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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor Amadeu Mortágua Velho da Maia Soares, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro e co-orientação científica do Doutor João Luís Teixeira Pestana, Investigador Auxiliar do Departamento de Biologia da Universidade de Aveiro e do Doutor Carsten Felix Nowak, Head of Section Conservation Genetics, Department of River Ecology & Conservation, Senckenberg Research Institute & Natural History Museum.

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Life is about catching the right waves
and riding them as long as you can.

O Júri

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Resumo

As populações que vivem em ecossistemas de água doce extremamente contaminados por metais podem estar sujeitas a forte seleção e deriva genética. Este processo de erosão genética poderá ameaçar a sua sobrevivência a longo prazo, uma vez que a capacidade de adaptação das populações a alterações das condições ambientais está diretamente relacionada com os níveis de diversidade genética. Neste sentido, a procura por novos bioindicadores, que aumentem a relevância ecológica da avaliação de risco ambiental, tem levado a um crescente interesse pela toxicologia evolutiva e por medidas de diversidade genética.

O trabalho aqui apresentado tem como objetivo último compreender de que forma os níveis de diversidade genética da espécie modelo em ecotoxicologia *Chironomus riparius* (Meigen) podem ser usados como indicadores de qualidade ecológica de sistemas de água doce. Para tal, avaliaram-se respostas microevolutivas à contaminação histórica por metais em populações de *C. riparius*, incluindo determinação dos níveis de diversidade genética, adaptação genética a metais e potenciais custos de *fitness*. A diversidade genética foi estimada com base na variação de sete marcadores de microssatélites enquanto que a adaptação genética a metais e potenciais custos de *fitness* foi avaliada através da tolerância aguda e crónica a diferentes stressores ambientais, medidas de balanço energético e mecanismos de defesa após manter as diferentes populações durante várias gerações em condições laboratoriais controlo. Por fim, as respostas microevolutivas de *C. riparius* à contaminação por metais foram comparadas com a diversidade e composição das comunidades de macroinvertebrados. Para determinar a relação de causa-efeito entre respostas microevolutivas e contaminação, os efeitos da poluição por metais foram investigados em diferentes locais historicamente contaminados por metais e comparados com várias referências.

Os resultados demonstraram elevados níveis de diversidade genética e uma considerável homogeneidade genética entre as populações monitorizadas em condições naturais. No entanto, observaram-se evidências de adaptação genética a metais nas populações de locais contaminados, incluindo maior tolerância à exposição aguda por metais e elevados níveis basais de glutationas e metalotioninas que possivelmente aumentam a capacidade de resposta das populações à exposição a metais. Além do mais, observaram-se maiores custos energéticos em populações de locais contaminados quando expostas a metais, enquanto que uma das populações de locais contaminados apresentou também custos de fitness em condições controlo. Finalmente, verificou-se que a diversidade e composição das comunidades de macroinvertebrados dos locais contaminados foi fortemente afetada e muitos grupos taxonómicos sensíveis à contaminação foram eliminados e substituídos por outros mais oportunistas, tais como *C. riparius*.

De um modo geral, as medidas de diversidade genética de populações naturais de *C. riparius* não mostraram ser ferramentas de biomonitorização particularmente vantajosas *per se* uma vez que não refletiram as respostas microevolutivas das diferentes populações à poluição histórica por metais. Tal facto poderá estar relacionado com a elevada densidade populacional e dinamismo da espécie em condições naturais, uma vez que se observou uma considerável perda de diversidade genética quando as populações foram mantidas em laboratório durante períodos de tempo relativamente longos. Não obstante, algumas linhas de evidência do presente trabalho sugerem o uso de medidas de diversidade genética de *C. riparius* em diversas situações experimentais como sejam: deteção de hibridização interespecífica; estabelecimento de níveis mínimos de diversidade genética em laboratório; e, finalmente, uso integrativo de medidas de diversidade genética em programas de biomonitorização com um foco mais direcionado para os efeitos ao nível da comunidade de macroinvertebrados.

Os resultados apresentados pretendem estimular a discussão acerca da adequabilidade de *C. riparius* como espécie modelo em toxicologia evolutiva bem como a sensibilidade e robustez das medidas de diversidade genética como indicadores de qualidade ambiental em avaliação de risco ecológico.

Keywords Evolutionary potential, evolutionary toxicology, genetic adaptation, genetic diversity, genetic erosion, metal contamination, microsatellites.

Abstract

Natural populations inhabiting heavily metal impacted freshwater ecosystems may face intense selection and genetic drift that conduct populations to severe reductions of genetic diversity, the so-called process of genetic erosion. Because the ability of populations to adapt to environmental change is directly related to the levels of genetic diversity, contaminant-driven genetic erosion may threaten the long-term survival of populations. The search for more robust and context-driven bio-indicators that add ecological relevance to the environmental risk assessments has increased interest in evolutionary toxicology and measures of genetic diversity.

The research described in the present thesis was performed with the ultimate goal of understanding whether the levels of genetic diversity of the model ecotoxicological species *Chironomus riparius* (Meigen) may be used as ecological indicators of the health of freshwater systems. For that, an integrative study was undertaken investigating microevolutionary responses of *C. riparius* towards historical metal pollution. This included assessments of levels of genetic diversity as well as determination of genetic adaptation to metals and associated fitness costs. Genetic diversity was estimated based on the variation of seven microsatellite markers. Genetic adaptation and associated fitness costs were investigated through acute and chronic exposures to different environmental stressors, measurements of energy budget and biochemical mechanisms of tolerance to metals, after maintaining populations for several generations under standard laboratory conditions. Microevolutionary responses of *C. riparius* to metal pollution were, afterwards, compared with macroinvertebrate diversity and composition metrics. To draw general conclusions of causal-relationship between microevolutionary responses and pollution history, effects were investigated across multiple metal polluted and reference site.

Globally, the levels of genetic diversity were globally high and there was a remarkable genetic homogeneity among all C. riparius populations in the field. However, *C. riparius* populations from metal polluted sites showed signs of genetic adaptation to metals as suggested by the increased tolerance to acute concentrations of metal and high basal levels of glutathiones and metallothioneins that likely enhance the fitness of populations to cope with metal toxicity. Furthermore, populations from metals contaminated sites had higher energetic costs when exposed to metals and one of the populations from contaminated sites showed also a poorer performance under control clean conditions. Finally, diversity and composition of macroinvertebrate communities from metal polluted sites was strongly affected and many sensitive taxonomic groups were eliminated and replaced by more opportunistic ones such as *C. riparius*.

Overall, measures of genetic diversity of *C. riparius* natural populations do not seem to be particularly advantageous biomonitoring tools *per se* once they did not reflect the underlying microevolutionary responses of natural populations to historical metal pollution. This is likely because of the large population densities together with the highly dynamic nature of *C. riparius* in the field as we observed genetic erosion in population reared under laboratory conditions over relatively long periods of time. However, several lines of evidence indicate that measures of genetic diversity may accrue valuable information in several experimental situations: detection of interspecific hybridization; establishment of minimum levels of genetic diversity in laboratory-reared *C. riparius* populations; finally, integrative use of measures of genetic diversity in biomonitoring programs with more community-level focus.

The results presented in this thesis aim to stimulate discussion on the suitability of *C. riparius* as a model species in evolutionary ecotoxicology studies as well as the sensitivity and robustness of genetic diversity measures as indicators of environmental quality in ecological risk assessment.

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CHAPTER I:

GENERAL INTRODUCTION

1. General introduction

Despite the fact that 75 percent of the Earth's surface is covered by water, less than one percent corresponds to fresh water. Freshwater ecosystems provide, nevertheless, suitable habitat to a disproportionately high biodiversity compared to their reduced spatial extent, amounting up to 10 percent of all known species in the world (Strayer and Dudgeon, 2010). However, the degradation of many freshwater ecosystems across the globe in recent decades and the loss of the biodiversity they support, strongly associated to the expansion of human populations, has increasingly attracted the interest of the scientific community and regulatory authorities (EC, 2006; Strayer and Dudgeon, 2010).

One of the major threats to the health of freshwater ecosystems is chemical pollution which is associated to a vast number of human activities including agriculture, industry, mining and wastewater discharges (Dudgeon et al., 2006; Strayer and Dudgeon, 2010). Environmental risk assessments (ERAs) are the procedures that estimate the likelihood of adverse effects occurring as a result of the release and accumulation of contaminants on natural ecosystems. They combine a component of hazard identification of the contaminants with a component of prediction of fate and exposure and are designed to produce fast and reproducible data, capable of providing simple answers about how contaminants affect natural ecosystems (Breitholtz et al., 2006).

Traditionally, ERAs have relied heavily on reductionist approaches conducted under standardized laboratory conditions that estimate the adverse toxic effects of contaminants based on short-term responses of a reduced number of short-lived, fast growing model organisms that are expected to be representative of the whole ecosystem (Breitholtz et al., 2006). Acute tests are designed for typically 1 or 2 days of exposure period while chronic tests evaluate the adverse effects of contaminants also at very short periods of time that typically range from 10 to 28 days [e.g. (OECD, 2004)]. Although the outcomes of these standardized tests provide valuable information to estimate the potential intrinsic hazard of chemicals, the simplification of the approach constrains the extrapolations to natural conditions (Breitholtz et al., 2006; Fischer et al., 2013). Therefore, they are no longer considered a solution to reliably predict the effects of contaminants under natural conditions and new and more holistic bioindicators that can quickly and inexpensively assess the ecotoxicological effects of contaminants in the dynamics of stressed biological communities are thus needed (Artigas et al., 2012; Beketov and Liess, 2012).

A major drawback of these traditional approaches is that the central role of the genetic diversity in population's response is largely neglected (De Coninck et al., 2014; Fischer et al., 2013) or, instead, seen as a source of undesirable statistical variation (Barata et al., 2002). Indeed, ecotoxicological effects of contaminants are frequently predicted based on dose-responses of clonal organisms such as *Daphnia magna* that reproduce asexually under laboratory favourable conditions (OECD, 2008). Likewise, even when sexually reproducing organisms such as the non-biting midge *Chironomus riparius* are used (OECD, 2004), predictions are often made based on long-standing laboratory populations with low levels of genetic diversity that are not representative of natural populations (Nowak et al., 2007c). As a result, considerable discrepancies in the tolerance towards environmental contaminants have been reported among different laboratory populations of different model ecotoxicological species introducing uncertainty in data interpretation (Barata et al., 2002; Nowak et al., 2007a).

1.1. Genetic diversity

In recent years paradigm shifted and increasing attention has been addressed to the topic of genetic diversity that has been proposed to represent the ultimate biomarker of effect (Bickham et al., 2000).

Genetic diversity constitutes the foundation for all biological diversity in Earth and is one of the three forms of biodiversity, along with species and ecosystems, which has been recommended for conservation and sustainable use by the International Union for Conservation of Nature (McNeely et al., 1990). Genetic diversity is defined as the variety of alleles and genotypes present in a population and provides the raw material for adaptation, evolution and, ultimately, long-term survival of populations and species (Frankham et al., 2002; Hughes et al., 2008). For this reason, high levels of genetic diversity are, in general, seen as indicators of population's health whereas, on the contrary, low levels of genetic diversity are indicative of poor fitness and increased vulnerability to environmental stress, meaning higher extinction risk (Reed and Frankham, 2003). Understanding the factors that influence the rate at which populations lose genetic diversity is, therefore, vital as genetic diversity is required to maintain both the short-term individual fitness and the long-term evolutionary potential of populations to respond to future, unpredictable environmental change (Hughes et al., 2008).

Theoretically, the genetic diversity harboured by a population at a given time depends on the balance between mutations, gene flow, natural selection and genetic drift.

Mutations and gene flow are sources of genetic variation, whereas natural selection and genetic drift act to reduce the genetic diversity of populations (Frankham et al., 2002; Hughes et al., 2008).

Several biotic and abiotic factors such as competition, predation, diseases, weather patterns, seasonal variations or geographic barriers, may influence the amount and distribution of genetic variation within and among populations (Frankham, 2005; Wang et al., 2013). However, empirical evidence has accumulated during the recent years showing that environmental contaminants may constitute highly disruptive selection pressures with the potential to impose dramatic losses of genetic diversity within very short periods of time (Athrey et al., 2007; Nowak et al., 2009; Ward and Robinson, 2005). Because this so-called process of contaminant-driven genetic erosion may contribute to major population declines and accelerate the extinction risk of populations, subtle effects of environmental contaminants in the genetic diversity of impacted populations must be taken into account in order to better predict their long-term effects and increase the ecological realism of ERAs (De Coninck et al., 2014; van Straalen and Timmermans, 2002).

Given the above, considerable efforts have been made to comprehensively understand how contaminants shape the overall levels of genetic diversity of populations, which evolutionary consequences they produce and in which manner changes in the genetic diversity of populations affect their susceptibility to environmental stress (Fasola et al., 2015; Morgan et al., 2007; Ribeiro and Lopes, 2013; van Straalen and Timmermans, 2002). Mounting evidence in this field has resulted in the emergence of evolutionary toxicology as a new research discipline that integrates the fundamental population genetics theories in risk assessment and whose purpose is to assess the long-term evolutionary impacts of contaminants in population's health over timescales that exceed the generation time of the standard ecotoxicological tests (Coutellec and Barata, 2011; Klerks et al., 2011).

1.2. Effects of environmental contaminants on the genetic diversity of populations

Environmental contaminants may impact the genetic diversity of populations owing to genetic drift and natural selection. The relative importance of these processes, however, depends largely upon the size of the exposed population. Whilst in small populations loss of genetic diversity is more likely to occur due to processes of genetic drift, natural

selection will prevail in larger populations (Ribeiro and Lopes, 2013; van Straalen and Timmermans, 2002).

Genetic drift is the process by which random fluctuations of gene frequencies occur in finite populations (Frankham, 1996). Therefore, genetic drift is expected to play a major role in the processes of genetic erosion of small populations particularly in cases that contaminant-exposure causes the elimination of a considerable portion of the population and, thereby, alleles are either fixed or lost in the population by chance events. Such a situation will lead to a reduction of the genetic diversity and will also augment the likelihood of inbreeding within the population. As a result, the expression of recessive deleterious alleles will likely increase which might depress the individual fitness and reduce survival and fecundity, leading to successive losses of genetic diversity and reductions in population densities in a downward spiral that may conduct to local extinction (Frankham, 2005; Reed and Frankham, 2003).

On the other hand, genetic diversity may also be altered due to selection for tolerant alleles that allow genetic adaptation of populations to the environmental adverse conditions. In this case, alleles that accrue fitness advantages and improve the ability to cope with contaminants will become more frequent within the population while the most sensitive ones will tend to be eliminated over generations (Medina et al., 2007; Ribeiro and Lopes, 2013; van Straalen and Timmermans, 2002). Such selection for increased tolerance may occur on pre-existing allelic variation or, alternatively, on alleles that appear through mutation events (Barrett and Schluter, 2008). However, according to Bickham et al. (2000) “there is only a small chance that a new mutation will increase the fitness of a population” (Bickham et al., 2000). In fact, while mutations in the non-coding region of the DNA may be eliminated or fixed by chance and so they may constitute an effective source of new genetic variation, most of the mutations in the coding region of the DNA are deleterious and, thereby, will be rapidly removed from the genetic background of the population (Bickham et al., 2000). This situation may be illustrated, for instance, by the work of Ellegren et al. (1997) who found increased germline mutation rates in a population of the barn swallow *Hirundo rustica* collected from Chernobyl in comparison to two reference populations. Authors found an increased frequency of partial albinism in the the exposed population that associated, however, with a lower probability of survival (Ellegren et al., 1997).

Although genetic adaptation to contaminants may increase the likelihood of short-term survival under such challenging environmental conditions, theory on adaptive changes predicts that the evolution of tolerance may carry detrimental fitness costs involving

physiological and/or genetic mechanisms that lower the fitness of genetically adapted populations under environmental changing conditions (Ribeiro and Lopes, 2013; van Straalen and Timmermans, 2002). Physiological costs are expected to occur because investment in enhanced defence mechanisms that confer better protection against contaminants is energy-costly and, thereby, the additional energy requirements may divert energy from fitness-related traits (such as growth and reproduction) and impose an unnecessary burden in the absence of the toxic stimulus (Klerks et al., 2011; Ribeiro and Lopes, 2013; Sokolova et al., 2012). Furthermore, fitness costs may also arise from either epistasis or antagonistic pleiotropy associated to negative genetic interactions between alleles that confer tolerance and fitness-related traits (Ribeiro and Lopes, 2013).

These evolutionary changes will depend, ultimately, on the relative genetic isolation of exposed populations. Extensive migration and gene flow from adjacent unexposed populations is normally expected to overcome the effects of genetic drift and natural selection and, thus, restore the levels of genetic diversity needed for the maintenance of demographic and evolutionary stability of populations (Chung et al., 2012; Medina et al., 2007).

1.3. Molecular markers of genetic diversity

Advances in molecular techniques allowed for robust and cost-effective assessments of populations' genetic diversity and structure and, for these purposes, neutral markers (i.e. sequences of DNA that are not under direct selection and, thus, have no expectable influence in the fitness) have been the markers of choice. They produce data sets that can be objectively analysed and used to determine the influence that main ecological and spatial factors have on the genetic structure and reproductive relationships of individuals, populations and, ultimately, species (Hoffmann and Willi, 2008a; Mussali-Galante et al.). By examining how genetic variation is partitioned within and among populations, they provide valuable information about the demographic and evolutionary history of populations such as the effective population size, degree of inbreeding, migration patterns, genetic bottlenecks or phylogenetic trees (Frankham, 2010; Hoffmann and Willi, 2008a).

Neutral markers have been used extensively in breeding programs and conservation management of many endangered and emblematic species (Andersen et al., 2015; Casas-Marce et al., 2013), to resolve taxonomic uncertainties (Ball et al., 2016; Pfenninger et al., 2007) and for phylogenetic purposes (Winkler et al., 2015; Zhao et al.,

2013). In evolutionary toxicology, neutral markers have been employed to understand how environmental contaminants lead to effective reductions of population sizes and/or conduct exposed populations to reproductive isolation in such a way they allow to document and anticipate long-term and evolutionary consequences of contaminant exposure (Paris et al., 2015; Santos et al., 2013).

A variety of different techniques is available to assess genetic diversity which differ in the way the genome is sampled and type of data they produce. Here, however, we will only refer to allozymes, Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP) and Microsatellites as they are among the most commonly used neutral markers in evolutionary toxicology studies. Briefly, allozymes are rapid and low cost co-dominant markers (i.e. distinguish heterozygotes from homozygotes) that detect genetic variation indirectly through protein variants in the same locus. However, the low degree of polymorphism is often referred as a major negative aspect of its use (Hedrick, 1999). RAPDs measure genetic variation based on the amplification of random DNA fragments produced by an arbitrary primer. They are also inexpensive and rapid to perform and do not require DNA sequence information of the genome of the organism. The major drawback of RAPDs is, however, its dominant nature that does not allow distinguishing homozygotes from heterozygotes and the low reproducibility of the DNA fragments (Sukumaran and Gopalakrishnan, 2015; Williams et al., 1990). RFLPs measure variation in the mtDNA sequences of restriction sites that are obtained by the cleavage of endonucleases. Although RFLPs are highly repeatable, major drawbacks are the fact that mtDNA is maternally inherited and the few loci screened per assay (Saiki et al., 1985; Sukumaran and Gopalakrishnan, 2015). AFLPs combine the strengths of RFLP and RAPD techniques. They measure genetic variation of DNA fragments that are digested by restriction endonucleases and amplified with enzymatic adapter ligands. AFLPs do not require sequence information but are more polymorphic, reproducible and robust compared to RAPD analysis (Sukumaran and Gopalakrishnan, 2015; Vos et al., 1995). Finally, Microsatellites are short DNA sequences of typically 2-5 base pairs that are repeated several times throughout the genome of the organism. Although these co-dominant markers require an initial effort and cost to develop specific primers for the species under study, they have become one of the most popular markers because of their hypervariability and abundance which allows the identification of patterns of genetic structure that are not possible to determine when using less polymorphic markers (Jarne and Lagoda, 1996; Sukumaran and Gopalakrishnan, 2015).

1.3.1. Application of neutral markers in evolutionary toxicology studies

Laboratory cases

The potential of environmental contaminants to alter the patterns of genetic variation of freshwater biota has been demonstrated by a number of multigenerational laboratory studies with both invertebrate and vertebrate aquatic species. For instance, Athrey et al. (2007) investigated the effects of eight generations of cadmium (Cd) selection in the overall levels of genetic diversity of the least killifish *Heterandria formosa* using seven Microsatellite markers and observed that the Cd-exposed groups had 10-20% lower genetic diversity compared to their respective controls (Athrey et al., 2007). Similarly, Ward and Robinson (2005) found higher loss of genetic diversity in a *Daphnia magna* population exposed to Cd during eight generations in comparison to populations maintained under control clean conditions. This loss of genetic diversity, assessed through AFLP markers, was accompanied by a three-fold increase of Cd tolerance which was attributed to an increased frequency of Cd-tolerant genotypes. No differences in the life-history endpoints life span, offspring production, time to first brood, number of offspring in the first brood or intrinsic population growth rate were found between the Cd-selection and control populations under clean conditions or under exposure to elevated temperatures or low food regimens but adults of the Cd-selection population were smaller compared to controls. The Cd-selection and control populations were equally sensitive to copper and malathion. However, the Cd-selection population was more tolerant to lead and more sensitive to phenol. Taken the results together, authors concluded that the increase in Cd-tolerance had a cost: loss of genetic diversity, reduced adult size and increased susceptibility to environmental changing conditions (i.e. phenol). Thus, although increased Cd-tolerance would allow persistence in metal-contaminated areas, the associated costs could represent an increased risk of population extinction and, thus, potentially compromise the long-term survival of the tolerant population under changing environmental conditions (Ward and Robinson, 2005).

Not all multigenerational selection experiments showed, nonetheless, contaminant-driven genetic erosion. For instance, Vogt et al. (2007a), using Microsatellite markers, failed to detect major losses of genetic diversity in the non-biting midge *Chironomus riparius* exposed during 11 generations to low TBT concentrations (Vogt et al., 2007a). These results contrasted with another multigenerational experiment in which genetic erosion in *C. riparius* was observed after 12 generations of TBT selection using the same Microsatellite markers (Nowak et al., 2009). These last authors registered increased mortality, delayed time to emergence and lower reproductive output in the TBT-selection

populations compared to the control populations but no clear evidences for increased TBT-tolerance. Authors concluded that genetic erosion had occurred mainly due to random genetic drift rather than due to selection for TBT-tolerant genotypes. Furthermore, the different effects that TBT had on the genetic diversity of *C. riparius* populations in the two different multigenerational experiments, even using the same molecular markers, were attributed to the combination of higher TBT concentrations and lower initial levels of genetic diversity in the later study. Therefore, the contrasting results of these two studies highlight that besides the importance of the intensity of the toxic stress, also the initial levels of genetic diversity harboured by populations play a decisive role in their responsiveness to environmental stress.

Indeed, whereas environmental contaminants have been shown to affect the levels of genetic diversity of exposed populations, there is also mounting evidence showing that reduced genetic variability may hamper population's responsiveness to environmental stress. This is well illustrated by a number of studies performed with lab populations of *C. riparius*. For example, Nowak et al. (2012) tested the effects of 11 generations of TBT-selection in two strains of *C. riparius* differing in their levels of genetic diversity and found that the outbred and genetically diverse strain was not clearly affected by TBT toxicity while, on the contrary, the genetically eroded and inbred strain showed a reduced population growth rate when exposed to TBT (Nowak et al., 2012). Similarly, Nowak et al. (2007a, 2008) also observed that genetically eroded and inbred laboratory populations of *C. riparius* were more adversely affected by Cd exposure than genetically diverse and outbred populations (Nowak et al., 2008; Nowak et al., 2007a).

Field cases

Going through the literature, one finds that most of the research on the effects of contaminants on the genetic diversity under natural freshwater ecosystems has focused on fish populations while fewer have explored the effects of environmental contaminants on invertebrates. Furthermore, the number of studies providing clear evidences for contaminant-driven genetic erosion is still limited. Among these, Paris et al. (2015) assessed the genetic diversity and structure of 15 populations of *Salmo trutta* collected from several reference and metal contaminated sites with a past-history of mining activity in the southwest of England. Employing Microsatellite markers, authors found that populations from metal contaminated sites were genetically less diverse compared to reference populations. Furthermore, they also found that populations from metal contaminated sites were genetically distinct from the references and also genetically

distinct from one another. Indeed, reference populations constituted a more homogeneous genetic group compared to populations from metal contaminated sites, despite the latter populations being geographically closer to each other without physical barriers in between. Loss of genetic diversity and increased genetic differentiation in metal-exposed populations was attributed to population bottlenecks, impairments of gene flow and extensive genetic drift. Also selection for tolerant genotypes was advanced as a possible explanation for the obtained outcomes, however, authors did not investigate genetic adaptation to metals in their study (Paris et al., 2015).

Also Kopp et al. (1992) found significantly lower levels of genetic diversity and consistent genotypic shifts (assessed at allozyme markers) in populations of mud minnows (*Umbra limi*) inhabiting a river segment of the Moose river (New York) impacted with low pH and high aluminium concentrations in comparison to reference populations (Kopp et al., 1992). Likewise, Murdoch and Hebert (1994), using RFLP markers, found that the genetic diversity of the brown bullhead *Ameiurus nebulosus* was consistently lower in contaminated sites compared to the selected references. Such genetic impoverishment was attributed to past stochastic reductions of population sizes (Murdoch and Hebert, 1994). Evidences of contaminant-driven genetic erosion were also reached by Maes et al. (2005) who observed a reduction of the genetic diversity of the eel *Anguilla anguilla* (at allozyme markers) with increasing metal contamination (Maes et al., 2005) or by Bourret et al. (2008) who found a negative correlation between the levels of genetic diversity of the yellow perch *Perca flavescens* (assessed at Microsatellite markers) and the bioaccumulation of Cd in the liver (Bourret et al., 2008). Krane et al. (1999) also found consistent lower levels of genetic diversity (assessed at RAPD markers) in rusty crayfish populations (*Orconectes rusticus*) collected from stream sediments with high concentrations of polycyclic aromatic hydrocarbons and/or polychlorinated biphenyl in comparison to populations collected from reference sites (Krane et al., 1999). Finally, in a study performed on the freshwater snail *Pleurocera canaliculatum* collected along a gradient of mercury (Hg) contamination, authors found altered genotype frequencies and lower heterozygote frequency at allozyme markers as well as increased DNA damage in snail populations located downstream the Hg-contamination hotspot (Benton et al., 2002).

In contrast with the above studies, no evidences for contaminant-driven genetic erosion were found by Santos et al. (2013) in populations of the three-spined stickleback *Gasterosteus aculeatus* collected from historically contaminated sites with industrial and/or domestic effluents compared to populations from uncontaminated sites. Absence of genetic erosion in exposed populations, assessed through Microsatellite markers, was

attributed to four major reasons: large effective population sizes, few genes involved in a potential genetic adaptation to contaminants that would not affect the overall levels of genetic diversity, extensive gene flow from uncontaminated sites and short generation times. However, authors found signs of genetic bottlenecks in populations from contaminated sites which contrasted with no or only weak signs of genetic bottlenecks in the two reference populations (Santos et al., 2013). Similarly, Knapen et al. (2009) found no signs of genetic erosion in stone loach populations (*Barbatula barbatula*) inhabiting chronically contaminated freshwater courses of Flanders using Microsatellite markers. Authors observed high levels of genetic diversity among all assessed populations, no evidences of recent genetic bottlenecks and the estimated effective sizes of all populations were very large. They argued that the little impact that contaminants had on the populations genetics would be due to the unique features of the species, namely the great ecological plasticity and the high tolerance to poor water quality conditions (Knapen et al., 2009). Likewise, Coors et al. (2009) did not observe significant effects in the levels of genetic diversity (at allozyme markers) of populations of the water flea *Daphnia magna* collected in ponds located along a gradient of agricultural land use. However, authors found a positive association between the acute tolerance to the pesticide carbaryl and the agricultural land use that was indicative of genetic adaptation to pesticides (Coors et al., 2009). Finally, Martins et al. (2009), investigating the effects of acid mine drainage in the populations genetics of *Daphnia longispina* residing the abandoned S. Domingos mining area (Portugal), found no differences in the levels of genetic diversity (at AFLP markers) and only weak genetic differentiation between the population collected from the impacted site and the two selected reference populations. While effects were hardly observed in the assessed neutral markers, authors found that within the impacted population there was a high frequency of tolerant and very tolerant organisms whereas, on the contrary, the percentage of tolerant and very tolerant organisms was low in the reference populations. Authors argued that the maintenance of high levels of genetic diversity in the impacted population would be due to a combination of: high gene flow from reference populations, existence of standing genetic diversity in dormant resting eggs, few genes involved in metal tolerance and, finally, loss of genetic diversity caused by metal contamination would be of the same magnitude of that caused by clonal frequency in the reference populations (Martins et al., 2009).

Whitehead et al. (2003) did not find any evidence of genetic impoverishment or increased genetic structuring (at both AFLP and Microsatellite markers) in populations of the endemic fish *Catostomus occidentalis* inhabiting several pesticide-contaminated sites located across two river basins in the Central California Valley, USA. However,

authors observed that, regardless of pesticide contamination, downstream populations had higher levels of genetic diversity in comparison to upstream populations (Whitehead et al., 2003). Similarly, Miller et al. (2012) did not observe any signs of genetic impoverishment or genetic structuring (assessed at Microsatellite markers) in populations of the walleye *Sander vitreus* inhabiting sites in the Upper Mississipi river (USA) contaminated with endocrine active compounds. However, they found a significant effect of geography in population genetics with populations located above the natural barrier of St. Anthony Falls being genetically distinct from populations located below the Falls (Miller et al., 2012).

Collectively, the reported studies show that generalization of contaminant-driven genetic erosion from one species to another is unlikely and the question whether the patterns of genetic variation of natural populations can be altered by contaminants in natural environments is complex and likely dependent on a number of interacting factors. These include intensity and duration of toxic stress, population size and dispersion capability and ecological features of the species under scrutiny. Furthermore, additional natural factors such as physical barriers may also affect the patterns of genetic variation of populations and further complicate the interpretation of the outcomes. In this sense, a common criticism to the application of neutral markers in evolutionary toxicology is that the detection of adverse effects of contaminants is highly dependent on reductions of the effective population sizes of exposed populations. Therefore, if such stochastic events do not occur, then effects will only be detectable using markers that are directly under selection. Due to this, it has been highly recommended a combination between neutral and selective markers in order to better understand the evolutionary effects of environmental contaminants (Hoffmann and Willi, 2008b).

