-382
- Ridley, M., 1996. Evolution. Blackwell Science, Inc., Cambridge. 719p.
- Robertson, A., Hill, W.G., 1984. Deviations from Hardy-Weinberg proportions - sampling variances and use in estimation of inbreeding coefficients. *Genetics* 107, 703-718.
- Robinet, T.T., Feunteun, E.E., 2002. Sublethal effects of exposure to chemical compounds: a cause for the decline in Atlantic eels? *Ecotoxicology* 11, 265-277.
- Roche, N., Buet, A., Ramade, F., 2003. Characterization and validation of ecotoxicological biomarkers in an eel population exposed to persistent organic pollutants in the Vaccares lake, French national reserve of Camargue. *Rev. D Ecol. - La Terre et La Vie*. 58, 127-141.
- Shaklee, J.B., Allendorf, F.W., Morizot, D.C., Whitt, G.S., 1990. Gene nomenclature for protein-coding loci in fish. *Transactions of the American Fisheries Society* 119, 2-15.
- Skoog, D.A., 1997. Principles of instrumental Analysis. Brooks Cole Eds., 5th edition, Florence, USA. 870p.
- Sokal, R.R., Rohlf F.J. 1997. Biometry, the principles and practice of statistics in biological Research. W.H. Freeman and Company, New York. 887p.
- Stanton, M.L., Roy, B.A., Thiede, D.A., 2000. Evolution in stressful environments. I. Phenotypic variability, phenotypic selection, and response to selection in five distinct environmental stresses. *Evolution* 54, 93-111.
- StatSoft, Inc. 2001. STATISTICA (data analysis software system), version 6. www.statsoft.com
- Tesch, F.W., 1977. The Eel, biology and management of Anguillid eels. Chapman & Hall, London. 434p.
- Theodorakis, C.W., Bickham, J.W., Lamb, T., Medica, P.A., Lyne, T.B., 2001. Integration of genotoxicity and population genetic analyses in kangaroo rats (*Dipodomys merriami*) exposed to radionuclide contamination at the Nevada test site, USA. *Environmental Toxicology and Chemistry* 20, 317-326.
- Thorpe, J.E., Koonce, J.F., Borgeson, D., Henderson, B., Lamsa, A., Maitland, P.S., Ross, M.A., Simon, R.C., Walters, C., 1981. Assessing and managing mans impact on fish genetic resources. *Canadian Journal of Fisheries and Aquatic Science* 38, 1899-1907.
- Van Ginneken, V.J.T., Van den Thillart, G.E.E.J.M., 2000. Eel fat stores are enough to reach the Sargasso. *Nature* 403 (6766), 156-157.
- Van Der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology* 13, 57-149.
- Van Straalen, N.M. 1999. Genetic biodiversity in toxicant-stressed populations. *Prog. Environ. Sci.* 1, 195-201.
- Van Straalen, N.M., Kammenga, J.E. 1998. Assessment of ecotoxicity at the population level using demographic parameters. In: Schüürmann, G., Markert, B. (eds.), *Ecotoxicology*. John Wiley & Sons, Inc. and Spektrum Akademischer Verlag, p.621-644.
- Van Straalen, N.M., Timmermans M.J.T.N., 2002. Genetic variation in toxicant-stressed populations: an evaluation of the "genetic erosion" hypothesis. *Human and Ecological Risk Assessment* 8, 983-1002.
- Wirth, T., Bernatchez, L., 2001. Genetic evidence against panmixia in the European eel. *Nature* 409, 1037-1040.

Appendix 1. Average heavy metal concentration per kg of wet *Anguilla anguilla* tissue per sampling site: n = number of individuals analysed for heavy metals. Values for As and Se correspond to one individual at the respective sampling site. Values for Hg, Cd, Pb, Ni, Cr, As and Se are expressed in $\mu\text{g.kg}^{-1}$. Values for Cu and Zn are expressed in mg.kg^{-1} .