1.4. *Chironomus riparius* (Meigen) as a model evolutionary toxicology species

The non-biting midge *Chironomus riparius* belongs to the Family Chironomidae, an ecologically important group of benthic freshwater invertebrates. Chironomids constitute one of the most productive, ubiquitous and diverse groups of benthic invertebrates in freshwater habitats in which they spend most of their lifetime as the aquatic larval stage (Armitage et al., 2012; Ferrington Jr, 2008). They are a major prey item for the diet of many invertebrate and vertebrate predators and play also an important role in detritus processing and organic matter cycling of limnic systems. Furthermore, *C. riparius* is a locally abundant opportunistic species that naturally occurs in a wide range of freshwater

habitats throughout the Holarctic (Armitage et al., 2012), including heavily contaminated areas from where other competitors and predators are often excluded (De Haas et al., 2005; Postma et al., 1995a). Larvae of *C. riparius* are tube-dwelling deposit feeders that live closely associated to the sediments where environmental contaminants accumulate more and where they burrow their cases and feed on particulate matter (Armitage et al., 2012).

C. riparius is also a model species in freshwater ecotoxicology and has been extensively used to assess the effects of stressors of environmental concern (Campos et al., 2014; Rodrigues et al., 2015). The species is sensitive to environmental stress, has short life-cycles, high reproductive output, small size, it is easy to culture under common-garden laboratory conditions and there are standard methods described to perform ecotoxicological assessments (OECD, 2004).

Given the above, *C. riparius* was chosen for the present study as it accomplishes basic ecological and ecotoxicological criteria that make the species particularly suited for evolutionary toxicology studies addressing the detrimental long-term consequences of environmental contaminants in freshwater ecosystems. Furthermore, earlier studies using laboratory-reared populations demonstrated that genetically eroded and inbred *C. riparius* populations may have reduced fitness and increased susceptibility to environmental pollution, i.e. lower evolutionary potential (Nowak et al., 2008; Nowak et al., 2007b). Likewise, contaminant-driven genetic erosion has been shown to occur within only few generations (Nowak et al., 2009). Finally, it has also been shown that *C. riparius* natural populations may genetically adapt to environmental contaminants (Postma et al., 1995a; Postma et al., 1995b). Altogether, these laboratory and field evidences emphasize the potential suitability of *C. riparius* as a model organism in evolutionary toxicology studies and the usefulness of measures of neutral genetic diversity of the species as bioindicators of ecological disturbance.

2. Objectives and outline of the thesis

Information on the long-term effects of contaminants in freshwater ecosystems is still scarce and scattered and, to date, comprehensive studies that simultaneously explore the long-term consequences of environmental pollution in the levels of neutral genetic diversity with populations' tolerance towards contaminants are rare, especially in freshwater ecosystems. Furthermore, while contaminant-driven genetic erosion has been demonstrated in several laboratory studies (Athrey et al., 2007; Nowak et al., 2009),

few studies have provided clear evidences of genetic erosion under real-field scenarios (Maes et al., 2005; Paris et al., 2015). Therefore, the degree at which environmental contaminants lead to evolutionary consequences in natural freshwater populations is not sufficiently well documented.

The research work described in this thesis aims to contribute with empirical foundation to the comprehensive understanding of the long-term evolutionary effects of historical metal contamination on natural freshwater biota. Here, an integrative study was undertaken, investigating the long-term effects of metal contamination across different levels of biological organization. Responses of natural populations of *C. riparius* towards historical metal contamination were determined in terms of genetic diversity (using Microsatellite markers) as well as in terms of microevolution of metal tolerance and associated fitness costs. Finally, the effects of historical metal contamination in terms of genetic diversity of *C. riparius* natural populations were related to the diversity of macroinvertebrate communities.

To draw general conclusions of causal-relationships between biological responses and contamination history, effects of metal contamination were investigated in multiple metal contaminated and reference sites located in the northern-centre part of Portugal. The research work presented in this thesis aimed to address the following fundamental questions:

- Does long-term metal contamination affect the overall levels of neutral genetic diversity of natural populations?

- Are *C. riparius* populations from metal contaminated areas adapted to metal contamination? And if so, are there fitness costs associated?

- Does genetic diversity affect population's susceptibility to other environmental stressors?

These questions were formulated with the ultimate goal of understanding whether neutral genetic diversity of *C. riparius*, measured in microsatellite markes, may be used as a suitable endpoint to biomonitor environmental disturbance of natural freshwater ecosystems.

The second chapter of the thesis (Chapter 2) investigates genetic erosion and metal adaptation caused by long-term metal contamination of freshwaters in *C. riparius*. For that, *C. riparius* populations were sampled and established in the laboratory as

permanent cultures: three populations were sampled from historically metal contaminated sites and three populations were sampled from nearby located reference sites. The patterns of neutral genetic diversity were estimated in the F0 generation using seven Microsatellite markers. Tolerance to cadmium (Cd) was investigated by performing acute (48-h immobilisation tests) and chronic (28-day partial life-cycle tests) laboratory standard tests after rearing all six populations for at least six generations under common-garden laboratory clean conditions in order to eliminate potentially confounding factors from acclimation to field conditions.

Since stress response is an energy-costly process that involves the mobilization of defense and repair mechanisms, differences in Cd tolerance between *C. riparius* populations from metal contaminated and reference sites should, ultimately, reflect differences at the sub-cellular level. In the third chapter of this thesis(Chapter 3), a biomarker approach was performed in order to comprehensively understand the differences in Cd-tolerance between populations. To this end, the two populations from metal contaminated sites with the highest acute Cd tolerance and the two most Cd-sensitive populations from reference sites were investigated for their protein expression profiles, levels of oxidative damage, cellular defences (including levels of glutathione, metallothioneins and enzymatic defences) and energy-related parameters after short-term Cd exposures.

The following chapter (Chapter 4) investigates whether a *C. riparius* population inhabiting a Hg-contaminated site evolved tolerance to Hg and whether there were fitness costs of tolerance under environmental changing conditions (i.e. salinity stress). Additionally, we also investigated the role of genetic diversity on the vulnerability of midge populations to Hg and salinity stress. For this purpose, the acute and chronic tolerance to Hg and salinity stress was experimentally assessed in four populations varying in their past-histories of selection pressures and levels of genetic diversity: two natural populations collected from a Hg-contaminated site and from a closely located reference site, one laboratory population and one "Gen^{+} population" generated from the cross-breeding of several *C. riparius* populations. The levels of genetic diversity of the two natural populations under common-garden laboratory conditions were also monitored in order to assess the degree of genetic impoverishment over time with laboratory rearing.

The fifth chapter (Chapter 5) investigates the importance of metal contamination, geographic isolation and temporal variation in the patterns of genetic diversity and genetic differentiation of *C. riparius* and its sibling sister species *Chironomus piger*.

Additionally, it is also investigated the genetic distinctiveness between the two species in the field. For that, larvae of *C. riparius* and/or *C. piger* were collected over a four-year period from a total of 18 sampling sites (5 river basins, NW of Portugal) varying in the degree of metal contamination. Seven Microsatellite markers were used to characterize patterns of genetic variation within and among species.

The sixth chapter (Chapter 6) investigates the suitability and robustness of measures of genetic variation of *C. riparius* natural populations as indicators of ecological disturbance of freshwater ecosystems. For that, the effects of historical metal contamination in the levels of genetic diversity of *C. riparius* were assessed and compared to those effects occurring at the community level. Genetic diversity of *C. riparius* populations was estimated based on the variation of Microsatellite makers. Community-level effects were assessed based on benthic macroinvertebrate community composition and diversity metrics.

Finally, the major findings and implications of the research work described in the present thesis as well as the implications for evolutionary toxicology studies and environmental risk assessment are discussed in the last chapter (Chapter 7 – General Discussion).

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CHAPTER II:

EVOLUTIONARY CONSEQUENCES OF HISTORICAL METAL CONTAMINATION FOR NATURAL POPULATIONS OF *CHIRONOMUS RIPARIUS* (DIPTERA: CHIRONOMIDAE)

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Abstract

Populations inhabiting metal-impacted freshwater systems located nearby industrial and urban areas may be under intense selection. The present study aims to address two fundamental microevolutionary aspects of metal contamination in the midge *Chironomus riparius* (Meigen): Are populations inhabiting historically metal contaminated sites genetically adapted to metals? And, are populations from these sites genetically eroded?

To answer these questions, *C. riparius* populations were sampled from three sites with well-known histories of metal contamination and three nearby-located references. Genetic adaptation to metals was investigated through acute and chronic exposures to cadmium (Cd), after rearing all populations for at least six generations under laboratory clean conditions. Genetic diversity was estimated based on the allelic variation of seven microsatellite markers.

Results showed higher acute tolerance to Cd in populations originating from metal contaminated sites compared to their respective references and significant differences in two out of three pairwise comparisons. However, there was a mismatch between acute and chronic tolerance to Cd with results of the partial life-cycle tests suggesting fitness costs under control clean conditions in two metal-adapted populations. Despite no evidences of genetic erosion in populations sampled from metal contaminated sites, our results suggest genetically inherited tolerance to Cd in populations inhabiting historically contaminated sites.

These findings lend support to the use of *C. riparius* as a model organism in evolutionary toxicology and highlight the importance of coupling measures of neutral genetic diversity with assessments of chemical tolerance of populations for a better understanding of contaminant-induced adaptation and evolutionary processes.

Keywords: Cadmium, freshwater, genetic diversity, invertebrates, metal adaptation, metal contamination.

1. Introduction

Anthropogenic contaminants have the potential to cause major detrimental effects on the health of aquatic biota (Campos et al., 2016; Rodrigues et al., 2015). However, a considerable number of natural populations may still persist in highly contaminated areas by physiologically acclimating and/or genetically adapting to contaminants (Janssens et al., 2009; Medina et al., 2007; Ribeiro and Lopes, 2013). Physiological acclimation is a reversible adaptive response that occurs at the individual level and organisms acquire tolerance through their exposure to contaminants (Adeyemi and Klerks, 2013; Farwell et al., 2012; Klerks et al., 1997). Genetic adaptation, in turn, involves changes in the genetic background and evolution of tolerance at the population level with selection of the most tolerant genotypes and elimination of the most sensitive ones (Agra et al., 2010; Lopes et al., 2004; Ward and Robinson, 2005; Xie and Klerks, 2003). Understanding whether resistance to chemicals is of transient nature or, instead, has a genetic basis that will persist over time even after the selective force is removed is critical as the later adaptive response may carry substantial fitness costs that lower the performance of populations under environmental changing conditions (Agra et al., 2010; Kliot and Ghanim, 2012; Salice et al., 2010; Xie and Klerks, 2004b).

In addition to the direct fitness costs, if population-level processes of genetic adaptation to contaminants are not counterbalanced by new genetic input, exposed populations may experience reductions in their levels of genetic diversity, the so-called process of contaminant-driven genetic erosion (Fasola et al., 2015; Ribeiro and Lopes, 2013; van Straalen and Timmermans, 2002). In this regard, small and isolated populations are expected to be particularly prone to genetic erosion since the probability of genetic drift and the increasing expression of recessive deleterious alleles is higher which, in turn, may conduct populations to further losses of genetic diversity and reduced reproductive fitness, thus, accelerating the risk of population extinction (Frankham, 2005; Reed and Frankham, 2003). Because the ability of natural populations to evolve and adapt to future environmental disturbance is directly related to the amount of genetic diversity (Frankham, 2005; Frankham, 2015), genetic erosion may compromise the long-term survival of populations.

During recent years, surveys of genetic diversity have gained increasing interest in evolutionary toxicology studies and neutral markers such as amplified fragment length polymorphisms or microsatellites (besides others) have been employed to understand and predict long-term ecological impacts of environmental contaminants (van Straalen

and Timmermans, 2002). By examining how genetic variation in the non-coding region of DNA (that is not expected to be under direct selection) is partitioned within and among populations, neutral markers allow to assess inferences about the current status, dynamics and past-history selection pressures acting on populations (Hoffmann and Willi, 2008).

Although their suitability to assess the long-term consequences of contaminants has been demonstrated under laboratory context (Athrey et al., 2007; Nowak et al., 2009), only few studies have provided clear evidence of contaminant-driven genetic erosion under natural conditions (Maes et al., 2005; Paris et al., 2015). Laboratory studies showed, for example, that exposure to tributyltin reduced genetic diversity of *C. riparius* populations within only few generations as a consequence of several life-history alterations including increased mortality and reduced fertility (Nowak et al., 2009). Similarly, laboratory populations of the least killifish *Heterandria formosa* showed 10-20% lower genetic diversity than control populations following eight generations of cadmium selection (Athrey et al., 2007). However, these laboratory evidences contrasted with no signs of genetic erosion in natural populations of the daphnid *Daphnia longispina* historically exposed to acid mine drainage (Martins et al., 2009), the killifish *Fundulus heteroclitus* inhabiting heavily contaminated urban harbors (McMillan et al., 2006) or the walleye *Sander vitreus* residing river segments contaminated with endocrine active compounds (Miller et al., 2012). Investigating genetic erosion during and after polluting events is difficult since genetic diversity of field populations is highly dynamic and is modulated by a multitude of interrelated biotic and abiotic components (Frankham, 2005; Moe et al., 2013; Pauls et al., 2013).

The non-biting midge *Chironomus riparius* Meigen is a model ecotoxicology species (OECD, 2004a, b) that naturally occurs in many freshwater ecosystems throughout Europe (Oppold et al., 2016). Chironomids are one of the most productive, ubiquitous and diverse taxonomic groups of benthic invertebrates inhabiting freshwater systems (Armitage et al., 2012; Ferrington Jr, 2008). Apart from being a major food resource for many invertebrates and vertebrates, it also plays a key role in nutrient cycling and organic matter processing of limnic systems (Armitage et al., 2012; Ferrington Jr, 2008). Furthermore, the benthic habits of the species along with the short life-cycles and ease rearing under laboratory conditions (OECD, 2004a), render *C. riparius* an ideal candidate to assess the long-term, evolutionary consequences of environmental contaminants in freshwater systems.

Integrative investigations that mechanistically link contamination under real-field scenarios to levels of genetic diversity of natural populations and genetic adaptation to contaminants are, to date, scarce, particularly in freshwater systems (Coors et al., 2009; Ribeiro et al., 2012). In this sense, the present study aims to address two fundamental microevolutionary aspects of historical metal contamination in the non-biting midge *C. riparius*: 1) Are *C. riparius* populations from metal contaminated sites genetically adapted to metals? 2) Does historical metal contamination reduce the genetic diversity of *C. riparius* populations?

To answer these questions, we sampled a total of six *C. riparius* populations using a pairwise design approach in a way that for each metal contaminated site, a reference site was also sampled within the same geographical area. This approach allows the separation of population effects due to historical metal contamination from those related with differences among the river systems in terms of, for instance, geochemical and climatic features that may differently influence the life-history traits of Chironomids and result in divergent evolutionary trajectories. Indeed, when investigating patterns of genetic variation in *C. riparius* populations using a much larger number of sampling sites, we found a significant effect of genetic isolation by geographic distance which indicates limited dispersal capability and genetic drift with increasing geographic distance and supports this experimental design (Pedrosa et al., *in press*).

The genetic basis of metal tolerance was investigated through laboratory testing of acute and chronic tolerance towards cadmium (Cd) after maintaining all populations for at least six generations under common-garden laboratory conditions. The patterns of genetic variation within and among populations were estimated using seven microsatellite markers.

2. Material and methods

2.1. Study area and selected populations

Chironomus riparius populations from historically metal contaminated and uncontaminated sites were sampled from three distinct geographical areas located in the Ave and Vouga river basins, northern Portugal (Figure 1; Supplementary Material S1).

The Ave basin consists in a drainage area of approximately 1388 km^2 with the Vizela river on the left bank (length: 47 km, area: 240 km^2) and the Este river on the right bank

(length: 51 km, area: 245 km^2) being the most important tributaries. Its middle and lower parts are significantly deteriorated mainly due to the presence numerous textile industries and other industries that during decades discharged their loads directly in the local streams without previous treatment (Gonçalves et al., 1992; Soares et al., 1999). Within the basin, the Este river is of major concern since intense urban and industrial pressures of the city of Braga resulted in the accumulation of high levels of Cd, Cu, Ni, Pb and Zn in the river sediments. The selected metal contaminated site in the Este river (site C_1) is located in the village of Celeirós, downstream the city of Braga, in which previous studies recorded Cd concentrations in the sediments of 5.96 – 144.00 mg/kg (Soares et al., 1999). Reference site R_1 is located in the village of São Mamede de Este, ca. 8 km upstream the contaminated site, near the source of the river and no past-history of metal contamination is known (Soares et al., 1999).

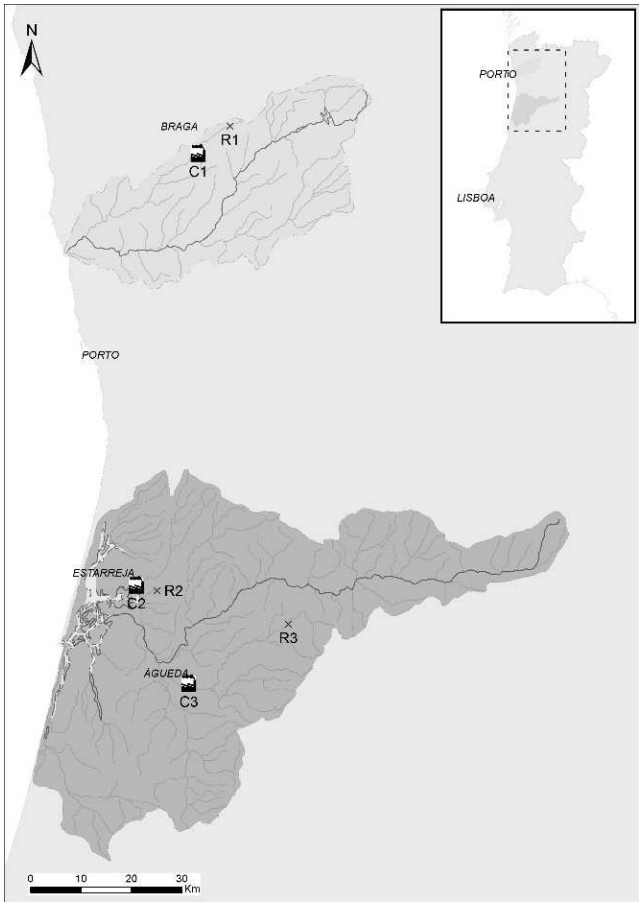

Figure 1 – Location of the metal contaminated (C_1, C_2, C_3) and reference (R_1, R_2, R_3) sites from where *C. riparius* populations were collected.

The Vouga basin consists in a catchment area of approximately 3362 km^2 and its main tributaries are the Sul, Caima, Antuã and Águeda rivers. It connects with the Atlantic Ocean through the vulnerable slow-flowing Ria de Aveiro lagoon which consists

in four main channels and several branches. S. Filipe Ditch (site C_2) is a manmade water stream that was constructed to carry effluents from the Chemical Industrial Complex of the Municipality of Estarreja to the Estarreja branch of Ria de Aveiro lagoon. From 1950 until 1975, the ditch received direct discharges from several chemical industries, including loads from a chlor-alkali electrolysis plant that released approximately 33 tons of mercury and other metals and metalloids associated to the industrial activities (Costa and Jesus-Rydin, 2001; Inácio et al., 1998). For this reason, the area is still highly contaminated with Hg as well as with As, Cd, Cr, Cu, Ni, Pb and Zn and the Cd concentrations in the sediments can reach up to 2.0 mg/kg (Lopes et al., 2014; Martins et al., 2011). The reference site R_2 is located in the Jardim river, in the village of Porto de Baixo, 4 km south-eastern from site C_2.

The Águeda river (length: 54 km; area: 433 km^2) is the largest tributary of the Vouga river. Its western area, located in the Municipality of Águeda, has an important Industrial Complex whose residuals are discharged to the Águeda river by several streams. Among these, Vale da Erva stream (site C_3) has previously been shown to be impacted by several metals (namely Cd, Cr, Cu, Ni, Pb and Zn) with Cd concentrations in the sediments reaching up to 2.0 mg/kg (Reis et al., 2005). The Alfusqueiro river (site R_3), situated in the village of Cercosa, 23 km eastern from metal contaminated site C_3, was selected as a reference site.

2.2. Establishment of pure *C. riparius* populations in the laboratory

In order to establish pure *C. riparius* cultures in the laboratory, a step-wise genetic-based procedure was followed. Larvae of *Chironomus* sp. were sampled from each of the six sites with the aid of a kick net (500 µm mesh). Once in the laboratory, each individual larva was morphologically checked with a stereo microscope and *Chironomus thummi* type larvae (which include *C. riparius*) were grown in breeding chambers, allowed to mate and lay the egg ropes. Each egg rope was then collected and cultivated individually. Several larvae per each egg rope were used to genetically identify the species according to mtDNA barcoding methodology (see below). A total of 10 *C. riparius* egg ropes was used to establish each single population in order to assure representativeness and comparability of populations. Establishment and maintenance of permanent cultures followed the recommendations for culturing *C. riparius* described in the Annex 2 of OECD guideline no. 218 (OECD, 2004a).

All populations were cultured for at least six generations (ca. 6 months) prior of testing for their Cd tolerance. Populations were maintained at 20 ± 1º C and 16: 8h (light: dark) photoperiod with large numbers of organisms in equal breeding chambers in plastic aquaria with 1.5 cm of inorganic fine sand (< 1 mm) and gentle aerated American Society for Testing and Materials (ASTM) hard water medium (ASTM, 1980). Grinded fish flake food (Tetramin®) was added *ad libitum* three times a week to the rearing aquaria and culturing media were renewed every week.

2.3. Assessment of cadmium tolerance in *C. riparius* populations

2.3.1. Test chemical

The ecotoxicological tests to assess the tolerance of the different populations towards cadmium (Cd) were performed using a single stock solution prepared by dissolving analytical grade $CdCl_2$ anhydrous (ACS grade, Sigma-Aldrich) in nanopure water and experimental solutions were directly prepared in ASTM by aliquoting appropriate volumes of the stock solution.

2.3.2. Acute toxicity tests

Acute tolerance of each single population was estimated by testing, in separate experiments, five egg ropes according to the OECD guideline 235 acute immobilisation test for *Chironomus* sp. (OECD, 2011). For each single egg rope, first instar larvae (<24h-old) were exposed to seven Cd treatments in a geometrical series (1.88; 3.75; 7.50; 15.00; 30.00; 60.00; 120.00 mg $Cd \cdot l^{-1}$) plus control treatment for a 48h-period without aeration or food supply. A total of 25 organisms (5 replicates with 5 organisms each) were used per treatment. Experiments were carried out in 6-well multiplates containing, each well, 10 ml of test solution. ASTM soft water medium (ASTM hard water diluted to 25% with nanopure water) was used in the acute tests and pH was adjusted to 6.00 in order to avoid Cd precipitation. Larval immobilisation was monitored after 48 hours of Cd exposure using a stereo microscope.

2.3.3. Chronic toxicity tests

Chronic tolerance was assessed through 28-day partial life-cycle tests according to the OECD guideline 219 (OECD, 2004b) and organisms of each population were obtained from 5 egg ropes hatching within a maximum admissible period of 6 hours. Newly hatched larvae were then transferred to plastic aquaria containing ASTM hard water, inorganic sand and food. After two days, larvae were collected and randomly assigned to plastic vials filled with 1.5 cm of inorganic sand and 250 ml of ASTM hard water with gentle aeration. Six Cd treatments (12.5; 25.0; 50.0; 100.0; 200.0 and 400.0 µg $Cd \cdot l^{-1}$) plus control treatment (ASTM hard water only) were tested. A total of 100 larvae (10 replicates with 10 larvae each) were used per population in each experimental treatment and organisms were fed every other day with Tetramin® (0.5 $mg \cdot larvae^{-1} \cdot day^{-1}$).

C. riparius imagoes were collected daily from emergence traps with the aid of an aspirator to assess the rate and time to emergence. Imagoes were preserved in 70% ethanol, individually dried for 24 h at 50º C, and weighed in a microbalance (Mettler Toledo UMT 2).

2.4. Assessment of genetic diversity of *C. riparius* populations

Genetic diversity of *C. riparius* populations was estimated based on the allelic variation of the imagoes used to establish the populations in the laboratory (i.e. F_0 generation). DNA extraction and genetic identification of *C. riparius* organisms was performed as in Pfenninger et al. (2007) adapted to 96-well multiplates (Pfenninger et al., 2007). Briefly, tissue was digested overnight (at 56 ºC) in 80 µl of standard cetyltrimethyl ammonium bromide (CTAB) buffer plus 2 µl of Proteinase K (20 µM) followed by treatment with 82 µl of standard 24:1 chloroform: isoamylalcohol. DNA was precipitated for at least 45 min (-20 ºC) in 125 µl of isopropanol, washed 2x in 150 µl of ethanol 70% and resolved in 30 µl of water for posterior use. Mitochondrial cytochrome oxidase I (mtDNA CO I) fragments were amplified in a T-Gradient thermocycler (Biometra, Göttingen, Germany) with the following cycling conditions: initial denaturing cycle of 3 min at 94º C followed by 36 cycles with 1 min at 92º C, 1 min at 55º C and 1 min at 72º C. Reaction mixture contained 3 mM $MgCl_2$, 1x reaction buffer (20 mM Tris-HCl, 50 mM KCl), 0.2 mM dNTP, 0.3 µM of each *Chironomus* specific primer (forward: 5' TCGAGCAGAATTAGGACGACC, reverse: 5' AGGA TCACCCCCACCAGCAGG) and 1 U *Taq* DNA polymerase in a total volume of 13 µl of reaction mixture plus 2 µl of DNA

sample. Sequencing of amplified mtDNA CO I was performed in an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). Sequences were assembled and aligned using the ClustalW alignment method (Thompson et al., 1994) and performed in sequence scanner software 2 (Applied Biosystems, Foster City, CA, USA) and Bioedit software version 7.0.5.2 (Ibis Therapeutics, Carlsbad, CA, USA). *Chironomus riparius* CO I haplotypes were identified according to reference sequences (GenBank accession numbers DQ910547-DQ910729 (Nowak et al., 2006)).

Genetically identified *C. riparius* organisms were, afterwards, used to estimate the allelic variation at 7 microsatellite loci (Supplementary Material S2). Microsatellite fragments were amplified with the following PCR conditions: initial denaturing cycle of 3 min at 94º C, followed by 36 cycles with 30 sec at 94 ºC, 30 sec at 55 ºC and 40 sec at 72º C and a final extension cycle with 5 min at 72º C. Reaction mixture contained 2.4 mM of $MgCl_2$, 0.25 mM of dNTP, 0.2 µM of each specific primer and 0.5 U of *Taq* DNA polymerase in a total volume of 8 µl of reaction mixture per microsatellite plus 2 µl of DNA sample. Microsatellite data were automatically scored using software GeneMarker® version 2.6.3 (Softgenetics, State College, PA) and corrected by eye whenever necessary.

2.5. Statistical analysis

2.5.1. Assessment of cadmium tolerance

According to our sampling design, we first compared the acute and chronic responses of *C. riparius* populations to Cd exposure within each geographical region: pairwise comparisons of C_1 vs R_1; C_2 vs R_2; C_3 vs R_3. This approach minimized differences in tolerance due to geographic factors. To investigate if there was a general pattern of tolerance to Cd among populations living in metal contaminated areas, we tested for differences in the average responses to Cd of the different populations originating from metal contaminated sites compared to the average responses of reference populations ("C_{pool}": C_1, C_2, C_3 versus "R_{pool}": R_1, R_2, R_3; n=3).

Acute tolerance to Cd was determined for each population through 48h-LC_{50}s (in mg $Cd \cdot l^{-1}$) derived from four-parameter logistic dose-response curves (equation: $Y = min + (max - min)/(1 - 10^{(logLC_{50} - X) \cdot HillSlope)})$, where Y = survival (%), min = 0% survival, max = highest survival and X = Cd concentration (mg $Cd \cdot l^{-1}$)). Chronic tolerance to Cd was assessed using emergence rate, time to emergence and weight of male and female imagoes as life-history endpoints. The relationship between emergence rate and Cd

concentration was analysed for each population by fitting four-parameter logistic curves as depicted for the acute tolerance of populations (where Y = emergence rate (%), min = 0, max = highest emergence rate and X = Cd concentration (µg $Cd \cdot l^{-1}$)), while exponential curves were used for time to emergence (equation: $Y = Y_0 \times e^{kX}$, where Y = time to emergence (days), Y_0 = average time to emergence at 0 µg $Cd \cdot l^{-1}$, k = estimated parameter of the exponential curve and X = Cd concentration (µg $Cd \cdot l^{-1}$)). Extra sums-of-squares F tests were used to compare dose responses between the different populations for acute (48h-LC_{50}s) and chronic endpoints (Motulsky and Christopoulos 2004). Complementarily, two-way analyses of variance (ANOVAs) followed by Tukey's post hoc tests were used to further explore the effect of the factors "population" and "Cd concentration" and of their interaction in the emergence rate, time to emergence and weight of male and female imagoes. These two-way ANOVAs and their multiple comparisons (Tukey post-hoc tests) were also used to compare *C. riparius* populations performance under control conditions (no Cd exposure) within each geographical area.

Significant diferences in time to emergence and weight of imagoes were statistically tested after excluding the highest Cd concentration tested (400 µg $Cd \cdot l^{-1}$) due to the low number of organisms that survived until emergence. Imagoes from the 100 µg $Cd \cdot l^{-1}$ treatment of population R_1 were accidentally lost and hence this treatment was excluded from the statistical analysis.

Assumptions of normality and homoscedascity were fulfilled for all life-history endpoints as confirmed by residual plot analysis. All statistical analyses were carried out with GraphPad Prism® (GraphPad Inc., San Diego, CA, USA) and significant differences were considered for *p*-values below 5%.

2.5.2. Assessment of genetic variation of natural populations of *C. riparius*

Standard descriptive measures of genetic diversity were estimated for all populations (mean number of alleles per locus [N_A], observed and expected heterozygosity [H_o and H_E] and deviations from Hardy-Weinberg equilibrium [HWE]) using the statistical package GenAlEx 6.501 (Peakall and Smouse, 2006). Deviations from HWE for each individual locus were performed using Chi-squared tests ($p < 0.05$).

Hierarchical analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was also applied to test partition of total genetic variation within and among populations of *C. riparius* and to estimate genetic differentiation between populations through Φ_{PT}.

Population pairwise Φ_{PT}-values were tested for a significance level of $p < 0.05$ followed by sequential Holm's Bonferroni correction for multiple comparisons between populations (Holm 1979). "Isolation-by-distance" was also evaluated using a Mantel test in which the correlation between geographical distance (log transformed) and population pairwise Φ_{PT}-values matrices were tested. Significance of AMOVA and Mantel test was tested by 999 random permutations of the data.

3. Results

3.1. Cadmium tolerance of *C. riparius* populations

3.1.1. Acute tolerance

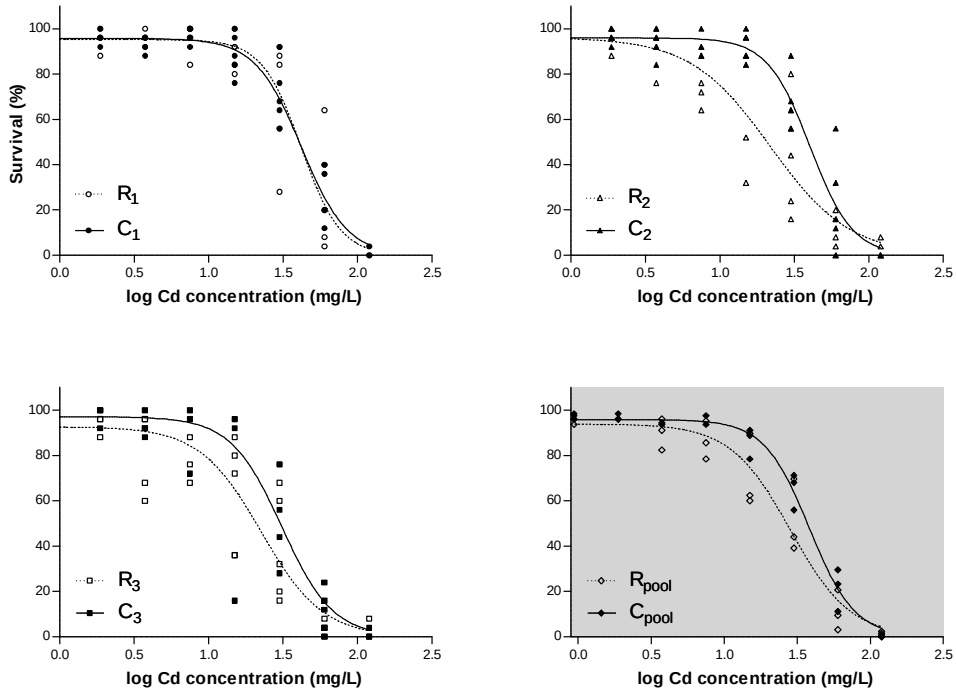

Figure 2 – Acute tolerance to Cd of populations from metal contaminated sites in comparison to nearby-collected reference populations: C_1 vs R_1, C_2 vs R_2 and C_3 vs R_3. Shown are also the data grouped according to contamination of the native environments: C_{pool} *vs.* R_{pool}.

The four-parameter logistic curves showed good fit to the data (R^2=0.87–0.96) and clear dose-response curves were obtained for all populations. Acute tolerance to Cd (i.e., 48h-LC_{50}s) varied within a two-fold factor, ranging from 21.83 mg Cd·l^{-1} (95% C.I. 16.82–28.34) in the population from reference site R_2 to 44.20 mg Cd·l^{-1} (95% C.I. 40.36–48.41) in the population from metal contaminated site C_1. Overall, the estimated LC_{50}s were

higher in the three populations from metal contaminated sites compared to their respective references (Figure 2; Table 1) with significant differences being noted for the population pairwise comparisons C_2 *vs* R_2 ($F_{1,84}$=22.79, p<0.001) and C_3 *vs* R_3 ($F_{1,84}$=4.60, p=0.035) (Supplementary Material S3).

This pattern was reinforced when comparing the pool of populations from metal contaminated sites against the pool of populations from reference sites (Figure 2; Table 1). Again the acute tolerance to Cd of the pool of populations from contaminated sites (LC_{50}=38.30 mg Cd·l^{-1}; 95% C.I. 35.47–41.35) was significantly higher compared to the pool of populations from reference sites (LC_{50}=28.50 mg Cd·l^{-1}; 95% C.I. 23.94–33.93; $F_{1,48}$=10.78, p=0.002) (Supplementary Material S3).

3.1.2. Chronic tolerance

Under control clean conditions, emergence rate of population C_1 was significantly lower and imagoes took more time to emerge than in population R_1. Furthermore, male imagoes of population C_1 were also significantly lighter compared to those from population R_1. No significant differences in the emergence rate or time to emergence were found between populations C_2 and R_2 and between populations C_3 and R_3 under control clean conditions. However, male and female imagoes of population C_3 were significantly lighter compared to those from population R_3 (Supplementary Material S4).

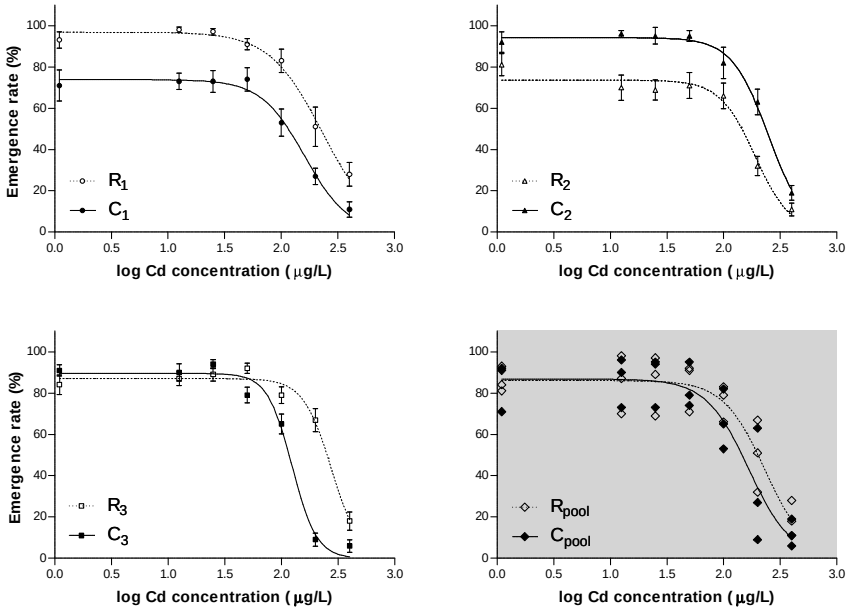

Figure 3 – Variation in the emergence rates (in %, mean ± SEM) with chronic exposure to Cd in populations collected from metal contaminated sites in comparison to the respective reference populations: C_1 vs R_1, C_2 vs R_2 and C_3 vs R_3. Shown are also the data grouped according to contamination of the native environments: C_{pool} *vs.* R_{pool}.

Exposure to increasing Cd concentrations significantly delayed and reduced the emergence of midges in all six populations (Figures 3 and 4; Supplementary Material S5 and S6), but no clear dose-responses were observed for the weight of imagoes (Figure 5; Supplementary Material S9).

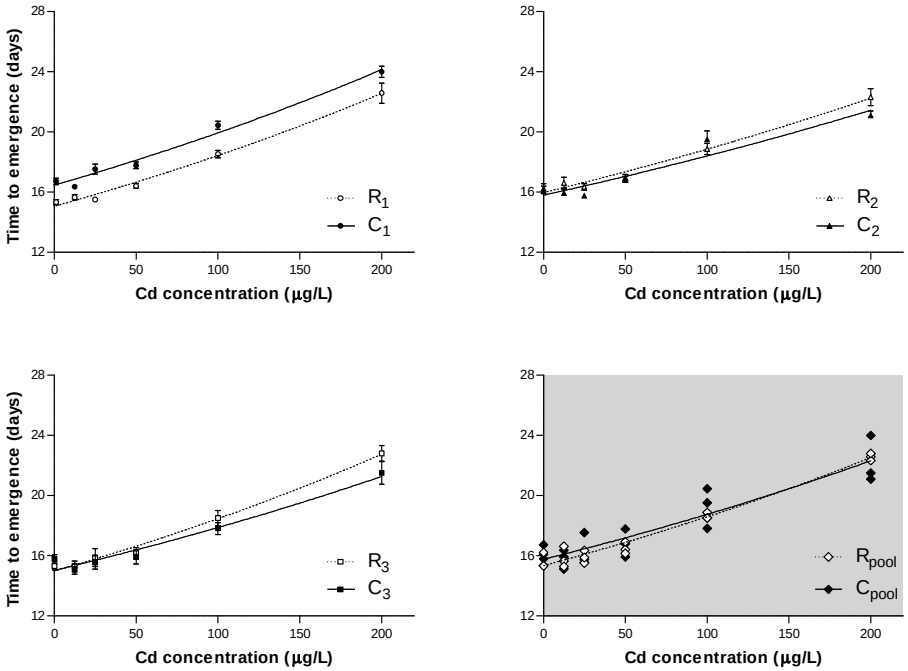

Figure 4 – Variation in time to emergence (in days, mean ± SEM) with chronic exposure to Cd in populations collected from metal contaminated sites in comparison to the respective reference populations: C_1 vs R_1, C_2 vs R_2 and C_3 vs R_3. Shown are also the data grouped according to contamination of the native environments: C_{pool} *vs.* R_{pool}.

Pairwise comparisons performed by extra sum-of-squares F tests revealed that the dose-response curves to Cd for emergence rates were significantly different for all pairwise comparisons (i.e. populations C_1 vs R_1; C_2 vs R_2; C_3 vs R_3) (Supplementary Material S7). Consistent with these results, two-way ANOVAs also detected a significant effect of "population" for emergence rate in all pairwise comparisons as well as a significant interaction between "population" and "Cd concentration" for population comparison C_3 vs R_3 ($p < 0.001$; Supplementary Material S5).

Concerning dose-response curves to Cd in terms of time to emergence, extra sums-of-squares F test revealed significant differences only for the comparison between populations C_1 and R_1 ($F_{2,116}=35.80$, $p<0.001$; Supplementary Material S8). These results were again in agreement with those from the two-way ANOVAs which detected a significant effect of "population" only when comparing populations C_1 vs R_1 (Supplementary Material S6).

Finally, results of the two-way ANOVAs showed a significant effect of "population" for the weight of male and female imagoes when comparing populations C_1 vs R_1 and populations C_3 vs R_3 (Supplementary Material S9).

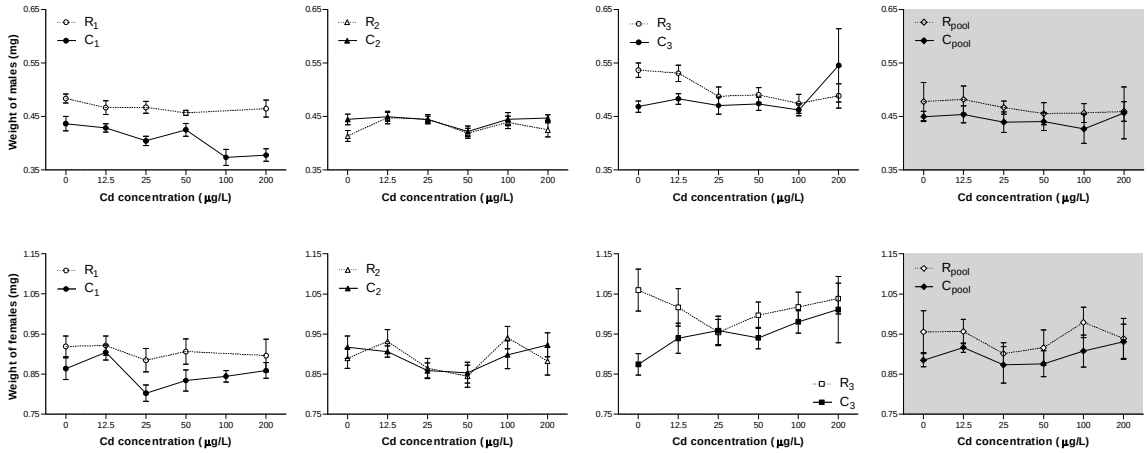

Figure 5 – Variation in the weight of male (top row) and female (bottom row) imagoes (mg, mean ± SEM) with chronic exposure to Cd in populations collected from metal contaminated sites in comparison to the respective reference populations: C_1 vs R_1, C_2 vs R_2 and C_3 vs R_3. Shown are also the data grouped according to contamination of the native environments: C_{pool} vs R_{pool}.

When populations from metal contaminated sites were pooled together and compared to the pool of populations from reference sites, no significant differences were observed in the dose-response curves to Cd for any of the adult traits analysed by the extra sum-of-squares F test while two-way ANOVAs revealed a significant effect of the factor "population" for emergence rate but accounting for only less than 1% of the total variation (Supplementary Material S5, S6 and S9).

In general, these results show no convergence between acute and chronic tolerance to Cd since only population C_2 performed better (higher emergence rate) than its respective reference population (i.e. R_2) across the Cd concentrations tested (Table 1).

Table 1 – Summary of the acute and chronic responses to cadmium for the pairwise comparisons between populations from reference (R) and metal contaminated (C) sites. Full statistical results are depicted in supplementary material S5, S6, S7, S8 and S9.

	R_1 vs C_1	**R_2 vs C_2**	**R_3 vs C_3**	**R_{pool} vs C_{pool}**
A. Acute response				
48h-LC_{50}	$R_1 = C_1$	$R_2 < C_2$	$R_3 < C_3$	$R_{pool} < C_{pool}$
B. Chronic response				
Emergence Rate	$R_1 > C_1$	$R_2 < C_2$	$R_3 > C_3$	$R_{pool} = C_{pool}$
Time to emergence	$R_1 < C_1$	$R_2 = C_2$	$R_3 = C_3$	$R_{pool} = C_{pool}$
Weight males	$R_1 > C_1$	$R_2 = C_2$	$R_3 > C_3$	$R_{pool} = C_{pool}$
Weight females	$R_1 > C_1$	$R_2 = C_2$	$R_3 > C_3$	$R_{pool} = C_{pool}$

3.2. Genetic diversity and differentiation of *C. riparius* populations

A total of 161 organisms were used to estimate genetic diversity and differentiation of *C. riparius* populations (Table 2). In general, H_E was higher in the three populations from metal contaminated sites in comparison to populations from reference sites. However, no clear patterns between populations from metal contaminated and uncontaminated sites were found for N_A or H_O. Furthermore, the estimators of genetic diversity (N_A, H_O and H_E) were consistently lower in the population from reference site R_1. Significant HWE deviations were observed in the population from contaminated site C_3 for two loci and in the three populations collected from reference sites for one locus (Table 2).

Table 2 – Population genetic parameters estimated for the six *C. riparius* populations. Shown is the number of individuals (n), mean number of alleles per locus (N_A), observed heterozygosity (Ho; loci with significant deviations from Hardy Weinberg Equilibrium are in parentheses) and expected heterozygosity (H_E). Pairwise genetic differentiation estimates between populations, as Φ_{PT}, is given on the right of the table based on 999 permutations.

	Genetic diversity				Genetic differentiation					
	n	**N_A**	**H_O**	**H_E**	**C_1**	**C_2**	**C_3**	**R_1**	**R_2**	**R_3**
C_1	27	3.71	0.50	0.53	-					
C_2	28	3.71	0.55	0.56	0.000	-				
C_3	31	4.14	0.60$^{(Msc4,6)}$	0.56	0.000	0.000	-			
R_1	28	3.57	0.46$^{(Msc6)}$	0.49	**0.038***	**0.034***	**0.070****	-		
R_2	25	4.00	0.49$^{(Msc5)}$	0.51	0.005	0.000	0.019	0.019	-	
R_3	22	3.71	0.55$^{(Msc2)}$	0.51	0.002	**0.027***	**0.032***	**0.065****	**0.064****	-

*Significant differences at $p < 0.05$

**Significant differences after Holm's sequential Bonferroni correction

Hierarchical analysis of molecular variance (AMOVA) revealed an overall weak but significant differentiation among *C. riparius* populations (Φ_{PT}=0.024, p=0.002; Supplementary Material S10) with pairwise Φ_{PT} analysis showing significant differences for 7 out of 15 pairwise comparisons. However, after Holm's Bonferroni correction, only 3 comparisons remained significant: R_1 *vs* C_3, R_1 *vs* R_3 and R_2 *vs* R_3 (Table 2). The Mantel test did not reveal a significant association between the geographical distance and population pairwise Φ_{PT}-values matrices (r_M=0.032, p=0.396).

4. Discussion

The present study addresses the role of metal contamination as a strong selection pressure acting on natural populations of the non-biting midge *Chironomus riparius*. Overall, our results suggest that persistence of *C. riparius* populations under historically metal contaminated sites may be related, at least in part, with adaptation to metals. However, no signs of genetic erosion were revealed by the microsatellite analysis in any of the populations tested.

Here, we investigated the tolerance of the different midge populations to Cd because previous studies had reported a past-history of Cd contamination that was common to the three metal contaminated sites: site C_1 (Soares et al., 1999), site C_2 (Martins et al., 2011) and site C_3 (Reis et al., 2005). Therefore, it is reasonable to assume that *C. riparius* populations inhabiting these sites have been continuously exposed to high levels of metal contamination.

To assess whether Cd-tolerance differed between *C. riparius* populations sampled from historical metal contaminated sites and nearby-located reference sites, all populations were cultured for at least six generations under laboratory clean conditions in order to minimize potential confounding effects of physiological acclimation to their native environments (Räsänen and Kruuk, 2007). In two out of the three pairwise comparisons, populations originating from metal contaminated sites were more tolerant to Cd than those collected from nearby reference sites. Therefore, our results suggest for differences in the genetic background of the experimental populations and an increased prevalence of Cd-tolerant genotypes in populations inhabiting metal contaminated sites which is in line with empirical evidences showing adaptation to metals in natural populations of freshwater invertebrates inhabiting metal contaminated sites. For example, populations of the aquatic oligochaete *Limnodrilus hoffmeisteri* inhabiting a heavily Cd-contaminated site showed greater tolerance to Cd than reference ones even after being cultured in the laboratory for several generations under control clean conditions (Klerks and Levinton, 1989). Similarly, Agra et al. (2010) and Lopes et al. (2004) also found evidences for adaptation to metals in a population of the cladoceran *Daphnia longispina* impacted with a metal rich acid mine drainage. Comparing multiple clonal lineages of the species collected from the metal-impacted site and a nearby-located reference site, both authors found that the most sensitive clonal lineages were only present in the reference population whereas the most tolerant ones were only present in the metal-exposed population (Agra et al., 2010; Lopes et al., 2004).

Interestingly, the population from reference site R_1 of our study, located upstream the metal contaminated site C_1, showed also high acute tolerance to Cd. This result might be related to the generalized pattern of metal contamination reported in the lower and middle parts of the Ave basin (Soares et al., 1999) that might have favored the presence of more tolerant genotypes in the whole basin including the selected reference site.

Contrary to our expectations, chronic responses of the studied populations did not consistently converge with acute responses. Indeed, only population C_2 that was more tolerant to acute exposures to Cd also showed higher emergence rates under chronic exposures to Cd when compared to reference population R_2. This apparent disagreement between acute and chronic tolerance has already been reported in other studies with aquatic invertebrates. For example, Barata et al. (2000) found no association between acute and chronic responses (measured as feeding rates) of different clonal lineages of *D. magna* towards copper and fluoranthene toxicity (Barata et al., 2000). Similarly, Lopes et al. (2005) and Saro et al. (2012) found no agreement between acute and chronic tolerance to metals (measured as feeding rates, growth and reproduction) in different clonal lineages of *D. longispina* (Lopes et al., 2005; Saro et al., 2012). Finally, Leppänen et al. (1998) compared acute and chronic responses (feeding activity measured as egestion rates) of metal-adapted and reference *C. riparius* natural populations and found that metal-adapted larvae, collected from contaminated sites, had higher acute tolerance to metals in comparison to those larvae from reference sites. However, no differences in the egestion rates of the different populations were observed (Leppänen et al., 1998).

The observed discrepancy between acute and chronic responses of the midge populations herein investigated may be related with the different exposure routes of Cd (Geffard et al., 2008; Irving et al., 2003). While acute tests evaluated waterborne Cd exposures only (as larvae were starved during the test period), the importance of dietary intake of Cd cannot be disregarded in the chronic assays. Dietary Cd exposure (but not waterborne Cd exposure) has been shown to significantly depress important components of the antioxidant system of freshwater invertebrates and, as such, Cd intake via food might elicit different effects relatively to waterborne exposures only (Xie and Buchwalter, 2011).

Alternatively, a plausible explanation for the obtained results is that different mechanisms are implicated in the acute and chronic responses to Cd in the selected midge populations. Acute responses to xenobiotics have generally been attributed to the involvement of specific mechanisms of tolerance that are likely controlled by few genes

of large effect while chronic responses have been associated to more general mechanisms of tolerance that are likely regulated by many genes of small effect and, thus, depend more on the overall fitness of the organisms (Barata et al., 2000). In the case of Cd, a major role has been ascribed to the cellular thiols metallothioneins (Toušová et al., 2016; Xie and Klerks, 2004c) and glutathione (Nair et al., 2013) due to their great capability to sequester free Cd ions. Interestingly, in a follow-up experiment, higher baseline levels of both thiols were observed in some of these populations from metal contaminated sites when compared with populations from reference sites (Pedrosa et al., 2017). Furthermore, Cd tolerance may also be achieved through changes in the patterns of uptake, accumulation and excretion of Cd (Xie and Klerks, 2004a) and previous studies have shown higher excretion efficiency and higher storage capability in the gut of Cd-adapted populations of *C. riparius* (Postma et al., 1996), where most of the Cd accumulates in waterborne and dietary Cd exposures (Leonard et al., 2009; Postma et al., 1996). Therefore, although results of the acute tests suggest that populations sampled from metal contaminated sites might have developed adaptive strategies to better cope with Cd, this does not necessarily confer enhanced tolerance under prolonged exposures to low levels of Cd stress as, in some cases, metal adaptation may carry important fitness costs (Ward and Robinson, 2005; Xie and Klerks, 2004b).

Based on our laboratory partial life-cycle experiments, and contrasting with previous results reported for other metal-adapted *C. riparius* populations (Postma et al., 1995), we could not observe any evidence of fitness costs associated to metal adaptation in population C_2. However, fitness costs were observed in populations C_1 and C_3. Population C_1, which showed to be the most tolerant population to acute Cd exposures (i.e. highest LC_{50}), exhibited poor performance under control clean conditions with lower emergence rates, longer developmental times and lighter imagoes. In the case of population C_3, no differences in terms of emergence rates or time to emergence were found in comparison to its reference population under control clean conditions but emerged imagoes were lighter which might suggests lower reproductive output (Sibley et al., 2001). It should be noted, however, that we did not assess effects of Cd exposure on reproduction directly and, therefore, we cannot exclude the possibility of fitness costs also in population C_2 in terms of, for instance, fecundity or egg rope fertility. Such fitness costs may be due to the additional energy requirements for improved defence mechanisms or due to negative genetic interactions between alleles that confer tolerance and fitness-related traits (Ribeiro and Lopes, 2013; van Straalen and Timmermans, 2002; Xie and Klerks, 2004b). Therefore, additional studies are necessary to better

understand the biochemical processes underlying the differential tolerance towards Cd among the different *C. riparius* populations of the present study.

Despite genetic erosion is widely accepted as a potential evolutionary outcome of long-term exposure to environmental contaminants (Bickham, 2011; Ribeiro and Lopes, 2013), the microsatellite analysis revealed no signs of genetic erosion in any *C. riparius* population collected from metal contaminated sites and only weak genetic differentiation among all experimental populations. These results contrasted with a previous multi-generation laboratory study demonstrating rapid contaminant-induced genetic erosion in *C. riparius* (Nowak et al., 2009). However, they are consistent with other studies showing low impacts of contaminants in the overall levels of genetic diversity of invertebrates (Costa et al., 2012; Gall et al., 2013), fishes (Miller et al., 2012; Whitehead et al., 2003) or mammals (Berckmoes et al., 2005; Wirgin et al., 2015) in natural environments. Furthermore, a lack of evidence for genetic erosion in pollution-tolerant natural populations has also been demonstrated. For example, populations of the springtail *Orchesella cincta* from metal-contaminated areas were found to be more tolerant to acute exposures of Cd in comparison to a springtail population from a reference site without any evidences of reduced genetic diversity due to metal contamination (Costa et al., 2012). Similar results were observed in natural populations of the barnacle *Amphibalanus variegates* for which no genetic erosion could be observed albeit higher acute tolerance to copper was registered in populations inhabiting metal-contaminated estuaries (Gall et al., 2013). Finally, no evidences of genetic erosion were shown in populations of the killifish *Fundulus heteroclitus* inhabiting highly PCB-impacted areas despite differences in acute tolerance to PCBs reaching up to 1000-fold factor in comparison to reference populations (McMillan et al., 2006; Nacci et al., 1999; Nacci et al., 2002).

Diminishments in the overall levels of genetic diversity are expected to occur when the effective population size is strongly reduced (Frankham, 1996; Hoffmann and Willi, 2008). Natural populations of *C. riparius* are typically very large reaching densities up to 70000 individuals$\cdot m^{-2}$ (Groenendijk et al., 1998). Therefore, even if metal contamination was sufficient to eliminate the most sensitive genotypes, the large effective population sizes in conjunction with the short life-cycles and the great dispersal capabilities of *C. riparius* likely prevented significant declines in the overall levels of genetic diversity (Groenendijk et al., 2002; Groenendijk et al., 1998).

5. Conclusions

Deciphering microevolutionary processes and outcomes in natural populations inhabiting heavily contaminated sites is essential to better understand the long-term impacts of environmental pollution. Our study shows genetically inherited tolerance to Cd in *C. riparius* populations inhabiting metal contaminated sites, despite no evidences of genetic erosion due to long-term metal contamination. The fact that increased tolerance to metals in populations collected from metal contaminated sites was not associated to losses of genetic diversity highlights the limitations of using neutral markers such as microsatellites as indicators of adaptation and microevolutionary responses to environmental contamination in species with large population sizes, such as the case of *C. riparius*. With the emergence of next generation sequencing technologies, it is now possible to analyse a large number of samples and screen an unprecedented number of markers (Pauls et al., 2014). Despite that it is still in its infancy, whole-genome studies may allow to identify loci that might be under selection and other genetic factors involved in altered metal tolerance (Altshuler et al., 2011; Gusev et al., 2014). Future studies should also consider spatially replicated sampling of multiple midge populations from both contaminated and uncontaminated environments.

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Supplementary material

Supplementary Material S1 – Location of *C. riparius* populations collection sites.

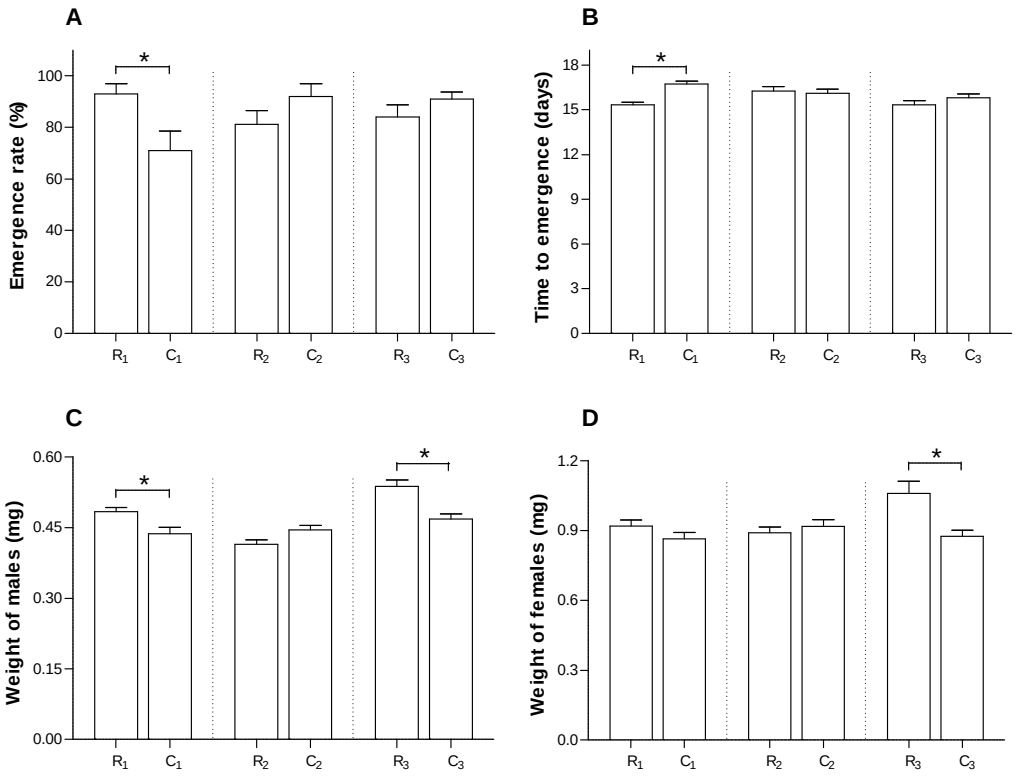
Site	Coordinate	Location	Watercourse	Subbasin	Basin
Contaminated site 1 (C_1)	41.522 N -8.435 W	Celeirós	Este river	Este	Ave
Contaminated site 2 (C_2)	40.750 N -8.577 W	Estarreja	S. Filipe Ditch	Antuã	Vouga
Contaminated site 3 (C_3)	40.575 N -8.453 W	Águeda	Vale da Erva Stream	Águeda	Vouga
Reference site 1 (R_1)	41.572 N -8.360 W	S. Mamede de Este	Este river	Este	Ave
Reference site 2 (R_2)	40.741 N -8.528 W	Porto de Baixo	Jardim river	Antuã	Vouga
Reference site 3 (R_3)	40.682 N -8.220 W	Cercosa	Alfusqueiro river	Águeda	Vouga

Supplementary Material S2 – Repeat motif, primer sequences, size range (in base pairs) and GenBank accession number of the microsatellite loci used to estimate the genetic diversity of *C. riparius* populations.

Locus	Motif	Primer sequence	Size (bp)	Bibliography	Accession no.
Msc1	CA_9	F: CATCATCCTTAACAACCCAC R: CTAGCTTTGCAGGCGAGTGC	95-103	(Nowak et al. 2006)	DQ408105
Msc2	$(TAA)_9,T_{10}$	F: AGACTAATGACCAGACTTGC R: CTTGTGATGCGAAAAGCCTG	114-141	(Nowak et al. 2006)	DQ408106
Msc3	$(GT)_{14}$, T_9, T_6	F: ACTACGCGTGCCTCAACAGC R: AGCTAATTCTCATGTTGGTC	168–176	(Nowak et al. 2006)	DQ408107
Msc4	$(TCA)_6$	F: TGACTGAACTTCCGCAATGGG R: CCGAGAATGCTGCGATCCAG	211–216	(Nowak et al. 2006)	DQ408108
Msc5	$(CA)_{11}$, A_9	F: AACATTTGAACGCACATCG R: ATTTGATTGTATGTCCTG	264–278	(Nowak et al. 2006)	DQ408109
Msc6	GA	F: TATCCCACCATATCGGCATC R: CACCCGCAAATGATATACACAA	168–229	(Soeter et al. 2010)	-
Msc7	GT	F: GCTGAATCGTGTGATGTGCT R: TGCTGCTTCTGTCGTATGCT	235–245	(Soeter et al. 2010)	-

Supplementary Material S3 – Four-parameter logistic dose-response curves used to estimate the acute tolerance of populations when exposed to Cd. Shown are the Hill slopes, the LC_{50}s in mg Cd/L (with 95% confidence intervals), the R^2 and the Sum of Squares (SS) of the logistic curves. Statistical analysis for pairwise comparisons among populations within each geographic area and between grouped populations in terms of LC_{50}s are presented: degrees of freedom (df), F-values (*F*), significance level (*p*).