River	Sampling site	n	Hg	Cd	Pb	Cu	Zn	Ni	Cr	As	Se
YSER	Y1	5	245.20	2.60	49.80	0.37	25.76	46.00	171.00	/	/
YSER	Y2	5	59.60	1.78	75.40	1.20	19.20	77.20	632.40	135.00	329.00
YSER	Y3	5	139.40	2.18	24.80	0.39	23.78	37.80	285.60	/	/
YSER	Y4	5	194.60	2.58	20.00	0.37	27.36	17.20	146.60	/	/
YSER	Y5	5	112.80	3.10	38.40	0.33	23.30	54.40	242.80	/	/
MEUSE	M1	6	152.50	23.15	10.33	0.68	27.22	55.67	823.67	263.00	1081.00
MEUSE	M2	5	215.40	22.72	39.20	0.53	30.24	94.00	138.40	168.00	743.00
MEUSE	M3	4	175.20	22.58	28.00	0.33	23.92	82.20	157.60	733.00	488.00
MEUSE	M4	5	144.20	5.16	83.60	0.36	23.84	27.00	197.20	321.00	342.00
SCHELDT	S1	3	70.33	1.67	85.67	0.83	32.50	92.00	183.67	243.00	667.00
SCHELDT	S2	3	142.00	1.50	95.33	0.70	23.83	37.33	135.33	257.00	913.00
SCHELDT	S3	5	91.20	3.86	5.00	0.58	19.74	27.00	139.00	229.00	1064.00
SCHELDT	S4	5	66.00	1.50	55.60	0.83	24.46	5.00	200.20	/	/
SCHELDT	S5	3	99.67	1.50	15.67	0.50	17.00	25.00	187.33	254.00	1166.00
SCHELDT	S6	5	66.20	6.98	85.60	0.57	28.90	90.00	174.60	/	/
SCHELDT	S7	4	127.75	1.50	73.75	0.67	24.75	61.25	181.25	704.00	1556.00

Appendix 2. Allele frequency of the 12 allozymatic and 8 microsatellite loci per sample; R : Allele range; N: number of individuals; A: number of alleles; H_E : expected heterozygosity, H_o : observed heterozygosity.

ALLOZYMES				MICROSATELLITES			
LOCUS	YSER	MEUSE	SCHELDT	LOCUS	YSER	MEUSE	SCHELDT
IDH-1*				AAN 01			
N	41	41	39	R	218-244	218-246	218-240
A	2	3	4	N	37	30	28
H_e	0.0705	0.0479	0.1903	A	9	10	10
H_o	0.0732	0.0488	0.2051	H_e	0.7356	0.7761	0.7353
GPI-1*				H_o	0.7838	0.7000	0.7143
N	40	39	40	AAN 02			
A	3	3	4	R	175-307	173-263	175-227
H_e	0.1403	0.1893	0.3231	N	34	38	36
H_o	0.1500	0.1282	0.2750	A	22	23	23
GPI-2*				H_e	0.9321	0.9224	0.9340
N	41	41	40	H_o	0.5588	0.6316	0.5278
A	4	4	2	AAN 05			
H_e	0.0714	0.1823	0.0247	R	177-199	177-197	177-197
H_o	0.0732	0.1463	0.0250	N	39	37	36
AAT-1*				A	9	11	9
N	41	41	38	H_e	0.7558	0.7513	0.7411
A	3	4	6	H_o	0.7692	0.8919	0.7500
H_e	0.1978	0.2023	0.1967	ARO 095			
H_o	0.2195	0.2195	0.1842	R	112-136	108-132	110-132
AAT-2*				N	35	29	33
N	41	41	38	A	12	13	12
A	2	2	2	H_e	0.8547	0.8740	0.8508
H_e	0.0241	0.0476	0.0260	H_o	0.8571	0.8893	0.5152
H_o	0.0244	0.0488	0.0263	ARO 054			
LDH-A*				R	142-170	150-172	144-166
N	41	41	39	N	39	39	41
A	1	1	1	A	14	11	11
H_e	0.0000	0.0000	0.0000	H_e	0.8586	0.8797	0.8507
H_o	0.0000	0.0000	0.0000	H_o	0.7436	0.8974	0.8780
LDH-B*				ANG 151			
N	41	41	39	R	164-202	160-196	158-186
A	1	1	1	N	39	32	38
H_e	0.0000	0.0000	0.0000	A	13	15	12
H_o	0.0000	0.0000	0.0000	H_e	0.8416	0.8936	0.8397
MPI-1*				H_o	0.8718	0.6875	0.8158
N	41	41	39	ANG 114			
A	3	3	1	R	191-263	190-351	190-233
H_e	0.1157	0.1579	0.0000	N	39	36	37
H_o	0.1220	0.1707	0.0000	A	19	29	17
MDH-1*				H_e	0.8958	0.9255	0.9069
N	41	41	41	H_o	0.7949	0.9444	0.8649
A	2	2	1	ARO 121			
H_e	0.0241	0.0241	0.0000	R	110-147	101-149	106-143
H_o	0.0244	0.0244	0.0000	N	39	31	36
MDH-2*				A	20	17	21
N	41	40	41	H_e	0.9293	0.9126	0.9317
A	3	4	4	H_o	0.8718	0.8065	0.8056
H_e	0.3067	0.1841	0.2439				
H_o	0.3171	0.1000	0.1463				
PGM-1*							
N	41	41	40				
A	1	1	1				
H_e	0.0000	0.0000	0.0000				
H_o	0.0000	0.0000	0.0000				
ADH-1*							
N	40	34	39				
A	3	3	2				
H_e	0.5116	0.5134	0.4970				