	Logistic curve parameters				Statistical significance			
Population	Hill Slope	LC_{50} (±95% C.I.)	R^2	SS	comparison	df	F	*p*
R_1	-3.252	40.21 (34.92-46.31)	0.90	6262	R_1 vs C_1	1,84	1.22	0.272
C_1	-2.887	44.20 (40.36-48.41)	0.96	2276				
R_2	-1.623	21.83 (16.82-28.34)	0.87	8597	R_2 vs C_2	1,84	22.79	<0.001
C_2	-3.043	40.38 (36.38-44.82)	0.94	3227				
R_3	-2.097	22.64 (18.21-28.14)	0.88	8171	R_3 vs C_3	1,84	4.60	0.035
C_3	-2.533	31.11 (25.94-37.30)	0.88	8262				
R_{pool}	-2.113	28.50 (23.94-33.93)	0.95	1828	R_{pool} vs C_{pool}	1,48	10.78	0.002
C_{pool}	-2.808	38.30 (35.47-41.35)	0.99	531				



Supplementary Material S4 – Variation in the life-history endpoints of the experimental populations under control clean conditions: (A) emergence rate (%, mean ± SEM), (B) time to emergence (days, mean ± SEM) and weight of (C) male and (D) female imagoes (mg, mean ± SEM). Asterisks denote significant differences within each pair of populations from two-way ANOVA post hoc Tukey tests.

Supplementary Material S5 – Two-way ANOVA results for the effect of cadmium in the emergence rate of *C. riparius* populations. Shown are degrees of freedom (df), sum of squares (SS), F-values (F), significance level (p) and percentage of variation (R^2) explained by each factor.

Pairwise comparison	Factor	df	SS	F	*p*	R^2
R_1 *vs* C_1	**Cd**	**6**	**83294**	**50.57**	**<0.001**	**60.99**
	Population	**1**	**18058**	**65.78**	**<0.001**	**13.22**
	Cd x Pop	6	637	0.39	0.89	0.47
R_2 *vs* C_2	**Cd**	**6**	**84382**	**56.04**	**<0.001**	**63.64**
	Population	**1**	**14178**	**56.50**	**<0.001**	**10.69**
	Cd x Pop	6	2232	1.48	0.19	1.68
R_3 *vs* C_3	**Cd**	**6**	**114217**	**130.60**	**<0.001**	**74.97**
	Population	**1**	**4803**	**32.96**	**<0.001**	**3.15**
	Cd x Pop	**6**	**14977**	**17.13**	**<0.001**	**9.83**
R_{pool} *vs* C_{pool}	**Cd**	**6**	**279156**	**138.10**	**<0.001**	**66.18**
	Population	**1**	**2425**	**7.20**	**0.008**	**0.57**
	Cd x Pop	6	4097	2.03	0.06	0.97

Supplementary Material S8 – Two-way ANOVA results for the effect of cadmium in the time to emergence of C. riparius populations. Shown are degrees of freedom (df), sum of squares (SS), F-values (F), significance level (*p*) and percentage of variation (R^2) explained by each factor.

Pairwise comparison	Factor	df	SS	*F*	*p*	R^2
R_1 *vs* C_1	**Cd**	**5**	**817.60**	**186.50**	**<0.001**	**83.22**
	Population	**1**	**64.61**	**73.68**	**<0.001**	**6.58**
	Cd x Pop	5	5.53	1.26	0.29	0.56
R_2 *vs* C_2	**Cd**	**5**	**517.30**	**86.87**	**<0.001**	**78.42**
	Population	1	3.79	3.18	0.08	0.57
	Cd x Pop	5	10.06	1.69	0.14	1.53
R_3 *vs* C_3	**Cd**	**5**	**579.40**	**61.91**	**<0.001**	**69.06**
	Population	1	4.06	2.17	0.14	0.48
	Cd x Pop	5	7.43	0.79	0.56	0.89
R_{pool} *vs* C_{pool}	**Cd**	**5**	**202.00**	**60.06**	**<0.001**	**91.91**
	Population	1	0.54	0.80	0.38	0.24
	Cd x Pop	5	1.11	0.33	0.89	0.50

Supplementary Material S7 – Four-parameter logistic dose-response curves used to estimate the emergence rate of populations when exposed to Cd. Shown are the Hill slopes, the EC_{50}s in mg Cd/L (with 95% confidence intervals), the R^2 and the Sum of Squares (SS) of the logistic curves. Statistical analysis for pairwise comparisons among populations in terms of emergence rates are presented on the right of the table: degrees of freedom (df), F-values (*F*), significance level (*p*).

	Logistic curve parameters				**Statistical significance**			
Population	Hill Slope	EC_{50} (± 95% C.I.)	R^2	SS	comparison	df	F	*p*
R_1	-1.866	230.50 (191.80-277.00)	0.72	16829	R_1 vs C_1	3,134	23.15	<0.001
C_1	-2.258	160.40 (128.20-200.80)	0.68	18724				
R_2	-2.775	190.60 (157.20-231.20)	0.67	17989	R_2 vs C_2	3,132	20.50	<0.001
C_2	-2.681	248.90 (217.20-285.20)	0.77	14600				
R_3	-3.485	276.00 (246.10-309.50)	0.78	11295	R_3 vs C_3	3,134	39.50	<0.001
C_3	-4.006	124.10 (113.00-136.20)	0.91	9089				
R_{pool}	-2.365	232.60 (185.30-291.90)	0.85	2100	R_{pool} vs C_{pool}	3,36	1.45	0.244
C_{pool}	-2.319	166.50 (125.20-221.50)	0.84	3178				

Supplementary Material S6 – Exponential curves used to estimate the time to emergence of populations when exposed to Cd. Shown are Y_0, k, R^2 and Sum of Squares (SS) of the curves. Statistical analysis for pairwise comparisons among populations in terms of the time to emergence are presented on the right of the table: degrees of freedom (df), F-values (*F*), significance level (*p*).

	Exponential curve parameters				Statistical significance			
Population	Y_0	K	R^2	SS	comparison	df	F	*p*
R_1	15.07	0.002	0.87	58.14	R_1 *vs* C_1	2,116	35.80	<0.001
C_1	16.47	0.002	0.90	46.90				
R_2	15.97	0.000	0.79	75.04	R_2 *vs* C_2	2,115	2.01	0.139
C_2	15.80	0.000	0.75	76.15				
R_3	14.99	0.002	0.79	113.00	R_3 *vs* C_3	2,112	2.74	0.069
C_3	15.03	0.002	0.67	94.23				
R_{pool}	15.34	0.002	0.97	3.18	R_{pool} *vs* C_{pool}	2,32	0.81	0.455
C_{pool}	15.77	0.002	0.85	15.93				

Supplementary Material S9 – Two-way ANOVA results for the effect of cadmium in the weight of male (A) and female (B) imagoes of *C. riparius* for pairwise comparisons. Shown are degrees of freedom (df), sum of squares (SS), *F*-values (*F*), significance level (*p*) and percentage of variation (R^2) explained by each factor.

		Males					Females				
Pairwise comparison	**Factor**	**df**	**SS**	***F***	***p***	**R^2**	**df**	**SS**	***F***	***p***	**R^2**
R_1 *vs* C_1	Cd	4,88	0.016	**3.24**	**0.016**	**8.01**	4,85	0.051	1.91	0.116	7.33
	Population	1,88	0.069	**57.18**	**<0.001**	**35.38**	1,85	0.065	**9.77**	**0.002**	**9.37**
	Cd x Pop	4,88	0.009	1.89	0.119	4.68	4,85	0.013	0.50	0.737	1.91
R_2 *vs* C_2	Cd	5,103	0.011	2.20	0.060	9.09	5,101	0.086	**2.47**	**0.037**	**10.58**
	Population	1,103	0.003	3.18	0.078	2.63	1,101	0.000	0.00	0.979	<0.10
	Cd x Pop	5,103	0.004	0.79	0.559	3.27	5,101	0.023	0.65	0.660	2.80
R_3 *vs* C_3	Cd	4,90	0.022	**2.73**	**0.034**	**9.07**	5,103	0.051	0.72	0.612	2.91
	Population	1,90	0.026	**13.22**	**0.001**	**10.99**	1,103	0.110	**7.78**	**0.006**	**6.31**
	Cd x Pop	4,90	0.012	1.54	0.196	5.13	5,103	0.106	1.49	0.198	6.06
R_{pool} *vs* C_{pool}	Cd	5,23	0.003	0.29	0.046	5.30	5,23	0.015	0.72	0.959	11.71
	Population	1,23	0.004	2.23	0.149	8.14	1,23	0.016	3.74	0.066	12.11
	Cd x Pop	5,23	0.001	0.09	0.993	1.68	5,23	0.004	0.20	0.613	3.26

Supplementary Material S10 – Hierarchical analysis of molecular variance (AMOVA) showing partitioning of genetic variation within and among populations of *C. riparius*.

Source of variation	df	Sum of Squares	Variation components	Variance (%)
Among Populations	**5**	**34.44**	**0.10**	**2.43**
Within Populations	**155**	**638.71**	**4.12**	**97.57**
Total	**160**	**673.05**	**4.22**	

CHAPTER III:

INVESTIGATING HERITABILITY OF CADMIUM TOLERANCE IN *CHIRONOMUS RIPARIUS* NATURAL POPULATIONS: A PHYSIOLOGICAL APPROACH

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Abstract

Physiological responses allow populations to cope with metal contamination and can be involved in the evolution of tolerance under historical metal contamination scenarios. Here we investigate physiological aspects that might be underlying the heritable high tolerance to cadmium (Cd) in two *Chironomus riparius* populations collected from historically metal contaminated sites in comparison to two populations from reference sites.

To evaluate differences in the physiological response to short-term Cd exposure, protein expression profiles, metallothioneins [MTs] and several antioxidant defences such as total glutathione (GSH_t), catalase (CAT) and glutathione-*S*-transferases [GSTs], were measured in all four populations reared for at least 8 generations under laboratory clean conditions. Cd-induced oxidative damage in lipids and energy related parameters (energy consumption and energy reserves) were also assessed.

Results showed two major gradients of protein profiles according to Cd concentration and population tolerance. Furthermore, Cd-tolerant populations showed higher baseline levels of MTs and GSH_t while Cd-sensitive populations, collected from reference sites, showed significant induction of GSH_t levels with Cd exposure that were nonetheless insufficient to avoid increased oxidative damage to lipids. Cd exposure had no clear effects on the antioxidant enzymes or energy reserves but triggered a general increase in energy consumption. Finally, energy consumption was higher in Cd-tolerant populations across experimental conditions.

Altogether, results demonstrate that inherited Cd-tolerance in these midge populations is related, at least in part, with different constitutive levels and plasticity of different defence mechanisms confirming the validity of using multiple physiological traits when studying evolution of tolerance.

Keywords: Energy allocation, glutathione, metal tolerance, metallothioneins, oxidative damage, protein profile.

1. Introduction

Plasticity in organisms' physiology together with genetic adaptation can play an important role in how natural populations can cope with environmental change (Bonduriansky and Day, 2008; Gienapp et al., 2008; Day and Bonduriansky, 2011; Schlichting and Wund, 2014; Stoks et al., 2014). This is not only because stress-induced physiological responses can facilitate short-term persistence of populations (i.e. before genetic adaptation can occur) (Pigliucci, 2005; Latta et al., 2007; Pestana et al., 2016), but also because variation in the direction and magnitude of the plastic responses is indicative of adaptive evolution to environmental stress, including chemical contamination (Sturmbauer et al., 1999; Haap et al., 2016; Oexle et al., 2016).

Metal contamination is widespread in many ecosystems and some populations are able to reduce their adverse effects through a suite of biochemical and physiological adjustments that allow them to live under such harsh conditions (Janssens et al., 2009; Sandbichler and Höckner, 2016). As such, evolution of tolerance and genetic adaptation to metals has been demonstrated in invertebrate populations under historical metal contamination (Agra et al., 2011; Ribeiro et al., 2012).

Yet, studies demonstrating the evolution of physiological responses to metals or altered patterns of the induced responses as the outcome of microevolution are rare. This is somewhat surprising given the considerable variation in the magnitude, direction and number of metal-induced physiological traits that have been demonstrated in a variety of organisms (Leiniö and Lehtonen, 2005; Xie et al., 2008). Moreover, variation in these responses has been also observed for conspecific populations (Gillis et al., 2014; Pain-Devin et al., 2014) highlighting the potential evolutionary importance of these physiological plastic traits. Nevertheless, it is always difficult to conclusively distinguish whether responses observed in different populations are genetically determined or are the result of plasticity in physiological traits and thus to disentangle the relative contribution of genetic adaptation and of phenotypic plasticity in the evolution of tolerance (Ghalambor et al., 2007; Merilä and Hendry, 2014).

In the present study we investigate whether evolution of cadmium (Cd) tolerance in the non-biting midge *Chironomus riparius* (Meigen, 1804) is associated to different physiological defense mechanisms that are usually triggered by metal exposure. *C. riparius* is a model species in ecotoxicology and several physiological responses have been previously demonstrated to confer tolerance to metals including Cd.

Firstly, it is well established that metallothioneins (MTs) are specifically implied in the cellular protection against metal toxicity and consequent oxidative stress (Kumari et al., 1998; Klaassen et al., 1999; Sandbichler and Höckner, 2016) and, thus, upregulation of the levels of MTs has been postulated to constitute adaptive responses of natural populations to metal exposure in aquatic environments (Gillis et al., 2014; Weng and Wang, 2014). These low molecular weight cysteine-rich proteins play a key role in the regulation of the homeostasis of essential metals and in the detoxification of non-essential metals such as Cd (Amiard et al., 2006; Janssens et al., 2009; Isani and Carpenè, 2014; Sandbichler and Höckner, 2016). Although other proteins such as heat shock proteins and phytochelatins have been implied in the physiological responses to metals in invertebrates (Planelló et al., 2010; Gonçalves et al., 2016; Sandbichler and Höckner, 2016), upregulation of the levels of MTs has been shown to be induced by Cd exposure in *C. riparius* (Fabrik et al., 2008; Toušová et al., 2016).

It has been also shown that metals, and Cd in particular, can induce oxidative stress in aquatic invertebrates (Valavanidis et al., 2006; Connon et al., 2008; Lushchak, 2011; Chandurvelan et al., 2013). In an attempt to protect the cellular integrity of organisms, several enzymatic and non-enzymatic antioxidant cellular defences are upregulated to cope with the increase of reactive oxygen species (ROS) caused by metal exposure (Valavanidis et al., 2006; Lushchak, 2011; Sandbichler and Höckner, 2016). Among these, relationships have been established between metal stress and the content of reduced glutathione (GSH) or the enzymatic activities of glutathione-*S*-transferases (GSTs) and catalase (CAT) (Barata et al., 2005; Gravato et al., 2006). GSH, the most abundant cellular thiol, is a radical scavenger that is used as a first line of defence to prevent the interactions of metals with main cellular structures. GSTs are a large family of enzymes that mostly catalyse the conjugation of various toxic compounds with the thiol group of GSH for subsequent elimination as less toxic and more water soluble and extractable compounds. Finally, CAT is an antioxidant enzyme that degrades hydrogen peroxide into water and oxygen (Valavanidis et al., 2006; Lushchak, 2011). Nonetheless, the excessive production of ROS caused by metal exposure may overwhelm defence responses and disrupt the cellular redox balance, causing oxidative damage to susceptible biological macromolecules including lipid, protein and DNA oxidation that can trigger serious cellular injuries (Livingstone, 2001; Bertin and Averbeck, 2006). Due to this, variations in the levels of lipid peroxidation (LPO) have been extensively used as indicators of the inability of antioxidant defence systems to adequately protect membrane integrity from free radical attacks (Boudet et al., 2013; Wu et al., 2013; Gillis et al., 2014).

The activation of cellular defence mechanisms, however, is a highly energy-demanding process and thus enhanced defence systems that improve the ability to cope with chemical stress are expected to have high metabolic requirements (Sibly and Calow, 1989; Sokolova et al., 2012). For this reason, additional analysis of the patterns of energy consumption and energy available for metabolism may provide important clues to better understand and predict population-level consequences of chemical stress (De Coen and Janssen, 2003; Rodrigues et al., 2015a; Campos et al., 2016) given the critical role of energy budget in stress adaptation and tolerance (Sibly and Calow, 1989).

The main goal of the present study was to demonstrate if these physiological responses were related to tolerance. For that, we used two *C. riparius* populations collected from historically metal-contaminated sites and for which higher tolerance towards acute Cd exposure was observed in comparison to two *C. riparius* populations collected from reference sites (Pedrosa et al. submitted for publication). We hypothesize that heritable differences in the physiological defence mechanisms against Cd exposure between Cd-tolerant and Cd-sensitive populations have to exist. Because all populations were cultured for several generations under common garden laboratory clean conditions before these ecotoxicological tests (i.e. before acute tests and the measurement of physiological responses to Cd exposure) we can exclude major acclimation or carry-over effects and assure that responses were the result of microevolution (Uusi-Heikkilä et al., 2015). By investigating the effects of short-term Cd exposure on protein expression profiles, levels of MTs, biomarkers of oxidative stress and energy related parameters, it is expected to shed light on the physiological processes mediating the differential Cd tolerance among these *C. riparius* natural populations.

2. Material and methods

2.1. Source populations

The four *C. riparius* populations used in all our experiments were collected from the Ave and Vouga river basins, Northern of Portugal. Population CEL was collected from a metal-impacted site located in the village of Celeirós, in the Este River, downstream the city of Braga. The river received direct urban and industrial discharges, without any previous treatment, during decades which resulted in the accumulation of great amounts of metals including Cd that can reach concentrations of 5.96 – 144.00 mg/kg in the local sediments (Soares et al., 1999). Population EST was collected from S. Filipe ditch, a

manmade stream that was constructed to receive the untreated loads of the chemical complex of the municipality of Estarreja. Despite the discharges were interrupted in the 1970s, the ditch is still contaminated with high levels of metals (Pereira et al., 1998), including Cd at concentrations of up to 2.0 mg/kg (Lopes et al., 2014). Population CER and population POR were collected, respectively, from the Alfusqueiro river (Cercosa, municipality of Vouzela) and the Jardim river (Porto de Baixo, municipality of Estarreja). To the best of our knowledge, there were no past-histories of metal contamination for the chosen reference sites.

Acute tolerance to Cd (i.e. 48h-LC_{50}) was previously determined in each population after 6 generations of laboratory rearing in common garden clean conditions according to the standard acute immobilisation test for *Chironomus* sp. (OECD, 2011). The 48h-LC_{50}s values obtained showed that the acute Cd-tolerance in populations from historically metal contaminated sites was approximately 2 fold higher in comparison to reference populations (Pedrosa et al., submitted for publication). Therefore, populations CEL and EST, collected from metal contaminated sites, were defined as being "Cd-tolerant" populations, whereas populations CER and POR, collected from reference sites, were defined as being "Cd-sensitive" populations.

The geographical location and acute Cd tolerance of the different populations is shown in Table 1 while the representation of the sequential approach used in this study is presented in Figure 1.

Table 1 – Site of collection and 48h-LC_{50}s (in mg Cd/L with 95% confidence intervals in parentheses) of the Cd-tolerant populations CEL and EST collected from metal contaminated sites (Cont) and the Cd-sensitive populations CER and POR collected from reference sites (Ref).

Code	Coordinate	Location	Watercourse	Subbasin	Basin	48h-LC_{50}
CEL (Cont)	41.522 N -8.435 W	Celeirós	Este river	Este	Ave	44.20 (40.36-48.41)
EST (Cont)	40.750 N -8.577 W	Estarreja	S. Filipe Ditch	Antuã	Vouga	40.38 (36.38-44.82)
CER (Ref)	40.682 N -8.220 W	Cercosa	Alfusqueiro river	Águeda	Vouga	22.64 (18.21-28.14)
POR (Ref)	40.741 N -8.528 W	Porto de Baixo	Jardim river	Antuã	Vouga	21.83 (16.82-28.34)

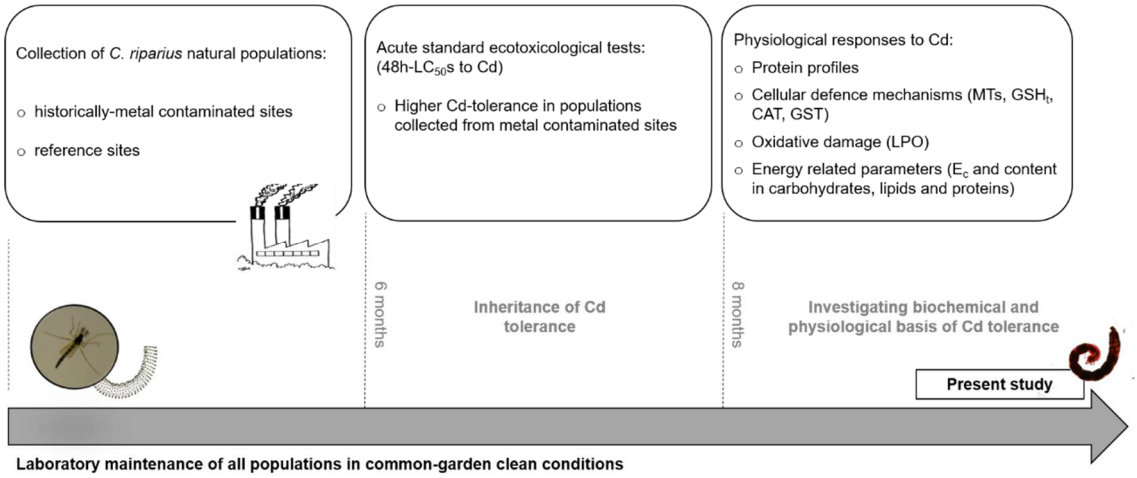

Figure 1 – Schematic representation of the research performed on the *C. riparius* populations collected from the metal contaminated sites CEL and EST and from the reference sites CER and POR.

2.2. Short-term Cd exposure for evaluation of physiological responses

After 8 months of laboratory rearing in common garden clean conditions, larvae of each population were obtained from at least 5 egg ropes hatching within a maximum admissible period of 6 hours. For each population, groups of 250 newly hatched larvae were allowed to grow for 12 days in plastic boxes containing 1.5 cm of inorganic fine sediment (<1 mm grain size) and 2 L of gently aerated ASTM hard water (ASTM, 1980). Grinded commercial fish food (Tetramin®) was added *ad libitum* every other day and ASTM medium was renewed after 6 days.

Afterwards, 12-day old larvae of each population (corresponding to 4th instar larval stage) were collected and randomly transferred to the experimental plastic beakers containing 250 mL of ASTM soft water (pH adjusted to 6.00 in order to avoid Cd precipitation). Three Cd treatments (0.2, 2.0 and 20.0 mg Cd/L prepared from a single stock solution of $CdCl_2$ anhydrous, ACS Grade, Sigma-Aldrich) plus a control treatment with ASTM soft water only, were tested without aeration or food supply. This wide gradient of concentrations was chosen since defence mechanisms that are specifically involved in the tolerance to a given stressor can only be clearly elucidated when organisms are tested to sufficiently high stress concentrations (Barata et al., 2000; Leitao et al., 2013). It is important to note that fourth instar *C. riparius* larvae are more tolerant to Cd (Williams et al., 1986) and as such these concentrations do not elicit mortality in such short exposures.

For each population, 15 replicates were used per concentration (5 replicates for SDS-PAGE protein separation and metallothionein quantification and 10 replicates for the analysis of oxidative stress and energy-related biomarkers), each replicate contained 15 larvae. After a 24-h exposure period, larvae of each replicate were weighted, frozen in liquid nitrogen and stored at -80 ºC for posterior biomarker assessments.

Room conditions of cultures and tests were 20 ± 1 ºC of temperature and 16: 8 h (light: dark) of photoperiod regimen.

2.3. Heat stable protein expression profile and metallothioneins quantification

Heat stable protein separation and metallothionein quantification was performed as in Velez et al. (2016) with only minor modifications. Briefly, frozen samples were thawed on ice and homogenized by sonication in 120 µL of homogenization buffer (0.1 mM Tris-HCl, 10 mM EDTA, 0.5% Triton X-100, 0.1 mM PVP). After centrifugation at 10,000 g for 10 min at 4 ºC, the supernatant was collected, transferred to a microtube, and incubated at 70 ºC for 10 min and centrifuged again with the same cycle conditions. The supernatant was suspended in 2x SDS-PAGE sample buffer (1:1, v:v) and boiled for 4 min at 95 ºC.

Proteins were afterwards separated by SDS-PAGE (Mini-PROTEAN® TGX precast gel – Bio-Rad) according to the Laemmli system (Laemmli, 1970). Molecular weight standards (protein marker II, NZYTech) were also included. Gels were stained with Coomassie brilliant Blue R-250 (Bio-Rad) and screened in a densitometric reader (Model GS 710, Bio-Rad). Protein content of each single protein band was calculated using Quantity One Program Software (Bio-Rad) and expressed as µg/mg wet weight tissue. After protein separation, each band was excised and the extraction was performed according to Shevchenko et al. (2006). Confirmation of metallothioneins (MTs) was derived through quantification of thiol groups according to the method described by Moron et al. (1979).

2.4. Oxidative stress and energy related biomarkers quantification

Frozen samples were thawed on ice and homogenized by sonication in 1600 µL of ultra-pure water. From each replicate, three aliquots of 300 µL were dispensed for the

quantification of energy-related parameters: one for carbohydrate and protein content; another for lipid content; and a third one for electron transport system (ETS) activity. An aliquot of 200 µL (plus 4 µL of 4 % butylated hydroxytoluene in methanol) was taken for the determination of lipid peroxidation (LPO), whereas the remaining tissue homogenate (~ 500 µL) was diluted in 500 µL of 0.2 M K-Phosphate buffer (pH 7.4) and centrifuged for 20 min at 10,000 g (4 ºC). The post-mitochondrial supernatant was afterwards collected for the analysis of total glutathione (GSH_t), catalase (CAT), glutathione-*S*-transferases (GSTs) and post-mitochondrial supernatant (PMS) protein.

All necessary measurements were performed in appropriate 96-well plates and absorbance of samples was read in a Microplate reader MultiSkan Spectrum (Thermo Fisher Scientific, USA). For more detailed methodology please see Rodrigues et al. (2015b).

2.4.1. Oxidative stress biomarkers

LPO levels were measured by quantifying thiobarbituric acid reactive substances (TBARS) at 535 nm and expressed as nmol TBARS/g wet weight tissue (Bird and Draper, 1984). GSH_t was determined at 412 nm using a recycling reaction of reduced glutathione (GSH) with DTNB in the presence of an excess of glutathione reductase and expressed as µM/mg protein (Tietze, 1969; Baker et al., 1990). The activity of GSTs was determined following the conjugation of GSH with CDNB at 340 nm (Habig et al., 1974) and the activity of CAT was determined following the decomposition of the substrate H_2O_2 at 240 nm using UV microplates (Claiborne, 1985). Enzymatic activities of GSTs and CAT were expressed as nmol/min/mg protein and µmol/min/mg protein, respectively. The amount of PMS protein was determined according to the Bradford's method (Bradford, 1976), using bovine γ-globulin as standard.

2.4.2. Energy-related biomarkers

For the quantification of carbohydrate and protein content, a volume of 100 µL of 15 % trichloroacetic acid was added to 300 µL of homogenized samples and then incubated for 10 min at -20 ºC. After centrifugation at 1,000 g for 10 min at 4 ºC, the supernatant was collected and used for carbohydrate measurements. The pellet was then re-suspended in 500 µL of NaOH, incubated for 30 min at 60 ºC and neutralized with 280

µL of HCl. This fraction was used for protein measurements. Carbohydrate content was determined by adding 200 µL of 5 % phenol and 800 µL of H_2SO_4 to the supernatant. Following incubation at 20 ºC for 30 min, samples and glucose (used as standard) were measured at 492 nm. Protein content was measured according to the Bradford's method (Bradford, 1976). Absorbance was measured after 30 min incubation in the microplate at 592 nm and bovine serum albumin was used as standard. Lipid content was determined by adding 500 µL of chloroform and methanol to 300 µL of homogenized samples. After centrifugation at 1,000 g for 5 min, the organic phase was transferred to clean glass tubes containing 500 µL of H_2SO_4 and samples were incubated at 200 ºC for 15 min. After cooling down to room temperature, 1500 µL of ultra-pure water was added to each tube and absorbance measured in the microplate at 375 nm. Tripalmitine was used as standard. Electron transport system (ETS) activity was determined by adding 150 µL of homogenization buffer (0.3 M Tris base; 0.45 % (w/v) PVP; 459 µM $MgSO_4$; 0.6 % (v/v) Triton X-100, pH 8.5) to 300 µL of homogenized samples. After centrifugation at 1,000 g for 10 min at 4 ºC, 50 µL of supernatant was transferred to the microplate and it was added 150 µL of buffer solution (0.13 M Tris base; 0.27 % (v/v) Triton X-100; 1.7 mM NADH; 274 µM NADPH) plus 100 µL of INT solution (p-iodonitrotetrazolium; 8 mM). The absorbance was measured kinetically at 490 nm over a 3-min period.

The conversion of lipid, carbohydrate and protein content into energetic values was calculated using the corresponding energy of combustion: 39,500 mJ/g lipid, 17,500 mJ/g glycogen, 24,000 mJ/g protein (De Coen and Janssen, 1997). The conversion of ETS activity into energetic values, to reflect energy consumption (E_c), was calculated using the specific oxyenthalpic equivalent for an average lipid, protein and carbohydrate mixture of 480 kJ/mol O_2 (De Coen and Janssen, 1997). Lipid, protein and carbohydrate content was expressed as mJ/mg wet weight tissue while energy consumption was expressed as mJ/h/mg wet weight tissue.

2.5. Analysis of Cd concentration in the water

Total Cd was determined after the 24-hour exposure period, in two water samples per population at each treatment in a Graphite Furnace AA (GFAA model Thermo X Series, Peltier Nebulizing Camera, Burgener Nebulizer; CETAC AS510 auto-sampler). Minimum 80 – 120% recovery percentages, blanks and ground water certified reference material (ERM-CA615) were used for analytical accuracy of laboratory measurements. Mean Cd concentrations in the water varied 83.1 – 90.6 % of the nominal concentrations and were

as follows: 0.18 ± 0.01, 1.66 ± 0.14, 17.50 ± 1.20 mg Cd/L. Cd concentration in the control treatments was below the detection limit (i.e. < 0.5 µg/L).

2.6. Data analysis

A Principal Coordinate Ordination (PCO) analysis was undertaken to reveal patterns of protein expression profiles between *C. riparius* populations across the gradient of Cd exposure. For that, a Bray-Curtis distance similarity matrix was submitted to the PCO analysis based on the square root-transformed expression of the electrophoretic protein bands of the different *C. riparius* populations across the Cd concentrations tested. Pearson correlation vectors (correlation > 0.60) were overlayed to the PCO diagram to identify which bands contributed more to the inter-protein profile differences. The PCO of the protein expression profiles was performed using PRIMER v6 software, with PERMANOVA + add-on software (Anderson et al., 2008).

Two-way analysis of variance (ANOVA) followed by Holm-Sidak post hoc test was used to statistically determine effects of “population” and “Cd concentration” in the assessed biomarkers: MTs, GSH_t, CAT, GSTs, LPO, as well as energy consumption (ETS activity) and content in carbohydrates, lipids and proteins. Normality and homoscedasticity of data were tested using Kolmogorov-Smirnov test and Levene’s test, respectively. All ANOVAs were performed using GraphPad Prism® and the significance level was set at 5 percent (GraphPad Inc., San Diego, CA, USA).

3. Results

3.1. Protein expression profiles across experimental conditions

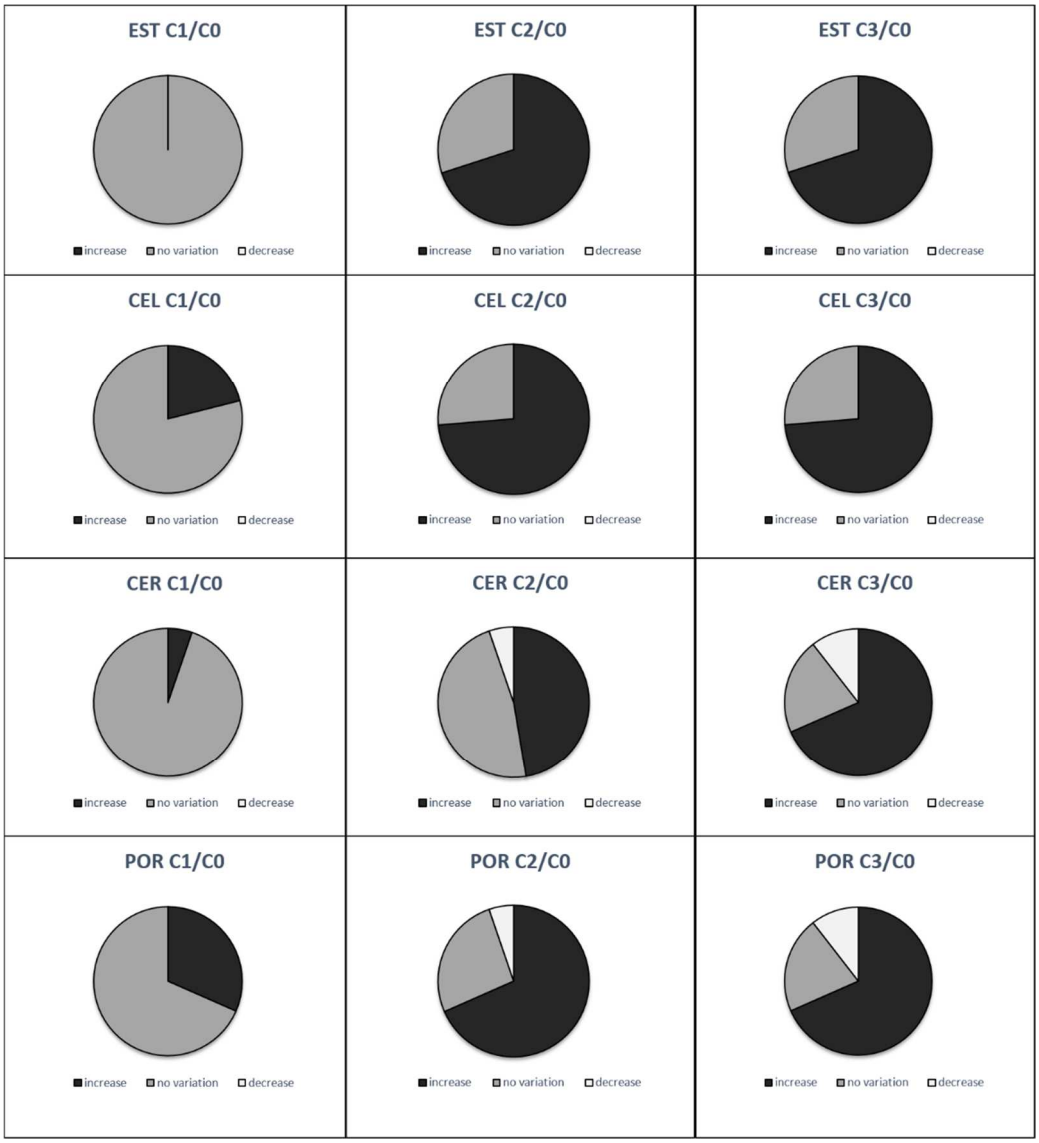

Figure 2 – Percentage of increase (black), no variation (grey) and decrease (white) of protein bands with Cd exposure (C1, C2 and C3 correspond to 0.18, 1.66 and 17.50 mg Cd/L, respectively) in Cd-tolerant (CEL and EST) and Cd-sensitive (CER and POR) populations with respect to control clean condition (C0). Proteins were considered to increase or decrease when protein bands at Cd exposures (C1, C2 and C3) were > 1.5-fold or < 0.5-fold than the control clean condition (C0), respectively.

SDS-PAGE of heat stable proteins allowed separation of 20 distinct protein bands across the experimental conditions (for further details see Table S1). From these, 19 bands were present in all populations, whereas one band (band xA) was present solely in Cd-tolerant population EST. Exposure to increasing Cd concentrations resulted in the

overexpression of most protein bands (Figure 2, Table S1). However, the number of bands overexpressed with Cd was higher in the two Cd-tolerant populations (Figure 2, Table S1). Decreases of protein expression with respect to control clean conditions (C0) were also observed in both Cd-sensitive populations at 1.66 mg/L Cd (C2) for one protein band and at 17.50 mg/L Cd (C3) for two protein bands (Figure 2, Table S1). Furthermore, for most electrophoretic bands, the lowest protein concentrations were found in the Cd-sensitive population POR. In contrast, the highest protein concentrations were generally found in the Cd-tolerant population EST and, more occasionally, in the Cd-tolerant population CEL (Table S1).

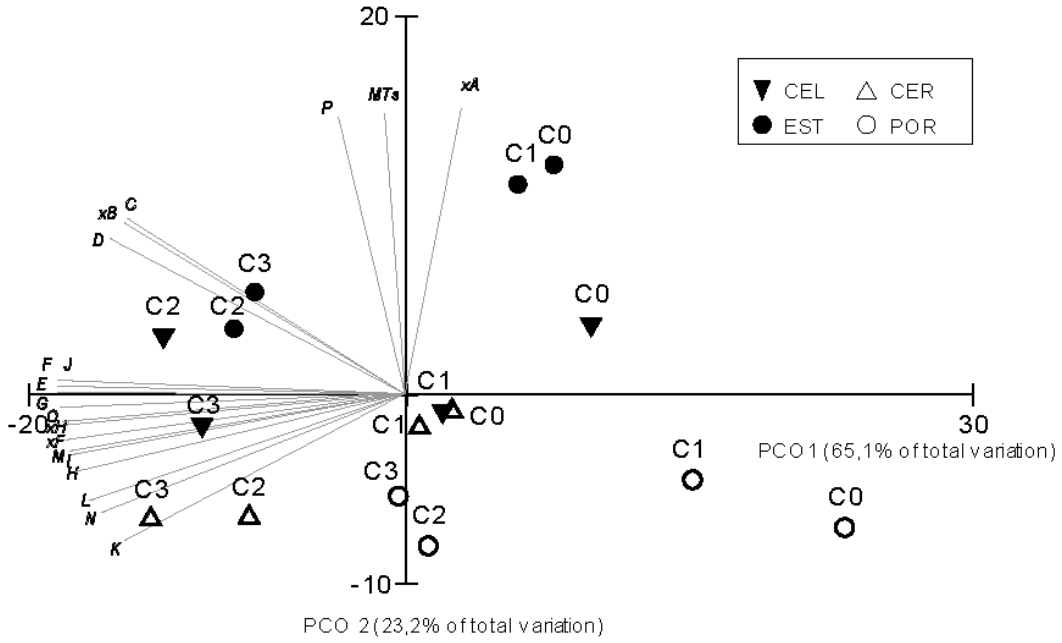

Figure 3 – Principal Coordinate Ordination (PCO) diagram based on the protein profile of Cd-tolerant (CEL and EST) and Cd-sensitive (CER and POR) populations under control clean conditions (C0) and Cd exposures (C1, C2 and C3 correspond to 0.18, 1.66 and 17.50 mg Cd/L, respectively). Pearson correlation vectors are superimposed using the protein expression of electrophoretic bands ($r > 0.60$).

These findings were reinforced by the results of the PCO analysis in which the two first axes explained 88.3% of the total variation of the data and allowed the identification of two major protein expression profiles according to Cd concentration and population tolerance (Figure 3). PCO1, which accounted for 65.1% of the total variation, had generally positive scores for the protein profiles of the different populations under control clean conditions and at the lowest Cd concentration and negative scores for the two highest Cd concentrations tested. PCO2 which accounted for 23.2% of the total variation,

had generally positive scores for the Cd-tolerant populations CEL and EST and negative scores for the Cd-sensitive populations CER and POR.

3.2. Metallothioneins, antioxidant cellular defenses and oxidative damage

Concerning MTs, results of the two-way ANOVA analysis revealed significant influence of the factor “population” (Figure 4, Table 2). MT’s content was consistently higher in larvae of Cd-tolerant populations than in larvae of Cd-sensitive populations across experimental conditions (Figure 4, Table 2). However, Cd exposure did not alter MT’s levels in any of the studied populations (Figure 4, Table 2).

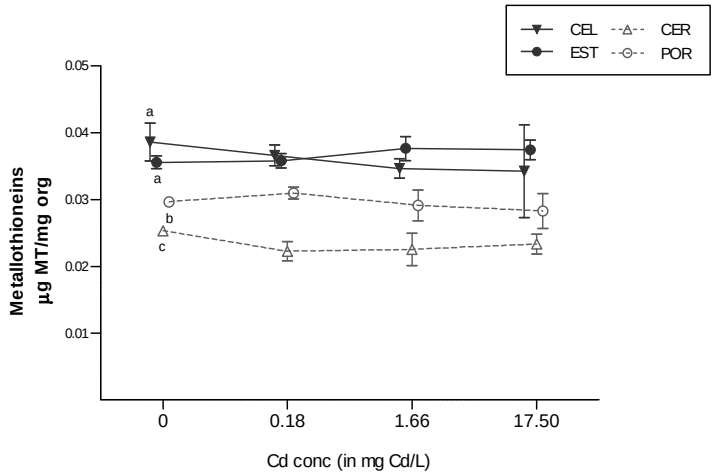

Figure 4 – The effect of short-term cadmium (Cd) exposure in the levels of metallothioneins (mean ± SEM) of Cd-tolerant (CEL and EST) and Cd-sensitive (CER and POR) populations. Different letters mean significant differences ($p < 0.05$) between populations.

The assessed antioxidant cellular defences and levels of oxidative damage in lipids had distinct patterns of variation across populations and exposures to Cd, which were supported by significant effects of population and interaction terms (Table 2). Levels of GSH_t were higher in Cd-tolerant and Cd-sensitive populations under control clean conditions and Cd exposure conditions, respectively. (Figure 5A, Table 2). CAT and GSTs enzymatic activities were not significantly altered by Cd exposure but varied among populations. The Cd-tolerant population CEL showed higher CAT activity across the experimental conditions (Figure 5B, Table 2), whereas both Cd-sensitive populations (CER and POR) showed generally higher GSTs activities compared to Cd-tolerant ones

across most Cd treatments (Figure 5C, Table 2). Levels of LPO were significantly higher in Cd-tolerant populations under control clean conditions but decreased with exposure to Cd. The opposite trend was observed in Cd-sensitive populations with an increase of the oxidative damage in lipids in both Cd-sensitive populations (Figure 5D, Table 2).

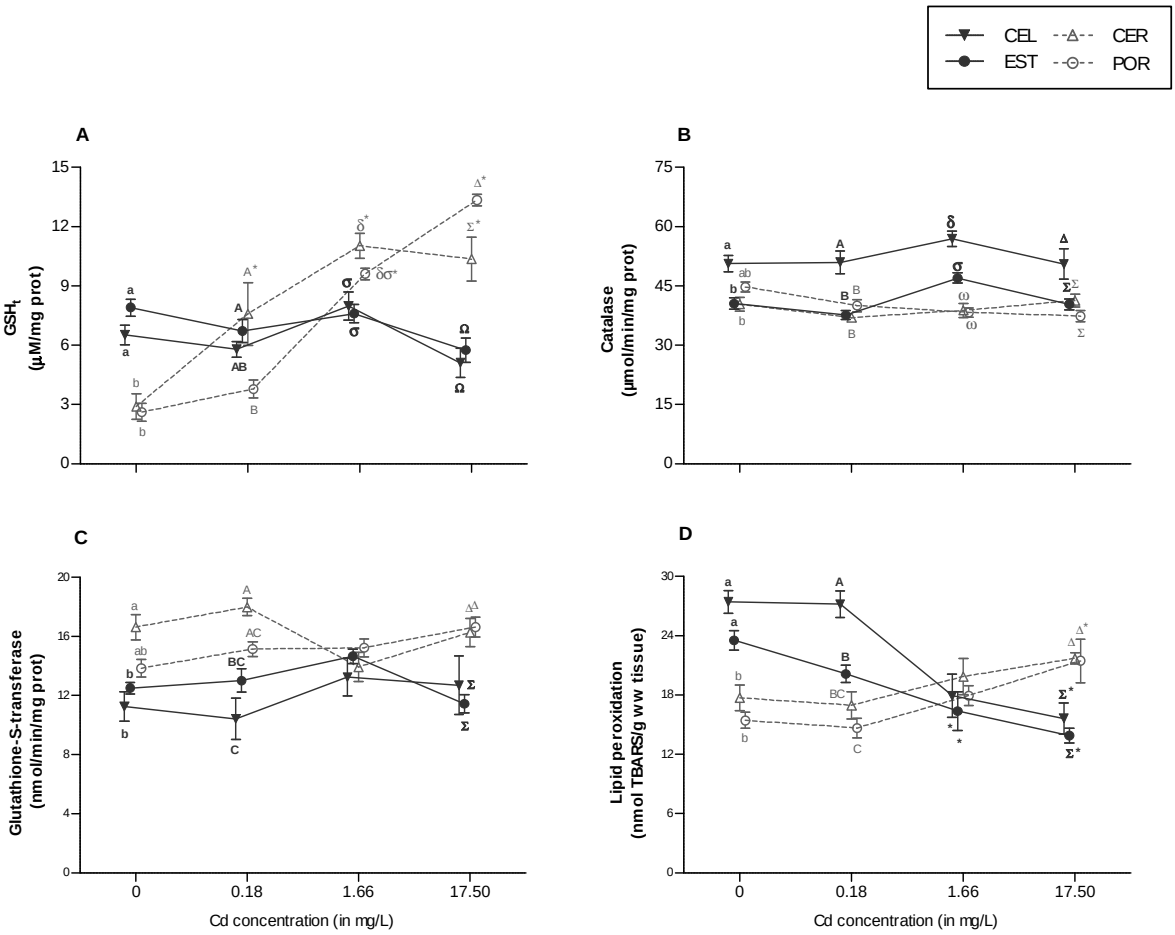

Figure 5 – The effect of short-term cadmium (Cd) exposure in the biomarkers of oxidative stress (mean ± SEM) of Cd-tolerant (CEL and EST) and Cd-sensitive (CER and POR) populations. Shown are mean levels of GSH_t (A), CAT activity (B), GSTs activity (C) and levels of LPO (D) across experimental conditions. Different letters denote significant differences ($p < 0.05$) between populations in each Cd treatment. Asterisks (*) denote significantly different Cd treatments in each population relatively to their respective control treatment (0 mg/L).

3.3. Energy related parameters

A concentration-dependent significant increase in cellular energy consumption (E_c) was observed in both Cd-tolerant populations (i.e. CEL and EST) as well as in Cd-sensitive population CER in response to Cd exposure (Figure 6, Table 2). In the case of the Cd-sensitive population POR, significant increases of E_c were observed only at the lowest Cd concentration tested. Furthermore, both Cd-tolerant populations showed

higher E_c than the two Cd-sensitive populations under exposure to the highest Cd-concentrations (Figure 6A; Table 2).

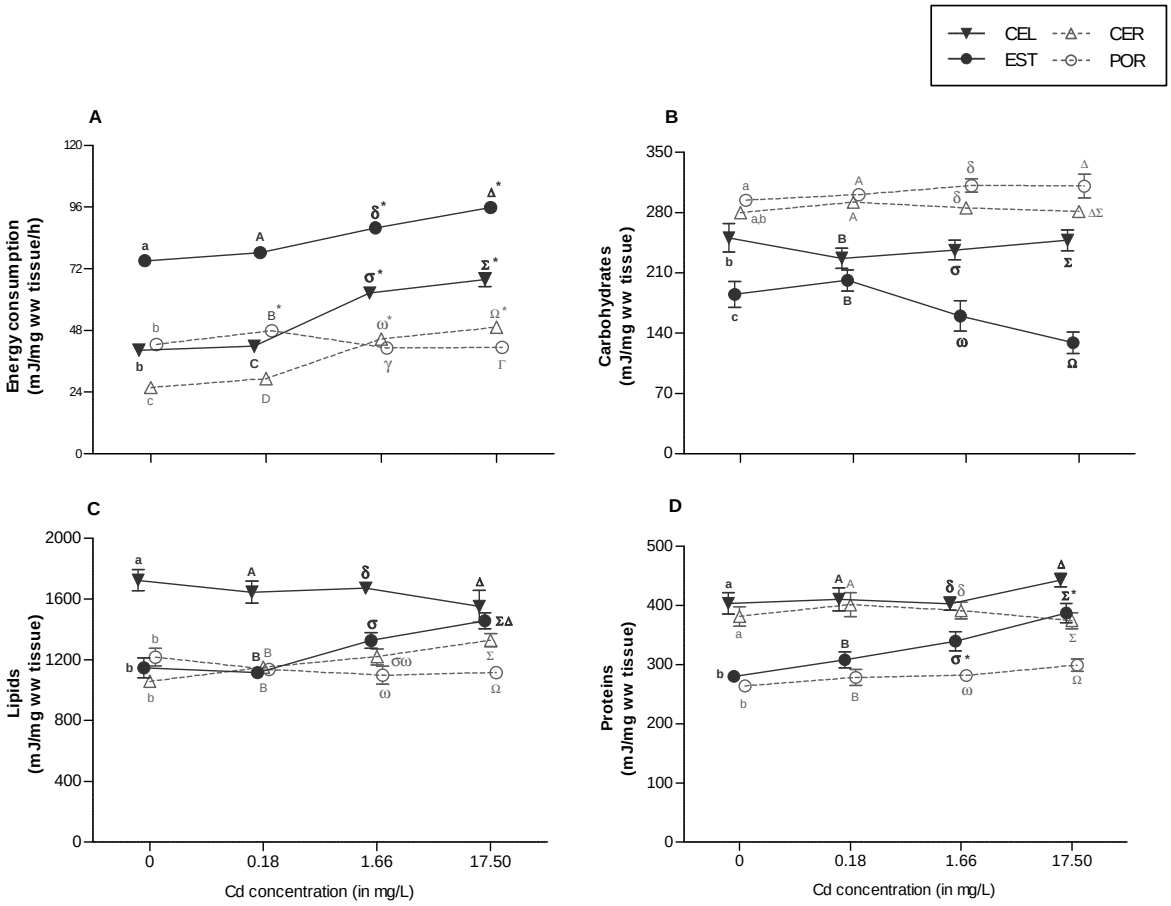

Figure 6 – The effect of short-term cadmium (Cd) exposure in the energy related parameters (mean ± SEM) of Cd-tolerant (CEL and EST) and Cd-sensitive (CER and POR) populations. Shown is the Energy consumption (A), Carbohydrate content (B), Lipid content (C) and Protein content (D). Different letters denote significant differences ($p < 0.05$) between populations in each Cd treatment. Asterisks (*) denote significantly different Cd treatments in each population relatively to their respective control treatment (0 mg/L).

Carbohydrate and lipid contents were not significantly altered by Cd exposure. However, Cd-tolerant populations had lower levels of carbohydrates than Cd-sensitive populations. Population CEL showed to have higher lipid content in comparison to all other populations across all experimental conditions (Figure 6B and 6C, Table 2). Protein content was only altered by Cd exposure in Cd-tolerant population EST (Figure 6D, Table 2).

Table 2 – Two-way ANOVA analysis of the assessed biochemical endpoints with F-values, significance levels (*p*) and percentage of variation (R^2) explained by the factors "Population", "Cadmium" and the interaction "Population x Cadmium". Statistically significant values at $p < 0.05$ appear in bold.

	Population			**Cadmium**			**Population x Cadmium**		
	F	***p***	**R^2**	**F**	***p***	**R^2**	**F**	***p***	**R^2**
1. Cellular defence mechanisms									
MTs	**24.96**	**<0.001**	54.91	0.26	0.853	0.57	0.33	0.962	2.17
GSH_t	**4.07**	**0.008**	2.90	**37.03**	**<0.001**	26.35	**20.38**	**<0.001**	43.52
CAT	**36.17**	**<0.001**	40.68	2.51	0.062	2.82	**2.51**	**0.011**	8.46
GSTs	**16.97**	**<0.001**	23.96	0.48	0.693	0.68	**2.51**	**0.011**	10.62
2. Oxidative damage									
LPO	**8.27**	**<0.001**	9.34	**4.11**	**0.008**	4.64	**10.54**	**<0.001**	35.70
3. Energy related parameters									
Energy consumption	**705.20**	**<0.001**	75.88	**110.00**	**<0.001**	11.84	**21.42**	**<0.001**	6.92
Carbohydrates	**117.20**	**<0.001**	67.68	1.03	0.381	0.60	**3.01**	**0.003**	5.21
Lipids	**69.44**	**<0.001**	53.13	2.62	0.054	2.00	**4.25**	**<0.001**	9.76
Proteins	**69.94**	**<0.001**	53.81	**6.36**	**<0.001**	4.89	**2.50**	**0.011**	5.78

4. Discussion

The purpose of our experimental design was to gain insights on the physiology in terms of cellular and oxidative stress defence underlying the heritable, differential tolerance of *C. riparius* natural populations to acute Cd stress. The overall outcomes of the present work provide evidences of different patterns of response concerning physiological defence mechanisms towards Cd exposure reflecting the observed differences in Cd tolerance between two Cd-tolerant *C. riparius* populations collected from historically metal contaminated sites and two Cd-sensitive populations collected from reference sites. Since all these populations had been cultured for several generations under common garden laboratory clean conditions, we show that differences in the baseline levels and patterns of response towards Cd exposure in terms of protein expression profiles, metallothioneins [MTs] and several antioxidant defences, are the result of microevolution in these physiological responses.

Results of the electrophoretic profile analysis of heat stable proteins showed that Cd exposure elicited protein overexpression in all four *C. riparius* populations which likely reflects the metabolic responses of the different populations to Cd stress (Lee et al., 2006b). Janssens et al. (2009) reported free Cd ions as inducers of a large variety of genes related to the mitogen-activated protein kinase cascade, the metal trafficking system (metallothioneins, phytochelatin synthase and ion pumps), the antioxidant defence system (superoxide dismutase, catalase and peroxidases), the sulfur salvage system (sulfate uptake proteins, synthesis of methionine, cysteine and glutathione), the iron metabolism (ferrotransferrin, iron transporters, ferritine), and the innate immune response (serine proteases and antimicrobial peptides). However, it was worth noting that protein overexpression was generally higher in the two Cd-tolerant populations CEL and EST than in the two Cd-sensitive populations CER and POR. Therefore, results suggest important differences in protein synthesis due to overexpression of genes related to Cd tolerance between Cd-tolerant and Cd-sensitive *C. riparius* populations. Although evidences of differential protein expression profiles have been found in invertebrates in heavily contaminated sites (Matranga et al., 2012; Thompson et al., 2012), we show that these differences are still evident after removing acclimation effects to their native environments.

Among proteins that were differently expressed, MTs showed greater constitutive levels in the two Cd-tolerant populations. This finding is consistent with other studies showing higher constitutive levels of MTs in invertebrate populations inhabiting heavily metal contaminated sites (Costa et al., 2012; Fisker et al., 2013). MTs are considered to play a key role in the detoxification of and protection from Cd and increments in their levels have been associated to an enhanced intracellular Cd ion chelation ability (Dallinger and Höckner, 2013). The observed lack of responses of MTs across Cd treatments is probably related to the short-term exposure period (24 h), that was not sufficient to induce *de novo* synthesis of MTs. Indeed, Wu et al. (2012) did not find any significant changes in the expression of MTs after a 1, 2 or 4-day exposures to high concentrations of Cd in the planarian *Dugesia japonica*, but only after 7 days of exposure. Similarly, Marie et al. (2006) did not observe significant increases of MTs in the bivalves *Dreissena polymorpha* and *Corbicula fluminea* following a 1-d or 3-d exposure to Cd. Therefore, although a central role in Cd detoxification is generally ascribed to MTs in *C. riparius* (Fabrik et al., 2008; Toušová et al., 2016), the upregulation of their levels seems to be relatively slow and longer exposures would be necessary to compare responses between *C. riparius* populations. Unfortunately, in this study we selected short-term exposures to ensure that larvae from the different populations and exposed to different

Cd concentrations were all in the same developmental stage. Future research thus has to focus on longer exposures.

Concerning the antioxidant cellular defences, our results revealed also distinct patterns of variation among Cd-tolerant and Cd-sensitive populations and allowed us to confirm the differential tolerance observed among populations. Cd-tolerant populations CEL and EST had higher levels of GSH_t under control clean conditions compared to Cd-sensitive populations CER and POR which is in agreement with the findings of Meyer et al. (2003) who also reported heritable, higher constitutive levels of GSH_t in a killifish population of *Fundulus heteroclitus* collected from a heavily contaminated site. Glutathione is a major source of intracellular thiols and is involved in cellular protection against oxidative stress and numerous xenobiotic compounds (Lushchak, 2011). Therefore, higher constitutive levels of GSH_t in Cd-tolerant populations might provide competitive advantage in their native environments. However, whilst GSH_t levels of Cd-tolerant populations remained unchanged with increasing Cd concentrations, Cd-sensitive populations markedly upregulated their GSH_t levels in response to Cd exposure. Low constitutive levels of MTs might explain increases of the tripeptide glutathione in Cd-sensitive populations that likely acted as a first line of defence against Cd and its secondary products. However, it is interesting to observe that the upregulation of GSH_t levels in Cd-sensitive populations was accompanied by concomitant increases of the LPO levels showing that the detoxification pathway of Cd through glutathione appears to be inefficient in combating the deleterious effects of Cd. Increases of LPO levels with metal exposure have been documented in many laboratory (Sornom et al., 2012; Vellinger et al., 2013; Wang et al., 2013) and field studies (Gillis et al., 2014; Machado et al., 2014), and have been associated to limited detoxification ability. A plausible explanation for the observed results is that the conjugation of Cd with GSH leads to the generation of oxidized glutathione (GSSG) that can be reversely reduced to GSH by glutathione reductase or exported from the cell (Canesi et al., 1999). However, under high levels of oxidative stress, ROS production may overwhelm the cellular defences of the organisms which causes an imbalance of the cellular redox status and, subsequently, initiates oxidative damage to susceptible macromolecules such as lipids (Novais et al., 2011).

CAT and GSTs were not responsive to Cd exposure. This lack of induction agrees with other studies showing that these enzymes were either unaffected or even inhibited with Cd (Geret et al., 2002; Li et al., 2014) and, thus, it is likely they are not involved in the short-term response to Cd of the different populations. However, there were

significant differences among populations. CAT activity was generally higher in the Cd-tolerant population CEL across the experimental conditions, suggesting a more efficient antioxidant metabolism that confers better protection against ROS (Livingstone, 2003). The higher GSTs activities observed in Cd-sensitive populations, on the other hand, might be related with the preferential use of GSH in the detoxification pathway of Cd in these populations since GSTs catalyse the conjugation of GSH with metal ions (Canesi et al., 1999; Wang et al., 2011; Corticeiro et al., 2013).

The decrease of the LPO levels in Cd-tolerant populations with increasing Cd concentrations, on the other hand, suggests that these populations are equipped with defence mechanisms that allow them to respond more effectively to Cd and even overcompensate the effects of metal-induced ROS attacks (Wang and Wang, 2010). As discussed above, the higher protein expression in tolerant populations under Cd exposure might also have contributed to the reductions in LPO levels, not only due to an increase of other detoxification and repairing proteins that were not evaluated, but also due to a concomitant increase of thiol groups within the cell (Janssens et al., 2009). As such, even if we observed a reduction in oxidative damage in lipids, this does not mean that other macromolecules such as proteins and DNA were not affected by ROS (Valavanidis et al., 2006).

Similar to our findings, Khan et al. (2011) observed that low mortality under metal exposure in an amphipod population (*Gammarus pulex*) collected from a historically metal impacted site was associated to low oxidative damage in lipids. Guilherme et al. (2008) also observed significant lower levels of LPO in a population of the golden grey mullet fish *Liza aurata* inhabiting the surrounding area of the metal-impacted site where *C. riparius* population EST was collected. In a follow-up study, authors found the low susceptibility to oxidative damage in this *L. aurata* population was related with higher activity of glutathione-dependent antioxidant enzymes and, as in our case, with higher GSH_t levels (Brandão et al., 2015).

In summary, the results confirm differential cellular defence mechanisms against Cd stress among the studied *C. riparius* populations, i.e., Cd-sensitive populations showed an increase in GSH_t levels and oxidative damage in lipids with Cd exposure. Cd-tolerant populations, on the other hand, showed higher constitutive levels of the cellular thiols GSHt and MTs that may provide higher chelation ability to Cd, thus minimizing lipid peroxidation. Furthermore, the activation of other defence mechanisms, as suggested by the results of the electrophoretic protein profiles, cannot be neglected. For example, heat shock proteins (HSPs) have been suggested to be rapidly induced by Cd (Lee et

al., 2006a; Planelló et al., 2010) and previous research on natural populations of *C. riparius* found strong association between levels of HSPs and metal contamination (Planelló et al., 2015). Finally, and although this was not addressed in the present study, tolerance to Cd in *C. riparius* natural populations has also been related to increased excretion of Cd (Postma et al., 1996).

Concerning effects of Cd on the energetic endpoints of *C. riparius* populations, results showed that ETS activity (used here as a proxy for cellular energy consumption) was generally induced with Cd exposure reflecting the allocation of energy resources for defence and repair mechanisms that are necessary to maintain the physiological homeostasis of exposed organisms (Choi et al., 2001; Rodrigues et al., 2015b). Concerning carbohydrate and lipid content, there was no clear pattern of response to Cd exposure for any of the populations tested which might be due to the short exposure period. However, Cd exposure caused a significant increase in protein levels of larvae from the Cd-tolerant population EST that might be related with protein synthesis (Lee et al., 2006b) as suggested by the results of the electrophoretic protein expression profile.

Bioenergetic costs of detoxification processes have been demonstrated in other invertebrate populations inhabiting metal contaminated sites (Rowe et al., 2001; Bednarska and Stachowicz, 2013). However, most of the research on this topic has not clearly disentangled the effects of physiological acclimation from inheritance of metal tolerance. For instance, Pook et al. (2009) demonstrated elevated energy demands in a metal-tolerant population of the polychaete *Nereis diversicolor* in which heritable, increased tolerance to Cu and Zn had been previously demonstrated (Grant et al., 1989; Pook et al., 2009). However, effects were determined directly on field collected organisms which limits the understanding of the role of the inheritance of tolerance itself on the energetic costs of exposed populations (Pook et al., 2009). Lagisz et al. (2005) studied Cd and Zn toxicokinetics in the F1-generation progeny of the beetle *Pterostichus oblongopunctatus* collected from reference and metal contaminated sites and found no differences in the metabolic rates, metal accumulation or metal excretion of populations. In our case, all *C. riparius* populations were cultured under common garden clean conditions for several generations and, thus, we can rule out any effects from physiological acclimation on the observed responses and confirm different inherited responses to Cd exposure in terms of energetic metabolism. Indeed, the higher energy consumption (i.e., ETS activity) observed for both Cd-tolerant populations may reflect higher energy demands of enhanced detoxification mechanisms to cope with Cd stress, which might be related with protein synthesis. In contrast, the higher GSH_t levels together

with the observed lower ETS activity among Cd-sensitive populations across Cd concentrations suggest that this glutathione mediated detoxification mechanism is not energetically costly but it is insufficient to avoid oxidative damage in lipids. Nevertheless, longer exposures would be necessary to elucidate these effects on *C. riparius* energetics under Cd exposure since other responses such as reduced locomotion and a general hypometabolism, that has been shown in other invertebrates under chemical stress, could also affect these responses (ETS activity, energy reserves and protein synthesis) (Hochachka and Lutz, 2001; Rodrigues et al., 2016).

Research on these populations provided also some evidences for negative fitness costs associated to enhanced Cd-tolerance, at least for population EST. Both Cd-tolerant populations showed constitutive overexpression of the cellular thiols MTs and GSH_t and population EST had also high energy consumption and low carbohydrate reserves under control clean conditions, the preferential energy resources that are rapidly mobilized for cellular metabolism (Choi et al., 2001). High costs of maintenance in Cd-tolerant population EST support, therefore, the hypothesis that inherited tolerance to environmental contaminants is energy-costly (Sibly and Calow, 1989). However, theory on adaptive responses to contaminants also postulates that the additional energy requirements for defence mechanisms may compete for internal energy resources and divert energy from fitness-related traits (Sibly and Calow, 1989; Ribeiro and Lopes, 2013). According to the results of our previous work, no fitness costs associated to Cd tolerance were found for Cd-tolerant population EST but only for Cd-tolerant population CEL which performed worse under non-toxic conditions exhibiting lower emergence rates, longer time to emergence and smaller imagoes (Pedrosa et al., submitted for publication). However, it should be noted that experiments were performed under standardized, optimal laboratory conditions and the negative consequences of, for example, food limitation or starvation on the fitness performance of populations were not tested (De Haas et al., 2006). Therefore, physiological compensatory mechanisms such as altered food intake or assimilation efficiency might have interacted and counterweighed the higher energy demands of Cd-tolerant population EST (Sokolova et al., 2012). Failure of population CEL to meet the energy cost hypothesis of tolerance, on the other hand, add to the many reported exceptions (Harper et al., 1997; Shirley and Sibly, 1999; Lagisz et al., 2005). According to Shirley and Sibly (1999) fitness costs are, in many cases, explained by negative pleiotropic effects on fitness-related traits of populations.

5. Conclusions

The results of the present study clearly showed different responses to Cd exposure between Cd-tolerant and Cd-sensitive populations. Differences in the inherited tolerance to Cd were evident since Cd-tolerant populations had higher baseline levels of MTs and GSH_t. Moreover, the results also showed important differences in the plasticity of these Cd-tolerant populations: higher inducible levels of protein expression and lack of inducibility of GSH_t with Cd exposure which suggests that also physiological and plastic responses evolved differently in *C. riparius* populations historically exposed to metals. Collectively, the findings of the present study highlight the importance of studying these physiological responses to understand the differential tolerance of natural populations.

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Supplementary material

Table S1 – Protein expression of the electrophoretic bands (mean ± SEM in µg/mg wet weight tissue) in Cd-tolerant (CEL and EST) and Cd-sensitive (CER and POR) populations under control clean conditions (0.00 mg/L Cd) and following Cd exposures of 0.18, 1.66 and 17.50 mg Cd/L. Increase of band expression with respect to control condition is marked with green and decrease of band expression is on red.

Protein band	0.00 mg/L Cd				0.18 mg/L Cd				1.66 mg/L Cd				17.50 mg/L Cd			
	EST	CEL	CER	POR	EST	CEL	CER	POR	EST	CEL	CER	POR	EST	CEL	CER	POR
MTs	0.036±0.001	0.039±0.003	0.025±0.001	0.029±0.000	0.036±0.001	0.037±0.001	0.022±0.001	0.025±0.006	0.038±0.002	0.035±0.001	0.023±0.002	0.029±0.002	0.037±0.001	0.034±0.007	0.023±0.001	0.028±0.003
xA	0.021±0.009	0.000±0.000	0.000±0.000	0.000±0.000	0.022±0.009	0.000±0.000	0.000±0.000	0.000±0.000	0.007±0.007	0.000±0.000	0.000±0.000	0.000±0.000	0.012±0.012	0.000±0.000	0.000±0.000	0.000±0.000
B	0.000±0.000	0.037±0.002	0.026±0.001	0.030±0.001	0.000±0.000	0.029±0.007	0.018±0.005	0.033±0.001	0.044±0.006	0.035±0.001	0.023±0.002	0.029±0.002	0.011±0.011	0.004±0.004	0.005±0.005	0.016±0.007
xB	0.035±0.001	0.011±0.011	0.005±0.005	0.000±0.000	0.035±0.001	0.013±0.008	0.010±0.006	0.000±0.000	0.039±0.003	0.034±0.001	0.027±0.004	0.013±0.008	0.037±0.001	0.028±0.010	0.024±0.002	0.021±0.009
C	0.036±0.001	0.011±0.011	0.015±0.006	0.000±0.000	0.036±0.001	0.029±0.007	0.015±0.006	0.000±0.000	0.044±0.005	0.035±0.001	0.028±0.004	0.006±0.006	0.042±0.003	0.030±0.012	0.026±0.003	0.027±0.007
D	0.038±0.001	0.036±0.001	0.016±0.007	0.000±0.000	0.038±0.001	0.030±0.008	0.024±0.002	0.000±0.000	0.058±0.012	0.039±0.002	0.031±0.004	0.019±0.008	0.053±0.004	0.034±0.013	0.038±0.005	0.032±0.008
E	0.036±0.001	0.034±0.019	0.030±0.001	0.006±0.006	0.036±0.001	0.044±0.007	0.036±0.007	0.027±0.007	0.066±0.015	0.044±0.002	0.041±0.004	0.040±0.005	0.064±0.011	0.054±0.022	0.052±0.009	0.041±0.004
F	0.038±0.001	0.034±0.019	0.030±0.001	0.006±0.006	0.037±0.001	0.052±0.010	0.035±0.007	0.027±0.007	0.084±0.023	0.047±0.004	0.041±0.003	0.042±0.007	0.070±0.010	0.066±0.030	0.059±0.011	0.045±0.004
xF	0.039±0.001	0.035±0.019	0.034±0.002	0.007±0.007	0.039±0.001	0.055±0.011	0.040±0.007	0.027±0.007	0.081±0.020	0.062±0.005	0.054±0.006	0.057±0.012	0.088±0.008	0.097±0.051	0.069±0.012	0.069±0.011
G	0.044±0.002	0.041±0.021	0.042±0.004	0.013±0.008	0.043±0.001	0.063±0.011	0.048±0.007	0.036±0.011	0.114±0.026	0.079±0.009	0.059±0.008	0.053±0.009	0.113±0.012	0.098±0.043	0.086±0.015	0.061±0.008
H	0.043±0.001	0.039±0.021	0.052±0.003	0.007±0.007	0.044±0.002	0.061±0.012	0.049±0.006	0.037±0.011	0.153±0.027	0.130±0.022	0.125±0.023	0.086±0.024	0.148±0.024	0.121±0.048	0.156±0.026	0.082±0.012
xH	0.043±0.001	0.056±0.013	0.059±0.009	0.032±0.012	0.051±0.007	0.072±0.010	0.047±0.007	0.037±0.011	0.141±0.026	0.184±0.034	0.101±0.019	0.061±0.010	0.159±0.019	0.181±0.065	0.130±0.027	0.080±0.009
I	0.047±0.002	0.064±0.013	0.066±0.009	0.043±0.015	0.057±0.006	0.084±0.011	0.058±0.008	0.039±0.012	0.192±0.033	0.176±0.024	0.116±0.021	0.093±0.027	0.181±0.027	0.172±0.069	0.149±0.029	0.114±0.020
J	0.061±0.001	0.095±0.017	0.085±0.009	0.052±0.018	0.076±0.008	0.115±0.014	0.072±0.010	0.050±0.015	0.163±0.029	0.190±0.026	0.110±0.022	0.066±0.014	0.168±0.031	0.155±0.046	0.119±0.024	0.068±0.028
K	0.035±0.000	0.054±0.009	0.038±0.006	0.042±0.007	0.044±0.007	0.058±0.011	0.038±0.006	0.026±0.007	0.088±0.018	0.101±0.014	0.121±0.026	0.049±0.004	0.060±0.011	0.115±0.051	0.154±0.029	0.053±0.013
L	0.049±0.007	0.054±0.012	0.050±0.007	0.039±0.013	0.054±0.006	0.072±0.015	0.053±0.006	0.040±0.005	0.146±0.023	0.204±0.038	0.125±0.021	0.113±0.025	0.092±0.016	0.206±0.079	0.136±0.023	0.109±0.019
M	0.066±0.008	0.062±0.010	0.094±0.015	0.051±0.017	0.077±0.006	0.117±0.033	0.111±0.014	0.070±0.015	0.192±0.024	0.292±0.026	0.153±0.018	0.123±0.024	0.158±0.034	0.269±0.071	0.129±0.009	0.102±0.021
N	0.134±0.020	0.111±0.008	0.178±0.035	0.101±0.024	0.175±0.020	0.217±0.070	0.224±0.034	0.157±0.034	0.322±0.029	0.487±0.059	0.348±0.084	0.262±0.053	0.239±0.042	0.389±0.110	0.364±0.071	0.215±0.023
O	0.247±0.021	0.177±0.013	0.311±0.095	0.205±0.063	0.281±0.036	0.489±0.188	0.325±0.061	0.368±0.092	0.526±0.074	0.857±0.181	0.289±0.080	0.278±0.065	0.344±0.032	0.619±0.174	0.298±0.062	0.285±0.071
P	0.754±0.063	0.457±0.082	0.303±0.029	0.109±0.029	0.837±0.065	0.323±0.100	0.246±0.060	0.171±0.051	0.751±0.124	1.006±0.547	0.098±0.057	0.042±0.012	0.690±0.086	0.315±0.098	0.060±0.033	0.020±0.009

CHAPTER IV:

THE ROLE OF GENETIC DIVERSITY AND PAST-HISTORY SELECTION PRESSURES IN THE SUSCEPTIBILITY OF *CHIRONOMUS RIPARIUS* POPULATIONS TO ENVIRONMENTAL STRESS

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Abstract

Natural populations experiencing intense selection and genetic drift may exhibit limited potential to adapt to environmental change. The present study addresses the following aspects of the “genetic erosion” hypothesis in the midge *Chironomus riparius*: does long-term mercury (Hg) contamination affect the Hg tolerance of midge populations inhabiting such impacted areas? If so, is there any fitness cost under changing environmental conditions? And does genetic impoverishment influence the susceptibility of *C. riparius* to cope with environmental stressful conditions? For this end, we tested the acute and chronic tolerance to Hg and salinity in four *C. riparius* populations differing in their levels of genetic diversity (assessed through microsatellite markers) and past-histories of Hg exposure.

Results showed that the midge population collected from a heavily Hg-contaminated site had higher Hg tolerance compared to the population collected from a closely-located reference site suggesting directional selection for Hg-tolerant traits in its native environment despite no genetic erosion in the field. No increased susceptibility under changing environmental conditions of salinity stress was observed. Moreover, results also showed that populations with higher genetic diversity performed better in the partial life-cycle assays providing evidence on the key role that genetic diversity plays as mediator of populations’ susceptibility to environmental stress.

Our findings are discussed in terms of the suitability of *C. riparius* as a model organism in evolutionary toxicology studies as well as the validity of ecotoxicological assessments using genetically eroded laboratory populations.

Keywords: *Chironomus riparius*, freshwater, genetic adaptation, genetic erosion, mercury, microsatellites, salinity.

1. Introduction

Evolutionary toxicology is a rapidly growing discipline whose purpose is to elucidate the long-term detrimental effects imposed by pollutants in natural populations over timescales that exceed the lifespan of the organisms (Bickham, 2011; Coutellec and Barata, 2011). Research in this field has mostly focused on disentangling the trans-generational and heritable effects of pollutants, how they shape the gene pool of populations and which negative effects they produce in the fitness and evolutionary potential of populations (Bickham, 2011; Ribeiro and Lopes, 2013).

Recently, mounting evidence on the long-term consequences of environmental pollutants has accumulated in the non-biting midge *Chironomus riparius* Meigen (Marinković et al., 2012; Müller et al., 2012; Nowak et al., 2009; Stefani et al., 2014; Vogt et al., 2007a). *Chironomus riparius* has great ecological relevance as it is an abundant and ubiquitous benthic species, that can occupy virtually all freshwater habitats within the Holarctic, where it constitutes a major food resource for the diet of many predators, and plays also an important role in detritus processing and nutrient recycling (Armitage et al., 2012). Furthermore, *C. riparius* is easy to culture under laboratory conditions, it has a short life-cycle, high reproductive output and is sensitive to different pollutants (Armitage et al., 2012; Campos et al., 2014; Rodrigues et al., 2015), which makes it a potential model organism for evolutionary toxicology studies. To date, however, little work has been carried out on natural populations of the species (Postma et al., 1995) and, thus, most of the empirical evidence has arisen from studies using laboratory populations (Marinković et al., 2012; Müller et al., 2012; Nowak et al., 2012; Stefani et al., 2014).

As part of a comprehensive case-study on the evolutionary responses of natural populations of *C. riparius* to historical metal contamination in Portuguese freshwaters, here we investigated the influence of both genetic background and levels of genetic diversity in the susceptibility of midge populations to environmental stress. Previous research performed in our laboratory showed that *C. riparius* populations collected from metal impacted sites had an overall higher tolerance to acute cadmium (Cd) stress in comparison to populations from reference sites. Since all populations had been maintained under clean laboratory conditions for several generations prior of performing the ecotoxicological tests, differences in Cd-tolerance were suggested to have a genetic basis, i.e., tolerance traits were conferred to midges via differences in their genetic background (Pedrosa et al., submitted for publication). Furthermore, we also found evidences for higher constitutive levels of the cellular thiols metallothionein and

glutathione in populations collected from metal contaminated sites suggesting, therefore, improved defence mechanisms that likely allow these populations to better cope with metal toxicity (Pedrosa et al., submitted for publication).

Among the experimental populations of our case-study, one *C. riparius* population was collected from S. Filipe ditch, a coastal man-made stream that received mercury-rich effluents from a chlor-alkali plant for decades. Although the discharges were interrupted in 1975, the ditch is still considered a hotspot of mercury (Hg) contamination (Costa and Jesus-Rydin, 2001; Reis et al., 2009) and the local surface sediments can reach concentrations of up to 17.40 $\mu g \cdot g^{-1}$ Hg (Pedrosa et al., submitted for publication). Therefore, we hypothesized that the *C. riparius* population collected from this site genetically adapted to Hg as a result of intense and continuous selection for Hg-tolerant traits. However, and although genetic adaptation to Hg may have increased the short-term survival of the population under such adverse environmental conditions, theory on micro-evolutionary responses predicts that pollutant-adapted populations may carry substantial fitness costs that cause them to become more vulnerable under changing environmental conditions (Kliot and Ghanim, 2012; Posthuma and Van Straalen, 1993; Ribeiro and Lopes, 2013). Therefore, studying evolutionary processes of pollution-tolerant populations gains increasing relevance at the light of the ongoing and future climate changes that are likely to alter the aquatic ecosystems and introduce a suite of new selective pressures (Hoffmann and Sgro, 2011; Pauls et al., 2013). In this sense, the projections of rising sea level associated with coastal erosion make saline intrusion a potential important perturbation for aquatic populations inhabiting coastal freshwater ecosystems (IPCC, 2014). Salinity has the potential to significantly affect the survival, growth and reproduction of freshwater organisms (Loureiro et al., 2013) and, hence, it is critical to better understand and anticipate how aquatic biota already impacted by chemical pollution can cope with such additional pressure.

Moreover, and besides the role played by the intrinsic genetic background of populations, also the amount of genetic diversity harbored by populations has the potential to influence the susceptibility to environmental stress (Frankham et al., 2002). High levels of genetic diversity are, in general, seen as indicators of population's health that potentially increase the responsiveness to environmental stress. Conversely, low levels of genetic diversity are suggestive of increased vulnerability to environmental stress (Nowak et al., 2012; Vogt et al., 2007b). As such, while the levels of genetic diversity of our experimental populations were found to be similarly high in the field (Pedrosa et al., submitted for publication), maintenance of midge populations in the laboratory for relatively long periods of time may result in the loss of considerable

amounts of genetic variation (Nowak et al., 2007b). Laboratory populations are established from a limited number of organisms, they are reproductively isolated and have comparatively much smaller population sizes which make them particularly prone to losses of genetic variation caused by chance events (i.e. random genetic drift) and, so, these effects need also to be considered when investigating the sensitivity of midge populations to environmental stress (Nowak et al., 2007b).

Against this background, the present study was undertaken to address the following questions: 1) Does long-term historical Hg contamination influence the Hg-tolerance of *C. riparius* populations inhabiting such impacted areas? If so, are there fitness cost under changing environmental conditions? 2) And, also, do levels of genetic diversity affect the susceptibility of *C. riparius* populations to cope with environmental stressful conditions?

For that, we assessed the acute and chronic tolerance to Hg and salinity in four *C. riparius* populations varying in their past-history selection pressures and levels of genetic diversity: two natural populations collected one from the Hg-contaminated site S. Filipe ditch and one from a closely located reference site, one laboratory population and a "Gen$^+$ population" generated from the cross-breeding of several *C. riparius* populations. In addition, we also monitored the levels of genetic diversity of the two natural populations under common-garden laboratory conditions in order to assess the degree of genetic impoverishment over time in the laboratory. We used microsatellite markers to characterize the levels of genetic diversity and the genetic divergence among the experimental populations.

2. Material and Methods

2.1. Experimental *C. riparius* populations

Four pure *C. riparius* populations were chosen for the present work: populations CONT, GEN$^+$, LAB and REF.

Population CONT was collected from the heavily Hg-contaminated site S. Filipe ditch (40º45'01''N, 8º34'37.48''W) and population REF was collected from a nearby reference site located in the Jardim river (40º44'29''N, 8º31'41''W). In order to establish pure *C. riparius* populations in the laboratory, each field-collected *Chironomus* sp. larva was morphologically checked with a stereo microscope and only *Chironomus thummi* type larvae (which include *C. riparius* and its sibling sister species *Chironomus piger*) were grown in breeding chambers, allowed to mate and lay the egg ropes. Each egg rope was

afterwards collected, cultivated individually and genotyped according to mtDNA barcoding methodology (Pfenninger et al., 2007). A total of 10 *C. riparius* egg ropes was used to establish populations CONT and REF. Both populations were cultured under common-garden laboratory conditions for ~40 months prior of determining their tolerance to Hg and salinity stress.

In addition, we tested also a laboratory population herein designated as population LAB that has been regularly used by our research group to perform ecotoxicological assessments of different stressors. This population has been reared in our laboratory for > 20 years and has received occasional genetic refreshments from other laboratories. Finally, population GEN^+ was established from the cross-breeding of five *C. riparius* populations including the three populations used in the present work (i.e. populations CONT, LAB and REF). Population GEN^+ was generated from four egg ropes of each of the five source populations two generations prior to performing the ecotoxicological tests in order to allow admixture of the genetic material.

All populations were cultured with large numbers of individuals in identical breeding cages according to the recommendations for *C. riparius* culturing described in the Annex 2 of OECD guideline no. 218 (OECD, 2004a). Larvae of each population were reared in plastic aquaria containing 1.5 cm of inorganic fine sand (<1 mm) and gently aerated American Society for Testing and Materials (ASTM) hard water (ASTM, 1980). Grinded fish flake food (Tetramin®) was added *ad libitum* three times a week and medium was renewed every week. Temperature regimen was 20 ± 1 ºC and photoperiod cycle was 16: 8h (light: dark).

The taxonomic identification of all experimental populations was confirmed genetically as *C. riparius* according to the microsatellite marker system used for the characterization of the levels of genetic diversity of populations (section below) that display species-specific alleles and, hence, allow species discrimination (Nowak et al., 2006).

2.2. Genetic diversity of *C. riparius* populations

The levels of genetic diversity of the four *C. riparius* populations were estimated based on the allelic variation of seven microsatellite loci in 29 – 30 individuals of each population (Table 1). DNA was extracted according to the DNA hotSHOT method (Montero-Pau et al., 2008). Tissue was homogenized in 30 µl of alkaline lysis buffer (25 mM NaOH, 0.2 mM Na_2EDTA, pH 8.00) for 30 min at 95 ºC followed by 5 min at 4 ºC. After thermal

treatment, 30 µl of neutralizing solution (40 mM Tris-HCl, pH 5.00) was added and samples were stored at 4 ºC for posterior use. Microsatellite fragments were amplified in a T-Gradient thermocycler (Biometra, Göttingen) according to the following cycling conditions: 36 x (30 s 94 ºC, 30 s 55 ºC, 40 s 72 ºC) and 1 x 5 min 72 ºC (for further information please see Supplementary Material S1). Reaction mixture contained 2.4 mM $MgCl_2$, 0.25 mM dNTP, 0.2 µM of each specific primer and 0.5 U Taq DNA polymerase in a total volume of 8 µl of reaction mixture per microsatellite plus 2 µl of DNA sample. Microsatellite fragments were then sequenced in an ABI 3730 capillary sequencer (Applied Biosystems, CA), automatically scored using software Genemarker© version 2.6.3 (Softgenetics, State College, PA) and manually corrected whenever necessary.

To assess the loss of genetic diversity over time in the laboratory in the two field-collected populations (i.e. populations CONT and REF), we also monitored the levels of genetic diversity when populations were first established in the laboratory (T_0 derived from Pedrosa et al., submitted for publication) as well as after 28 and 40 months under laboratory conditions. Genetic diversity after 40 months under laboratory conditions corresponds to the levels of genetic diversity of the populations when the ecotoxicological tests of the present study were performed.

2.3. Mercury and salinity tolerance of *C. riparius* populations

2.3.1. Test chemicals

Mercuric (II) chloride ($HgCl_2$; Merck KGaA, Darmstadt, Germany) and sodium chloride (NaCl; Sigma-Aldrich, St. Louis, MO, USA) were used to prepare the appropriate mercuric and saline stock solutions, respectively, with nanopure (18 MΩ) water.

2.3.2. Acute ecotoxicological tests

Acute tests were performed according to the OECD guideline 235 acute immobilisation test for *Chironomus* sp. (OECD, 2011) and test organisms of each population were obtained from four egg-ropes hatching within a 6h-period. Newly hatched first instar larvae (< 24 h-old) were then exposed to a range of Hg and NaCl concentrations for a 48 h-period without aeration or food supply. A total of seven mercuric treatments (nominal Hg concentrations corrected to the measured stock solution concentration were: 1.12, 2.23, 4.47, 8.94, 17.87, 35.74 and 71.53 mg $Hg \cdot l^{-1}$), seven salinity treatments (0.5, 1.0, 2.0, 4.0, 8.0, 16.0 and 32.0 mg $NaCl \cdot l^{-1}$) and controls (ASTM

hard water only) were tested. Each treatment consisted of 6 replicates, each replicate containing 5 larvae.

Experiments were carried out in 6-well plastic multiplates, each well containing a total volume of 10 ml of test solution. Larval immobilisation was monitored after 48 h of exposure period using a stereomicroscope.

2.3.3. Chronic ecotoxicological tests

Static, 28-day partial life-cycle tests were performed according to the OECD guideline 219 (OECD, 2004b) and test organisms of each population were obtained, again, from four egg-ropes hatching within a 6h-period. Newly hatched larvae were then transferred to plastic aquaria and allowed to grow for two days in gently aerated ASTM hard water, inorganic sand (< 1 mm) and grinded fish flake food (Tetramin®). Afterwards, larvae were collected and randomly assigned to the test vials containing 250 ml of gently aerated test solution and 1.5 cm of inorganic sand. Mercuric (actual Hg concentrations: 16.58; 29.34; 47.31 µg $Hg \cdot l^{-1}$) and salinity treatments (actual concentrations: 1.3, 2.5, 5.0 mg $NaCl \cdot l^{-1}$) as well as the controls (ASTM hard water only) were prepared 24 h prior of assigning the larvae to the test vials. A total of six replicates were tested per treatment, each replicate containing 10 larvae. Organisms were fed every other day with Tetramin® at a daily rate of 0.5 mg *per* larvae.

Emergence was recorded daily and imagoes were collected from emergent traps, dried at 50 ºC for 24 h and weighted in a microbalance (Mettler UTM2).

2.3.4. Mercuric and saline concentrations

Total Hg was determined in an Advanced Mercury Analyser model AMA 254 (LECO, MI, USA) by atomic absorption spectrometry (AAS) with thermal decomposition of the sample and collection of the Hg vapor on a gold amalgamator (Costley et al., 2000). The operational conditions used were: drying time of 60 s, decomposition time of 150 s and waiting time of 45 s, for solid samples and a drying time of 100 s for water samples. Mercuric concentration was determined in the stock solutions as well as in three replicates of each Hg treatment at the beginning (i.e. after a 24h rest period) of the chronic test. Analytical accuracy was assessed using the certified reference material TORT-2 (lobster hepatopancreas) from the National Research Council Canada. The

recovery values achieved for TORT-2 analyses ranged from 92.4 to 103.4% with standard deviations below 3%.

Salinity was determined using a digital refractometer HI 96822 (HANNA Instruments, Woonsocket, USA) and concentrations were adjusted to the required working solutions whenever necessary by adding appropriate volumes of ASTM hard water.

2.4. Statistical analysis

Population genetic parameters [mean number of alleles per locus [N_A], observed heterozygosity (H_O), expected heterozygosity (H_E) and deviations from Hardy-Weinberg equilibrium (HWE)] were calculated using the statistical package GenAlex 6.501 (Peakall and Smouse, 2006). Hierarchical analysis of molecular variance (AMOVA) was also performed to estimate the genetic divergence (as Φ_{PT}) among populations. Pairwise Φ_{PT} were tested using a permutation procedure with 999 runs and significant differences were tested for $p < 0.05$. Genetic relationship among populations was also graphically assessed through Principal Coordinate Analysis (PCoA).

Acute tolerance of the different *C. riparius* populations to Hg and NaCl was evaluated in terms of 48h-LC_{50}s derived from four-parameter logistic curves. Significant differences between populations in the estimated LC_{50}s were statistically compared following the method described in (Sprague and Fogels, 1977). Chronic tolerance to Hg and NaCl was analysed through two-way analysis of variance (ANOVA) followed by Holm-Sidak post-hoc tests to statistically assess the influence of the factors “population” and “toxic stress” (as Hg and NaCl) in the life-history endpoints emergence rate, time to emergence and weight of male and female imagoes. The highest Hg treatment (43.72 $\mu g \cdot l^{-1}$) was excluded from the statistical analyses of time to emergence and weight of imagoes due to the low emergence rate within all four populations (0.00 – 6.67%) that could result in statistical biases. Assumptions of normality and homoscedascity were met for all life-history endpoints as confirmed by residual plot analyses and statistical significance was considered for $p < 0.05$. All statistical analyses were performed using GraphPad Prism® version 6.00 for Windows (GraphPad Software, La Jolla, California, USA).

3. Results

3.1. Genetic diversity of *C. riparius* populations

Results of the population genetic parameters showed considerable differences in the genetic diversity of the four experimental *C. riparius* populations (Table 1). Generally, mean number of alleles per locus (N_A), observed heterozygosity (H_O) and expected heterozygosity (H_E) were highest in population GEN$^+$ and lowest in population REF. In addition, populations CONT and GEN$^+$ were polymorphic across all microsatellite loci whereas no allelic variation was found in population LAB at locus Msc 3 and in population REF at loci Msc 1, 5 and 7. No significant HWE deviations were found within populations CONT, LAB and REF after Bonferroni correction. However, population GEN$^+$ showed significant HWE deviations at two loci (heterozygosity excess at locus Msc 1 and heterozygosity deficiency at locus Msc 6; Table 1).

Table 1 – Summary of the *C. riparius* population genetic parameters based on a total of 29-30 individuals per population (n). Number of alleles per locus (N_A), observed heterozygosity (H_O) and expected heterozygosity (H_E) are shown as mean values ± SEM. Asterisks (*) indicate significant deviations from Hardy-Weinberg equilibrium at microsatellite loci after Bonferroni correction. Estimates of population pairwise genetic differentiation (as Φ_{PT}) are shown below diagonal (*p* values < 0.001 for all pairwise comparisons).

		Genetic diversity			Genetic differentiation			
	n	N_A	H_O	H_E	CONT	REF	GEN$^+$	LAB
CONT	29	3.00±0.31	0.42±0.04	0.48±0.05	-			
REF	29	1.51±0.20	0.21±0.09	0.21±0.08	0.644	-		
GEN$^+$	30	3.57±0.48	0.49±0.06^{*Msc1,6}	0.56±0.06	0.189	0.485	-	
LAB	29	2.43±0.48	0.43±0.09	0.40±0.08	0.437	0.742	0.315	-

Analysis of molecular variance revealed that genetic divergence among the four populations was very high (Φ_{PT} = 0.50, p < 0.001) and pairwise population genetic divergence ranged from 0.19 (GEN$^+$ *vs* CONT) to 0.74 (REF *vs* LAB; Table 1). This pattern was reinforced by the results of the principal coordinate analysis (PCoA) that clearly discriminated populations CONT, LAB and REF as distinct genetic entities while population GEN$^+$ showed considerable overlapping with populations CONT, LAB and REF (Figure 1).

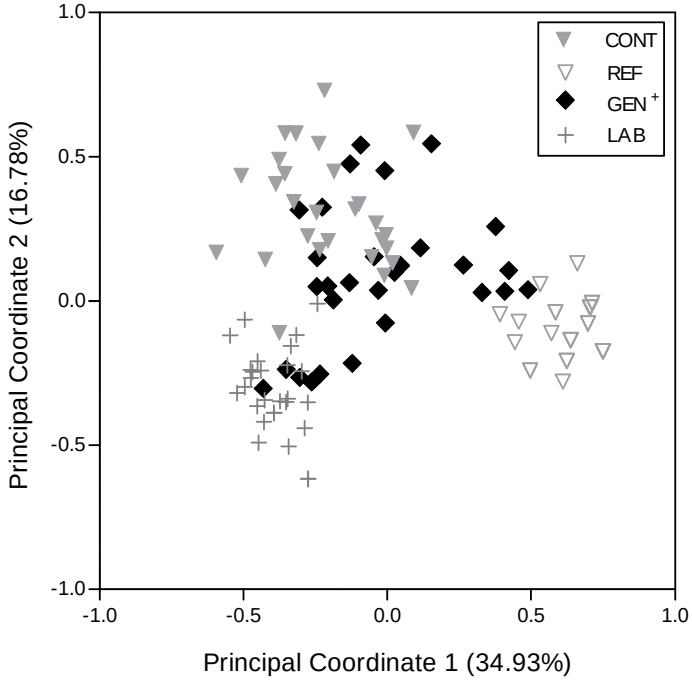

Figure 1 – Principal Coordinate analysis (PCoA) of microsatellite data from the individuals analysed in the four *C. riparius* populations: CONT, GEN^{+}, LAB and REF.

Furthermore, results also showed there was a decrease in the levels of genetic diversity of populations CONT and REF after a 40 month-period of laboratory rearing (Figure 2A, B). However, the decrease in the levels of genetic diversity was noticeably higher in population REF (~58.5% loss of H_E and ~60.7% of N_A) than in population CONT (~15.0% loss of H_E and ~19.2% of N_A).

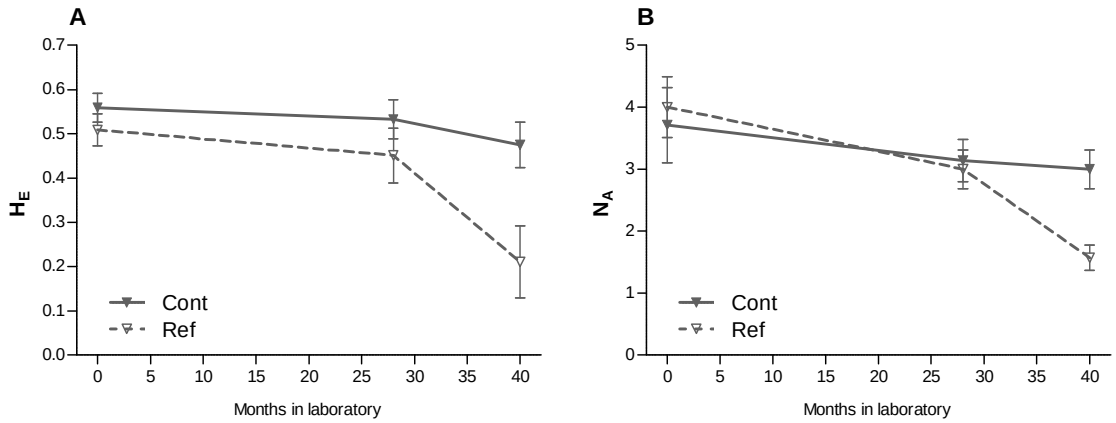

Figure 2 – Loss of genetic diversity in terms of expected heterozygosity (A) and number of alleles per locus (B) over time in the laboratory (mean ± SEM) in the *C. riparius* populations collected from Hg-impacted (CONT) and reference (REF) sites.

3.2. Acute tolerance of *C. riparius* populations

No mortality in the control treatments was observed for any of the experimental populations. The four-parameter logistic curves showed good fit to the data ($R^2 > 0.86$) and clear dose-response curves were obtained for all populations across the tested concentrations of Hg and NaCl.

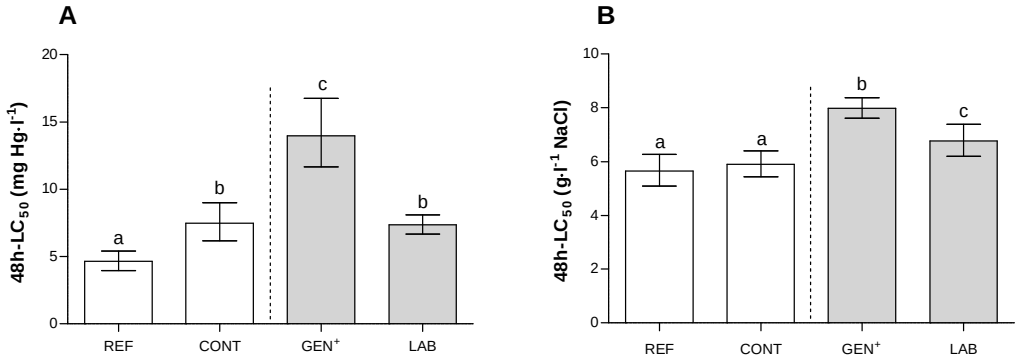

Figure 3 – Acute tolerance to Hg (A) and NaCl (B) of the four *C. riparius* populations determined as 48h-LC_{50} (± 95% confidence intervals). Different letter mean significant differences between populations for a significance level of $p < 0.05$.

Individual toxicity values for each population, i.e. 48h-LC_{50}s, are depicted in Figure 3. Overall, population GEN^+ appeared to be the most tolerant population to acute concentrations of either Hg or NaCl while, on the contrary, population REF was the most sensitive one (Figure 3). Concerning Hg, the estimated 48 h-LC_{50}s (± 95% CI) of the different populations varied within a threefold factor, ranging from 4.64 $mg \cdot l^{-1}$ Hg (3.97 – 5.43) in population REF to 13.97 $mg \cdot l^{-1}$ Hg (11.66 – 16.75) in population GEN^+ and were ranked as follows from most to least sensitive population: REF < LAB = CONT < GEN^+ (Figure 3A). With respect to NaCl, the LC_{50}s varied from 5.66 $g \cdot l^{-1}$ NaCl (5.09 – 6.28) in population REF to 7.99 $g \cdot l^{-1}$ NaCl (7.62 – 8.38) in population GEN^+ and the ranking of population's sensitivity (from most to least sensitive) was: REF = CONT < LAB < GEN^+ (Figure 3B).

3.3. Chronic tolerance of *C. riparius* populations

Emergence rates in the controls were above 80% (Figures 4A and 5A) and, therefore, all *C. riparius* populations fulfilled the validity criteria of OECD guideline 219 (OECD, 2004b). Exposure to Hg and NaCl reduced the emergence rate and delayed the time to emergence of imagoes within all populations (Figures 4A-C and 5A-C). No clear effects

in the weight of imagoes were observed for Hg exposures, but there was a concentration-dependent reduction in the weight of male and female imagoes of the different populations across NaCl exposures (Figures 4D-E and 5D-E).

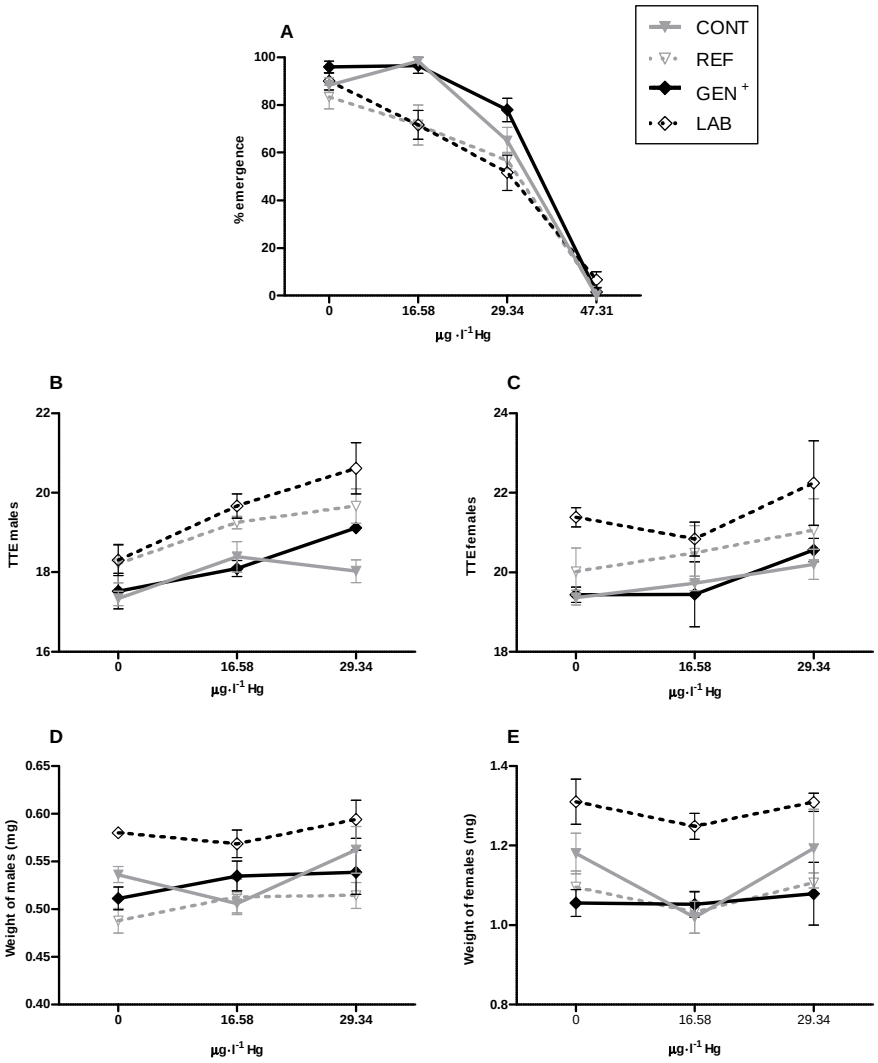

Figure 4 – Life-history trait responses (Mean ± SEM) of the four *C. riparius* populations exposed to Hg: (A) emergence rate (%), (B, C) time to emergence of males and females (days), (D, E) weight of adult males and females (mg).

Two-way ANOVA analyses revealed significant effects of “population” and of “Hg” and “NaCl” in the assessed life-history endpoints (Table 2). The only exception was the weight of male imagoes that was not significantly affected by Hg exposure. There were also significant interactions between factors for emergence rate, indicating the different populations responded differently to the increase in Hg and NaCl concentrations. Likewise, significant interactions between “population” and “NaCl” were found for time to emergence of males and for weight of female imagoes.

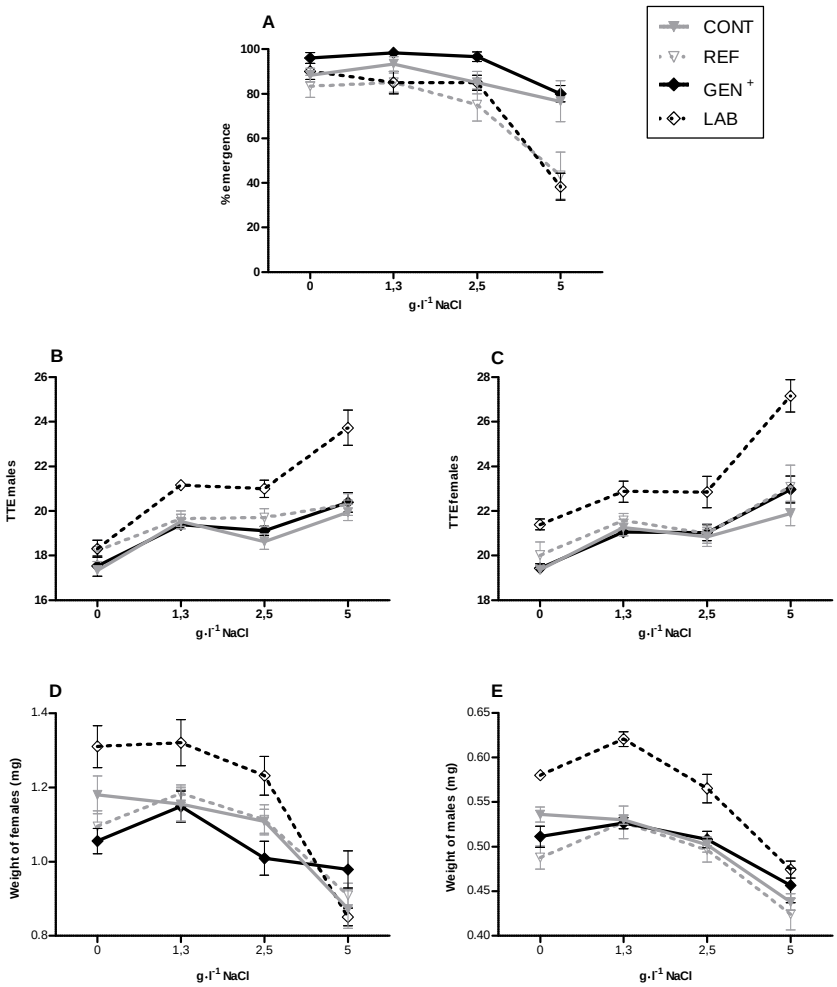

Figure 5 – Life-history trait responses (Mean ± SEM) of the four *C. riparius* populations exposed to NaCl: (A) emergence rate (%), (B, C) time to emergence (days), (D, E) weight of adult males and females (mg).

Concerning Hg exposures, at low and intermediate Hg concentrations, the emergence rates of populations CONT and GEN$^+$ were higher compared to populations LAB and REF (Figure 4A). Conversely, time to emergence and weight of imagoes of population LAB were higher across Hg treatments compared to populations CONT and GEN$^+$ as well as compared to population REF (Figure 4B-E; Table 2). With respect to NaCl exposures, emergence rates of populations CONT and GEN$^+$ were, again, higher compared to populations LAB and REF (Figure 5A). Furthermore, male and female imagoes from population LAB took again more time to emerge across NaCl treatments (including controls) but were heavier than those from the other three populations experimentally assessed (Figure 5B-E; Table 2).

Table 2 – Two-way ANOVAs for the life-history traits under Hg and salinity exposures for the four *C. riparius* populations. Shown are degrees of freedom (df), F-values, significance level (*p*) and percentage of variation explained by stress (as Hg and NaCl), population and interaction between factors for emergence rate, time to emergence (TTE) for males and females and weight of male and female imagoes.

		Two-way ANOVA Hg				Two-way ANOVA Salinity			
Endpoint	**Factor**	**df**	**F**	**p**	**% var**	**df**	**F**	**p**	**% var**
1. Emergence rate	Stress	3	326.30	< 0.001	88.04	3	29.94	< 0.001	38.35
	Pop	3	9.42	< 0.001	2.54	3	13.37	< 0.001	17.12
	Stress x Pop	9	3.39	0.002	2.75	9	2.79	0.007	10.72
2. TTE males	Stress	2	17.13	< 0.001	25.12	3	47.16	< 0.001	46.09
	Pop	3	11.72	< 0.001	25.76	3	25.13	< 0.001	24.56
	Stress x Pop	6	1.11	0.368	4.88	9	2.55	0.013	7.48
3. TTE females	Stress	2	3.69	0.031	1.67	3	34.79	< 0.001	45.18
	Pop	3	6.31	0.001	5.15	3	22.03	< 0.001	28.61
	Stress x Pop	6	0.24	0.963	35.36	9	1.59	0.132	6.21
4. Weight males	Stress	2	2.80	0.069	5.15	3	54.77	< 0.001	46.57
	Pop	3	12.83	< 0.001	35.36	3	30.94	< 0.001	26.31
	Stress x Pop	6	0.98	0.445	5.42	9	1.74	0.094	4.44
5. Weight females	Stress	2	3.17	0.050	6.14	3	32.75	<0.001	44.51
	Pop	3	12.18	< 0.001	35.42	3	6.05	<0.001	8.22
	Stress x Pop	6	0.54	0.778	3.12	9	2.54	0.013	10.36

4. Discussion

By exposing different populations of the non-biting midge *Chironomus riparius* to Hg and salinity gradients, the overall outcomes of the present study provide empirical evidence on the central role that both the intrinsic genetic background and the levels of genetic diversity play on the susceptibility of midge populations towards environmental stress. Here, we showed that despite the reduced sensitivity towards lethal and sub-lethal Hg concentrations exhibited by the population collected from a historically Hg-contaminated site in comparison to the population collected from a closely located reference site, there were no observable fitness costs of tolerance under salinity exposure. In addition, our results highlighted also the importance of genetic diversity for the tolerance of *C. riparius* populations to environmental stress.

Among the four experimental populations, the levels of genetic diversity of populations CONT and GEN$^+$ were found to be high and within the range of genetic diversity reported for natural populations of *C. riparius* (Nemec et al., 2013; Nowak et al., 2007b) whereas the levels of genetic diversity of the other two populations (LAB and REF) were substantially lower and no allelic variation was observed at some of the assessed microsatellite loci. Deviations from Hardy-Weinberg equilibrium were not observed in populations CONT, LAB and REF but only in population GEN$^+$ which is likely due to the

recent admixture of genetic material from distinct source populations. Furthermore, genetic divergence among populations was very high compared to the weak genetic divergence that has been reported for midge populations under natural conditions (Nemec et al., 2013; Pfenninger and Nowak, 2008) suggesting, therefore, remarkable differences in the genetic backgrounds of the four experimental populations of our study.

A reduction in the levels of genetic diversity of populations CONT and REF was also evident following a 40-month period of laboratory rearing under common-garden clean conditions. This is consistent with previous studies showing genetic impoverishment in laboratory populations of *C. riparius* (Nowak et al., 2007b) as well as in laboratory populations of other species that are routinely used for ecotoxicological assessments of environmental stressors, such as the springtail *Orchesella cincta* (Costa et al., 2012), the least killifish *Heterandria formosa* (Athrey et al., 2007) or the zebrafish *Danio rerio* (Coe et al., 2009). However, it was worth noting that genetic impoverishment was considerably higher in population REF than in population CONT. This finding was surprising since both populations had equally high initial levels of genetic diversity and were maintained under similar laboratory conditions. However, at least two non-mutually excluding explanations can be advanced: maladaptation of population REF to laboratory conditions and differences in their breeding histories. While the former explanation cannot be excluded, results of this and previous work did not suggest major differences in the fitness performance of the two populations under control clean conditions in terms of emergence rate, time to emergence or weight of imagoes. Therefore, differences in the degree of genetic impoverishment of the two populations are most likely related with their distinct breeding histories in the laboratory rather than potential maladaptation of population REF to laboratory conditions.

As already stated, because laboratory populations are established from a limited number of organisms and are genetically isolated, they are particularly prone to the effects of random genetic drift that can impose dramatic losses of genetic diversity within just a few generations (Lagisz et al., 2011; Santos et al., 2013). Thus, the cumulative genetic drift due to chance events likely led to the elimination of considerable amounts of allelic variation from population REF. This, in turn, likely increased the frequency of mating among close relatives (i.e. inbreeding) and the expression of deleterious recessive alleles that further accelerated the loss of genetic diversity over time in this population. On the contrary, population CONT was probably less affected by genetic drift and inbreeding and, thus, was still able to retain higher levels of genetic diversity (Lagisz et al., 2011). Therefore, and regardless of the causes, our data show that the rate of genetic diversity loss in the laboratory may be distinct among populations and, as such,

merely maintaining relatively large population sizes may not effectively prevent genetic erosion (Briscoe et al., 1992).

A major goal of the present study was to search for evidences of genetic adaptation to Hg and for potential fitness costs of Hg tolerance under changing environmental conditions in population CONT, collected from an historically Hg contaminated site, in comparison to population REF that had been collected from a closely located reference site. Overall, our results showed that population CONT exhibited higher acute and chronic tolerance towards Hg toxicity compared to population REF. Because both populations had been reared under laboratory clean conditions for many generations prior of performing the ecotoxicological tests, differences in Hg-tolerance are likely related to the fact that population CONT contained more Hg-tolerant genotypes than population REF. Therefore, our results agree with previous studies showing the role of metals as major selective pressures that can impose important changes in the genetic background of exposed invertebrate populations (Agra et al., 2011; Costa et al., 2012; Timmermans et al., 2005; Vidal and Horne, 2003). For example, the freshwater oligochaete *Tubifex tubifex* rapidly acquired Hg tolerance when exposed to Hg contaminated sediments (Vidal and Horne, 2003). Similarly, springtail populations of *Orchesella cincta* inhabiting historically metal contaminated sites displayed heritable high metal tolerance compared to reference populations as a consequence of selection on metal-tolerant traits (including higher constitutive levels of metallothioneins) that interact in complex gene networks and confer enhanced tolerance to metals (Timmermans et al., 2005; Van Straalen et al., 2011).

The increased tolerance of the midge population CONT to Hg, however, did not seem to involve increased susceptibility to salinity stress, a potentially important selective pressure that population CONT (as well as population REF) may have to cope with in their natural environments in response to the impacts of climate change (IPCC, 2014). Indeed, results showed no significant differences in the acute tolerance to NaCl and, under chronic exposures to NaCl, population CONT performed even better than population REF. Therefore, our results contrast with a number of previous studies (Leitao et al., 2013; Salice et al., 2010; Vigneron et al., 2015) but add to existing evidence suggesting that tolerance to contaminants does not always involve increased susceptibility to changing environmental conditions (Haap et al., 2016; Hoffmann et al., 2014). It should be noted, however, that fitness costs of Hg tolerance were investigated only to a single environmental stressor which might not be sufficient to fully understand the long-term implications of Hg tolerance. For example, evolution of tolerance to polychlorinated biphenyls in the killifish *Fundulus heteroclitus* was associated to reduced

survival under clean laboratory conditions and reduced tolerance to hypoxia (Meyer and Di Giulio, 2003) but did not involve increased susceptibility to bacterial pathogen (Nacci et al., 2009) or to neurotoxic insecticides (Clark and Di Giulio, 2012). Similarly, evolution of Cd-tolerance in a laboratory population of *Daphnia magna* did not involve increased susceptibility to clean laboratory conditions, lead, elevated temperatures, low food regimens, copper or malathion but only increased susceptibility to phenol stress (Ward and Robinson, 2005). Therefore, although population CONT did not display a fitness cost in terms of tolerance to salinity, we cannot rule out increased susceptibility to other environmental stressors.

A further analysis of the data showed that genetic diversity was also an important factor determining Hg and NaCl tolerance of populations. Indeed, the genetic diversity of populations CONT and GEN^{+} was higher and both populations had higher emergence rates across chronic exposures to Hg and NaCl than populations with lower genetic diversity, i.e. populations LAB and REF. Because the contribution of the emergence rates to the reproductive success and the growth rates of *C. riparius* populations is quite high (Heye et al., 2016; Nowak et al., 2012), in a context of reduced survivorship in which only a reduced number of larvae is able to cope with the environmental stress and reach the adult stage, this may lead to a rapid decrease in the population sizes. In this regard, we can state that genetically eroded populations LAB and REF were more susceptible to chronic exposures of Hg and NaCl than populations CONT and GEN^{+}. Therefore, our results agree quite well with the evolutionary premise that the cumulative deleterious effects of genetic erosion and inbreeding will cause a decline in the overall fitness of populations that become increasingly expressed when populations are faced with more stressful environmental conditions (Reed and Frankham, 2003). Empirical evidence on this topic has been provided, for example, by the work of Spielman et al. (2004) who observed that genetic erosion and inbreeding reduced the ability of *Drosophila melanogaster* populations to cope with different pathogens. Similarly, Markert et al. (2010) found that the majority of genetically eroded populations of the crustacean *Americamysis bahia* became extinct under stressful saline conditions whereas most of the genetically diverse populations were still able to survive. Finally, Nowak et al. (2007a, 2008) also observed that genetically eroded and inbred *C. riparius* lab populations were more sensitive to chronic exposures of Cd exhibiting lower emergence rates, longer developmental times to reach the adult stage and lower reproductive output in comparison to populations with higher genetic diversity.

In our case, although both genetically eroded *C. riparius* populations had reduced emergence rates when exposed to Hg and NaCl, longer developmental times compared

to the genetically diverse populations CONT and GEN^+ were found solely for population LAB. However, male and female imagoes of this population were consistently heavier across the experimental conditions (including under control clean conditions). Therefore, our data suggest that population LAB had a different emergence strategy that was characterized by longer developmental times but heavier imagoes. A possible explanation for this finding may be that population LAB has been reared in our laboratory facilities for more than two decades and, thus, successive generations under optimal, relatively constant environments may have resulted in significant alterations in some life-history traits, a phenomenon that has been documented in other long-standing laboratory insect populations as well (Griffiths et al., 2005; Harshman and Hoffmann, 2000; Sgro and Partridge, 2000).

It was also worth noting that the chronic and acute tolerance of populations to both Hg and NaCl exposures did not always converge, a finding that has been often reported in other works (Barata et al., 2000; Leitao et al., 2013; Saro et al., 2012) and has been attributed to the different mechanisms that govern acute and chronic responses. According to Barata et al. (2000), acute tolerance is mainly determined by specific mechanisms of tolerance [such as metallothioneins in the case of Hg (Gimbert et al., 2016) or the enzymatic activity of ion pump ATPases in the case of NaCl (Jonusaite et al., 2013)] whereas sub-lethal tolerance is more dependent on general mechanisms of response and, hence, it is closely associated to the overall fitness of the population (Barata et al., 2000). This means that genetically eroded and inbred populations are particularly threatened when chronically exposed to sub-lethal levels of environmental stressors (Nowak et al., 2012; Pekkala et al., 2014) while their acute tolerance will also depend on their specific genetic background and, thus, on the existence of tolerant genotypes (Barata et al., 2000). In this regard, it is important to state that populations GEN^+ and CONT, besides exhibiting the highest levels of genetic diversity were also the only populations containing genotypes from the Hg contaminated site, i.e. tolerant genotypes which might also contribute to higher tolerance to Hg. At the same time, the results observed for population REF should be analysed bearing in mind that this population suffered a substantial loss of genetic diversity while reared in common-garden laboratory conditions and, so, might not be fully representative of the genetic background of the population under natural conditions.

Finally, because the amount of genetic diversity harbored by the different experimental populations was found to influence their susceptibility towards environmental stress, results of the present study provide further evidence about the utmost importance of preventing losses of genetic diversity in *C. riparius* laboratory

populations (Nowak et al., 2007a; Nowak et al., 2012). It is critical to regularly monitor the levels of genetic diversity and proceed to occasional genetic refreshments with new genetic input in order to alleviate the effects of genetic drift and inbreeding. It is clear from this and previous works [e.g. (Nowak et al., 2008)] that minimum threshold levels of genetic diversity should be established to improve the predictive power of the ecotoxicological assessments and reduce uncertainty among laboratories. According to our data, this procedure might be particularly important to improve the outcomes of chronic ecotoxicological tests that evaluate the deleterious effects of the exposure to low, but often ecologically relevant, concentrations of environmental contaminants. Similarly, evolutionary toxicology studies concerned with the effects of contaminants in the long-term population's health should also put efforts in using genetically diverse populations rather than long-standing lab populations, often genetically eroded and inbred, in order to increase the ecological realism of the assessments (Nowak et al., 2012). In this context, the microsatellite markers used in the present study provide a good basis to achieve such goal.

5. Conclusion

Investigating the effects that pollutants exert on the genetic background of natural populations is complex but of utmost importance to comprehensively understand their long-term detrimental impacts (Ribeiro and Lopes, 2013). Here, we found higher tolerance to Hg in a midge population that had been collected from a metal polluted site in comparison to a nearby-collected reference population but no increased susceptibility under changing environmental conditions. Likewise, we also found increased tolerance to environmental stressful conditions of Hg and NaCl in populations with higher genetic diversity than in genetically eroded midge populations.

Collectively, our findings lend support to the use of *C. riparius* as a model organism in evolutionary toxicology studies and highlight the influence that processes of natural selection and genetic drift have in shaping the genetics and the susceptibility of midge populations to environmental stress. In this regard, the use of *C. riparius* seems to be particularly attractive in evolutionary toxicology given that chironomids live closely associated with river sediments, where pollutants tend to accumulate more, and are able to persist under heavily contaminated habitats from where most species are eliminated (Clements et al., 2000; Smolders et al., 2003), which constitutes an indispensable prerequisite for evolutionary change.

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Supplementary Material

Supplementary Material S1 – Repeat motif, primer sequences, size range (in base pairs) and GenBank accession number of the microsatellite loci used to estimate the genetic diversity of *C. riparius* populations.

Locus	Motif	Primer sequence	Size (bp)	Bibliography	Access no.
Msc1	CA_9	F: CATCATCCTTAACAACCCAC R: CTAGCTTTGCAGGCGAGTGC	95-103	Nowak et al. 2006	DQ408105
Msc2	$(TAA)_9,T_{10}$	F: AGACTAATGACCAGACTTGC R: CTTGTGATGCGAAAAGCCTG	114-141	Nowak et al. 2006	DQ408106
Msc3	$(GT)_{14}$, T_9, T_6	F: ACTACGCGTGCCTCAACAGC R: AGCTAATTCTCATGTTGGTC	168–176	Nowak et al. 2006	DQ408107
Msc4	$(TCA)_6$	F: TGACTGAACTTCCGCAATGGG R: CCGAGAATGCTGCGATCCAG	211–216	Nowak et al. 2006	DQ408108
Msc5	$(CA)_{11}$, A_9	F: AACATTTGAACGCACATCG R: ATTTGATTGTATGTCCTG	264–278	Nowak et al. 2006	DQ408109
Msc6	GA	F: TATCCCACCATATCGGCATC R: CACCCGCAAATGATATACACAA	168–229	Soeter et al. 2010	-
Msc7	GT	F: GCTGAATCGTGTGATGTGCT R: TGCTGCTTCTGTCGTATGCT	235–245	Soeter et al. 2010	-

CHAPTER V:

POPULATION GENETIC STRUCTURE AND HYBRIDIZATION PATTERNS IN THE CRYPTIC SISTER SPECIES *CHIRONOMUS RIPARIUS* AND *CHIRONOMUS PIGER* ACROSS DIFFERENTLY POLLUTED FRESHWATER SYSTEMS

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Abstract

Chironomids are an integral and functionally important part of many freshwater ecosystems. Yet, to date, there is limited understanding of their microevolutionary processes under chemically polluted natural environments.

In this study, we investigated the genetic variation within populations of the ecotoxicological model species *Chironomus riparius* and its cryptic sister species *Chironomus piger* at 18 metal-contaminated and reference sites in northwestern Portugal. Microsatellite analysis was conducted on 909 samples to answer if metal contamination affects genetic variation in natural chironomid populations as previously suggested from controlled laboratory experiments.

Similarly high levels of genetic diversity and significant but weak genetic substructuring were found across all sites and temporal replicates, with no effects of metal contamination on the genetic variation or species' abundance, although *C. piger* tended to be less frequent at highly contaminated sites. Our results indicate that high levels of gene flow and population dynamic processes may overlay potential pollutant effects. At least for our study species, we conclude that the "genetic erosion hypothesis", which suggests that chemical pollution will reduce genome-wide genetic variability in affected populations, does not hold under natural conditions. Interestingly, our study provides evidence of successful hybridization between the two sister species under natural conditions.

Keywords: Genetic erosion; genetic diversity, metal contamination, interspecific hybridization, microsatellites.

1. Introduction

Since natural environments are largely heterogeneous and the multitude of biotic and abiotic factors may considerably vary even at fine spatial scales, populations inhabiting different habitats will often experience different selection pressures (Kubisch et al., 2014; Wang et al., 2013). Thus, limited dispersal capability in combination with geographical isolation, physical barriers or distinct environmental conditions can result in substantial differences in the genetic architecture of populations (Miller et al., 2012; Sexton et al., 2014). Elucidating patterns of genetic variation within and among populations has, therefore, broad implications for understanding the microevolutionary dynamics of populations and unravel their demographic and breeding histories (Pauls et al., 2013).

Members of the dipteran family Chironomidae, commonly known as non-biting midges, are an integral and functionally important part of many freshwater ecosystems (Armitage et al., 2012). Chironomids constitute one of the most abundant, species-rich and ecologically diverse groups of benthic invertebrates in freshwater habitats in which they spend most of their lifetime as aquatic larval stages (Armitage et al., 2012; Ferrington Jr, 2008). Furthermore, they are involved in the cycling of organic matter and represent a major prey item for the diet of many invertebrate and vertebrate species (Armitage et al., 2012). However, and despite their great ecological relevance, chironomids are often neglected in most biomonitoring studies due to taxonomic difficulties related to their morphologically cryptic nature (Pfenninger et al., 2007).

Chironomus riparius Meigen 1804 and *Chironomus piger* Strenzke 1959, for instance, are morphologically cryptic sister species (Pfenninger et al., 2007). These opportunistic tube-dwelling deposit feeders are commonly found in ditches, ponds, puddles and streams throughout Europe (Michailova et al., 2015; Oppold et al., 2016a), where they frequently co-occur in sympatry (Pfenninger and Nowak, 2008). However, while laboratory studies have shown that *C. riparius* may interbreed with *C. piger* and produce fertile progeny (Hägele, 1984, 1999), niche partitioning together with different swarming behaviors and dysgenesis syndromes have been argued to prevent effective hybridization under field conditions (Hägele, 1999; Miehlbradt and Neumann, 1976; Schmidt et al., 2013).

Chironomus riparius, in particular, has received considerable interest among the scientific community as it is a well-established standard test species in ecotoxicology (OECD, 2004). However, despite its extensive use in traditional laboratory and field ecotoxicology studies (Campos et al., 2014; Faria et al., 2006; Rodrigues et al., 2015)

as well as in the rapidly growing scientific field of evolutionary toxicology (Nowak et al., 2009; Stefani et al., 2014), the microevolutionary responses to varying environmental conditions, including anthropogenic-induced disturbances, are poorly documented.

To the best of our knowledge, one single study investigated the genetic population structure in *C. riparius* and *C. piger* at a fine spatial scale (Pfenninger and Nowak, 2008). In this study, authors found weak genetic structure across the study area in both species and related it to their great dispersal potential. However, sampling was performed along a single river basin and larvae were collected at a single occasion. Therefore, essential questions such as if populations from geographically close river basins are reproductively isolated or frequently admix due to active or passive dispersal of adults remain unknown to date. Furthermore, at a fine spatial scale, anthropogenic disturbances such as metal contamination may constitute major selection pressures that rapidly depress the gene pool of exposed populations through their impacts on the survivorship and reproduction (Athrey et al., 2007; Nowak et al., 2009; Ward and Robinson, 2005). Because genetic erosion has been predicted to diminish the evolutionary potential of populations to adapt to environmental change and, ultimately, increase the risk of extinction, documenting ongoing processes of contaminant-driven genetic erosion has been a subject of long-standing concern (Ribeiro and Lopes, 2013; van Straalen and Timmermans, 2002).

Based on laboratory studies showing that contaminants at environmentally relevant concentrations may indeed impact the genetic population structure of *C. riparius* at neutral loci (Nowak et al., 2009; Vogt et al., 2007), we are currently conducting a comprehensive research work to evaluate microevolutionary dynamics and genetic erosion in pollution-affected *Chironomus* populations (Pedrosa et al., 2017a; Pedrosa et al., 2017b). Here, we employed microsatellite markers to search for patterns of neutral genetic variation within and among the two cryptic sister species *C. riparius* and *C. piger*, in a region with high levels of metal contamination located in northwestern Portugal. Larvae of both species were collected over a four-year period from 18 sampling sites located across five river basins and varying in their levels of metal contamination. The following questions were addressed: 1) Is there a spatial or temporal genetic heterogeneity in *C. riparius* or *C. piger* at a regional scale, suggesting restrictions to gene flow by spatial or environmental factors? 2) Does metal contamination affect population genetic characteristics such as genetic diversity and differentiation among populations due to processes acting on genome-wide neutral diversity, as suggested by previous studies for a wide range of organisms? 3) Does metal contamination impact interspecific competition or reproductive isolation between the morphologically cryptic sister species?

2. Material and methods

2.1. Study area

Larvae of either *C. riparius* or *C. piger* were collected between the autumn of 2010 and the autumn of 2013 from the Ave (4 sites), Vouga (11 sites), Lis (1 site), Moel (1 site) and Tejo (1 site) river basins, NW of Portugal, in a total of 32 sampling surveys. Sampling sites represent a range from low to high levels of metal contamination and were chosen from a set of more than 200 candidate sites as the only ones in which there was a sufficient number of individuals to perform the genetic analyses (Figure 1; for further details about the sampling surveys please see Supplementary Material S1).

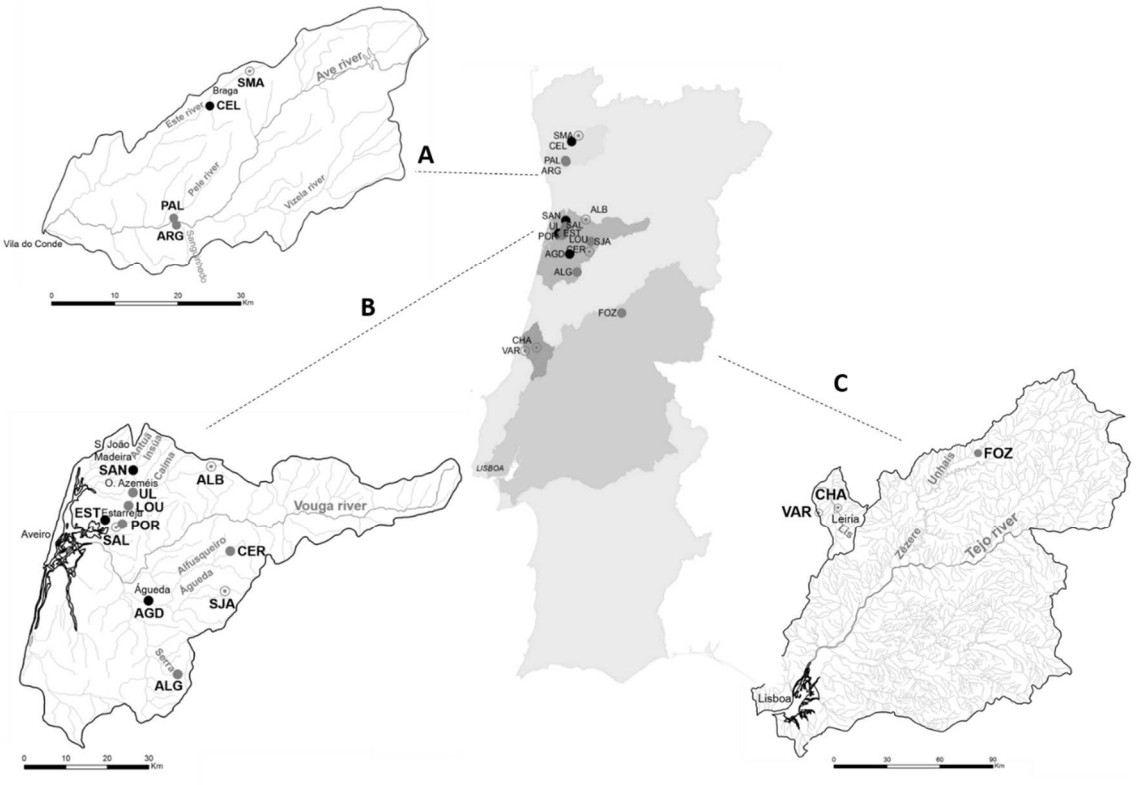

Figure 1 – Geographical location of the sampling sites of the study area from where *C. riparius* and *C. piger* were collected.

The Ave basin covers a drainage area of ca. 1388 km^2 and its middle and lower parts are severely degraded mainly due to the presence of numerous textile industries and other industrial plants that, during decades, discharged their untreated sewages directly to the local water bodies. Site SMA and site CEL locate in the Este river, respectively, near the source of the river and downstream the city of Braga where high levels of metal

contamination have been reported. Site ARG and site PAL locate, respectively, in the Sanguinhedo and Pele streams and were chosen because previous works indicated also high levels of metal contamination (Gonçalves et al., 1992; Soares et al., 1999).

The Vouga basin has a catchment area of ca. 3362km^2 and drains into the Atlantic Ocean through the slow-flowing lagoon Ria de Aveiro (Van der Weijden and Pacheco, 2006). Within the basin, important water quality impairments have been identified in several river stretches situated near industrial areas: site EST locates in S. Filipe Ditch, a manmade stream constructed in the 1950s to carry the sewages from the Chemical Complex of Estarreja (including sewages from a chlor-alkali electrolysis plant that released ca. 33 tons of mercury in the area) to the Ria de Aveiro lagoon (Costa and Jesus-Rydin, 2001; Inácio et al., 1998); site SAN locates in the Antuã river, downstream the towns of São João da Madeira and Oliveira de Azeméis in which there are placed several metallurgic, metallomechanic, footwear and textile industries (Costa and Monteiro, 2016); site UL locates in the Insúa river, the major tributary of the Antuã river, downstream Oliveira de Azeméis; site LOU locates in the Antuã river after the confluence of the Insúa river; site AGD locates in the Vale da Erva stream, downstream the Industrial Complex of Águeda where high levels of metal contamination have been reported (Reis et al., 2005). The remaining sites surveyed in the basin (i.e. sites ALB, ALG, CER, POR, SAL, SJA) locate in more rural landscapes dominated by forestry and small agricultural fields.

Site CHA, in the Milagres stream (Lis basin), has been subjected to continuous ecological disasters over the last 30 years due to successive discharges of animal husbandry wastewaters (Vieira et al., 2009). Site VAR, in the Várzea stream (Moel basin), and site FOZ, in the Unhais river (Tejo basin), locate in rural areas dominated by forestry and small agricultural fields.

2.2. Sediment sample collection and chemical characterization

Chemical characterization of the sampling sites was performed in terms of total content of arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), mercury (Hg), nickel (Ni) and zinc (Zn). Sediment samples from the upper 5 cm surface layer of each site were collected from the area where chironomids were surveyed. Once in the laboratory, samples were homogenized, dried and sieved through a 1mm mesh sieve. Total Hg was measured with no previous digestion of the material by thermal decomposition atomic absorption spectrometry with gold malgamation (LECO® model

AMA-254). For the analysis of the remaining chemical elements, sediments were acid digested according to standard procedure Method 3052B for Acid Digestion of sediments (USEPA, 1996). Total As, Cd, Cr, Cu, Ni and Pb was analysed by Graphite Furnace AA (GFAA model Thermo X Series, Peltier Nebulizing Camera, Burgener Nebulizer; CETAC AS510 auto-sampler), whereas Zn was analysed by Flame Atomic Absorption Spectrometry (FLAA model Jobin Yvon Activa M). Total metal and As content of sediment samples was determined in triplicate and results expressed as mean values. Minimum 80-120% of recovery percentages, blanks and certified reference material (NRC MESS-3) were used for analytical accuracy of laboratory measurements.

2.3. Assessment of genetic diversity and structure of populations

Extraction of genomic DNA and genetic identification of *C. riparius* and *C. piger* was performed according to (Pfenninger et al., 2007) with some minor modifications. Larval tissue was digested overnight at 56 ºC in 96-well plates, each well containing 80 µl of standard cetyltrimethyl ammonium bromide (CTAB) buffer and 2 µl of Proteinase K (20 µM). Samples were afterwards treated with 82 µl of standard 24:1 chloroform:isoamylalcohol and DNA was precipitated for at least 45 min (-20 ºC) in 125 µl of isopropanol, washed 2x in 150 µl of ethanol 70% and resolved in 30 µl of water for downstream analysis.

Mitochondrial cytochrome oxidase I (CO I) fragments were then amplified in a T-Gradient thermocycler (Biometra, Göttingen, Germany) with the following cycling conditions: one initial denaturation cycle of 3 min at 94º C followed by 36 cycles with 1 min at 92º C, 1 min at 55º C and 1 min at 72º C. Reaction mixture contained 3 mM $MgCl_2$, reaction buffer (20 mM Tris-HCl and 50 mM KCl), 0.2 mM dNTP, 0.3 µM of each *Chironomus* specific primer (forward: 5' TCGAG CAGAATTAGGACGACC, reverse: 5' AGGATCACCCCCACCAGCAGG) and 1 U *Taq* DNA polymerase in a total volume of 13 µl of reaction mixture plus approx. 30 ng of template DNA. Sequencing of amplified mtDNA CO I was performed in forward direction on an ABI 3730 DNA Analyzer (Applied Byosystems, Foster City, CA). Sequences were assembled and aligned using the ClustalW alignment method (Thompson et al., 1994) and performed in sequence scanner software 2 (Applied Biosystems, Foster City, CA, USA) and Bioedit software version 7.0.5.2 (Ibis Therapeutics, Carlsbad, CA, USA). *Chironomus riparius* and *C. piger* haplotypes were identified according to reference sequences (GenBank accession numbers DQ910547-DQ910729 (Pfenninger et al., 2007)).

Allelic variation of CO I-identified *C. riparius* and *C. piger* individuals was subsequently measured at seven microsatellite loci (Nowak et al., 2006). Microsatellite fragments were amplified with the following PCR conditions: initial denaturation cycle of 3 min at 94º C, followed by 36 cycles with 30s at 94 ºC, 30s at 55 ºC and 40s at 72º C, followed by a final extension cycle with 5 min at 72º C. Reaction mixture contained 2.4 mM of $MgCl_2$, 0.25 mM of dNTP, 0.2 µM of each specific primer and 0.5 U of *Taq* DNA polymerase in a total volume of 8 µl of reaction mixture per microsatellite plus approx. 30 ng of template DNA (further details about the repeat motifs, primer sequences and size range of microsatellite alleles are provided in Supplementary Material S2). Microsatellite data were automatically scored using software GeneMarker® version 2.6.3 (Softgenetics, State College, PA) and corrected manually whenever necessary.

2.4. Data analysis

Two Bayesian clustering methods, namely STRUCTURE 2.3.4 (Pritchard et al., 2000) and NewHybrids (Anderson and Thompson, 2002), were used to investigate hybridization between *C. riparius* and *C. piger*. For STRUCTURE, the length of *burn-in* period as well as the number of Markov Chain Monte Carlo (MCMC) iterations was 10^6. Ten iterations were run for a k = 2 with independent allele frequencies and admixture and results of independent replicates were merged using CLUMPP 1.1.2 software (Jakobsson and Rosenberg, 2007). NewHybrids was applied with no prior information using the uniform priors and 10^6 MCMC sweeps after 10^6 *burn-in* steps.

After excluding potential hybrids, genetic diversity was estimated for both species across sampling survey in GenAlex 6.503 (Peakall and Smouse, 2006, 2012) based on the mean number of alleles per locus (N_A) and the observed (H_O) and expected heterozygosity (H_E). In the case of *C. piger*, no allelic variation was found at microsatellite locus Msc04 and, therefore, the locus was excluded from all subsequent genetic analyses. The existence of private alleles within sampling surveys was also checked and considered for a frequency ≥ 5%. Departure from Hardy-Weinberg equilibrium (HWE) was tested for each individual locus by formal Chi^2 tests. The presence of null alleles, scoring errors and large allele dropouts was verified in Microchecker 2.2.3. (Van Oosterhout et al., 2004).

Hierarchical analysis of molecular variance (AMOVA) was performed in GenAlex 6.503 for each species in separate to assess the partitioning of allelic variation within and among sampling surveys. Significant genetic differentiation among pairwise

comparisons was assessed through F_{ST} estimates using 9999 permutations of the datasets and applying sequential Holm's Bonferroni corrections to account for multiple comparisons. To visualize the genetic relationships among individuals and among sampling surveys, two principal coordinate analyses (PCoAs) were undertaken for each species using Nei's genetic distance among individuals and population pairwise F_{ST} values. Complementarily, independent AMOVAs were performed for each single population of *C. riparius* and *C. piger* monitored for at least two seasons in order to assess genetic structure over time. Finally, STRUCTURE analyses were performed for *C. riparius* and for *C. piger* in separate (with k values up to 10 and 3 iterations each) to further investigate the presence of genetic clusters in each species. Analyses were performed both with and without population information using the same conditions set for the detection of interspecific hybridization with the exception of correlated allele frequencies.

One-way analysis of variance (ANOVA) was carried out in Graphpad 5.00 (Graphpad software, San Diego, CA, USA) to determine whether the degree of metal contamination of the sites (i.e. low, moderate and high) influenced the abundance and the genetic diversity (N_A, H_O and H_E) of *C. riparius* and *C. piger* populations. Complementarily, Pearson correlation tests were performed to assess the association between the metal concentration of the sediments and the abundance and genetic diversity of the two species. A Pearson correlation test was also used to assess the relationship between the abundance of *C. riparius* and *C. piger* across the study area.

Simple Mantel tests were performed in GenAlex to explore, independently, the influence of metal contamination and geographic distance on the extent of genetic structure of the two species. For Mantel tests, the following distance matrices were tested using 9999 randomizations of the datasets: pairwise F_{ST} values, geographic distance (in km, log transformed) and difference in metal concentration between sediment samples (total and metal-by-metal concentrations, log transformed).

The likelihood of recent effective population size reductions (bottleneck events) was tested in BOTTLENECK 1.2.02. For that, the stepwise mutation model and the two-phase mutation model (with 95% single-step mutations and variance set to 12), which is an intermediate model between the infinite allele model and the stepwise mutation model, were employed using 100,000 iterations of the datasets. Statistical significance of heterozygosity excess (indicative of recent bottlenecks) was determined through one-tailed Wilcoxon signed rank test that is the most powerful test when using less than 20 loci (Piry et al., 1999).

The potential existence of loci under selection was investigated using BAYESCAN 2.1. This Bayesian hierarchical method decomposes locus-population F_{ST} coefficients into a population-specific component (beta) shared by all loci and a locus-specific component (alpha) shared by all populations. Departure from neutrality at a given locus is assumed for alpha significantly different from 0 with positive values of alpha suggesting directional selection and negative values suggesting balancing selection. Analyses were conducted for a sample size of 5,000 and a thinning interval of 10, resulting in a total number of 100,000 iterations. Four independent runs were performed to check the consistency of the results and the false discovery rate was set at 0.10. Loci with Bayes factors >10 were retained as outliers since they represent "strong" evidence for selection (Foll and Gaggiotti, 2008).

3. Results

3.1. Metal contamination of the stream sediments

Results showed remarkable differences in the total metal concentration and in the measured concentrations of As, Cd, Cr, Cu, Hg, Ni, Pb and Zn of the surface sediments of the sampling sites (Supplementary Material S1).

To evaluate the ecological risk posed by metals, measured concentrations of each single metal were compared to the consensus-based sediment quality guidelines of MacDonald et al., (2000) which predict metal toxicity to bottom-dwelling aquatic organisms based on two levels of metal contamination: a lower threshold effect concentration (TEC) below which harmful effects are unlikely to occur and an upper probable effect concentration (PEC) above which harmful effects are likely to occur. According to this, sites ALB, CHA, SAL, SJA, SMA and VAR were considered to be uncontaminated by metals as they did not exceed the sediment quality guidelines for none of the metals. Sites ALG, ARG, CER, FOZ, LOU, PAL, POR and UL were considered moderately toxic as they exceeded the protective TEC values (but not PECs) for some of the metals analysed. Lastly, four sites exceeded the PEC values for some of the metals and were considered to pose a high ecological risk: site AGD exceeded PEC for Ni; site CEL exceeded PECs for Cu and Zn; site EST exceeded PECs for As and Hg; and site SAN exceeded PEC for As.

3.2. Genetic identification of *C. riparius* and *C. piger*

Mitochondrial CO I fragment sequences (approx. 596-bp region) were obtained for a total of 1730 individuals, with 1085 individuals being undoubtedly assigned to *C. riparius* and 645 to *C. piger* (Supplementary Material S3). While *C. riparius* and *C. piger* co-occurred in 27 out of the 32 sampling surveys, there was a negative correlation between their abundances (Pearson correlation r = -0.469, p = 0.007; Supplementary Material S4). No single *C. riparius* individual was recorded in ALB in spring 2011 and in SJA in spring 2011 and no single *C. piger* individual was found in CEL in spring 2013, LOU in winter 2012 and SMA in autumn 2011.

3.3. Hybridization between *C. riparius* x *C. piger*

Due to the maximum number of individuals analysed in each sampling survey (set at n < 40) and the insufficient number of individuals in other surveys, patterns of genetic variation in *C. riparius* were determined based on the microsatellite variation of 562 individuals collected from 15 sites of the Ave, Lis, Moel and Vouga basins. For the same reasons, patterns of genetic variation in *C. piger* were based on 347 individuals collected from 11 sites of the Ave, Lis, Tejo and Vouga basins (Table 1).

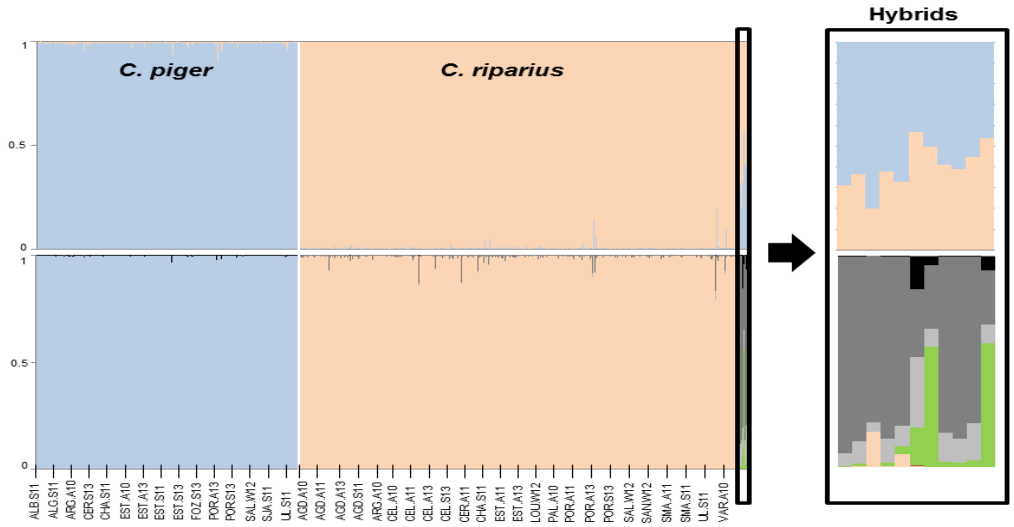

Figure 2 – Genetic differentiation between *C. piger* (n=347) and *C. riparius* (n= 562) shown as individual assignment probabilities (y axis) using Structure (A) and NewHybrids (B) software based on Bayesian clustering. Hybrid categories assigned in NewHybrids for 11 individuals of site POR (spring 2013) are shown in dark grey (backcross *C. piger*), light grey (F1), green (F2) and black (backcross *C. riparius*).

Both STRUCTURE and NewHybrids analyses grouped *C. riparius* and *C. piger* individuals in separate genetic clusters, which harmonized with the findings of the mtDNA analysis (data not shown). In site POR in spring 2013, nonetheless, 11 larvae showed intermediate genotypes in STRUCTURE (q_i value < 0.8 to each cluster), suggesting the existence of *C. piger* x *C. riparius* hybridization. NewHybrids confirmed this finding, with hybrid genotypes being mostly assigned to *C. piger* backcrosses or F1 hybrid categories (Figure 2). All hybrid individuals showed mitochondrial *C. piger* haplotypes and were removed from the subsequent genetic analyses.

3.4. Genetic diversity in *C. riparius* and *C. piger* across the study area

Due to low marker variability and the sequencing approach, which was intended to be used for species identification only, we decided to focus solely on the nuclear microsatellite patterns for estimations of genetic variability. No evidence for the presence of null alleles was found in *C. riparius* or *C. piger*. In *C. riparius*, a total of 40 alleles were identified, with an overall mean number of alleles per locus of 3.72 and a mean H_O and H_E of 0.53. In *C. piger*, a total of 55 alleles were identified, the N_A was 5.34, the H_O was 0.49 and the H_E was 0.52 (Table 1).

Genetic diversity of *C. riparius* and *C. piger* did not markedly differ across sampling surveys (Table 1, Table 2). In *C. riparius*, N_A varied from 3.00 (site CEL in spring 2013) to 4.71 (site VAR in autumn 2010). H_O was lowest in SAL in winter 2012 (H_O = 0.44 ± 0.06) and highest in EST in autumn 2013 (H_O = 0.65 ± 0.04). H_E was lowest in SMA in autumn 2011 (H_E = 0.49 ± 0.03) and highest in VAR in autumn 2010 (H_E = 0.58 ± 0.04) (Table 1). In the case of *C. piger*, N_A varied from 4.17 (site SAL in winter 2012 and site ARG in autumn 2010) to 6.17 (site FOZ in spring 2013). H_O was lowest in site CER in spring 2013 (H_O = 0.41 ± 0.13) and highest in UL in spring 2011 (H_O = 0.64 ± 0.14). H_E was lowest in SAL in winter 2012 (H_E = 0.47±0.12) and highest in ARG in autumn 2010 (H_E = 0.60 ± 0.08) (Table 1).

Significant Hardy-Weinberg disequilibria were found in different sampling surveys of the two species at different microsatellite loci (Table 1). No evidences of private alleles were found in both species at frequencies ≥ 0.05.

Table 1 – Genetic diversity of *C. riparius* and *C. piger* across the sampling sites. Shown are number of individuals analysed (n), mean number of alleles (N_A ± SE) and observed (H_O ± SE) and expected (H_E ± SE) heterozygosity. Asterisks indicate microsatellite loci under significant Hardy-Weinberg disequilibria.

Chironomus piger							*Chironomus riparius*						
Site	**Season**	**Cont**	**n**	**N_A**	**H_O**	**H_E**	**Site**	**Season**	**Cont**	**n**	**N_A**	**H_O**	**H_E**
ALB	Spring 2011	Low	30	5.50±1.09	0.53±0.11^{*Msc3}	0.58±0.11	CHA	Spring 2011	Low	30	4.57±0.95	0.58±0.06^{*Msc2}	0.55±0.05
CHA	Spring 2011	Low	30	5.17±1.08	0.44±0.12^{*Msc2,5}	0.47±0.12	SAL	Winter 2012	Low	13	3.43±0.48	0.44±0.06^{*Msc6}	0.49±0.04
SAL	Winter 2012	Low	12	4.17±1.05	0.47±0.14^{*Msc6}	0.47±0.12	SMA	Spring 2011	Low	29	3.43±0.48	0.53±0.05^{*Msc4}	0.52±0.05
SJA	Spring 2011	Low	27	5.83±1.25	0.46±0.13^{*Msc2}	0.52±0.09	SMA	Autumn 2011	Low	28	3.29±0.52	0.45±0.02^{*Msc6}	0.49±0.03
							VAR	Autumn 2010	Low	30	4.71±0.87	0.56±0.05	0.58±0.04
ALG	Spring 2011	Moderate	28	5.17±1.08	0.48±0.13^{*Msc3,5}	0.47±0.12	ARG	Autumn 2010	Moderate	24	3.43±0.43	0.58±0.06	0.53±0.03
ARG	Autumn 2010	Moderate	10	4.17±0.70	0.55±0.09	0.60±0.08	CER	Autumn 2011	Moderate	22	3.71±0.61	0.55±0.07^{*Msc2}	0.51±0.05
CER	Spring 2013	Moderate	21	5.83±1.38	0.41±0.13^{*Msc1,3,7}	0.51±0.12	LOU	Winter 2012	Moderate	27	3.86±0.74	0.53±0.04	0.53±0.04
FOZ	Spring 2013	Moderate	28	6.17±1.25	0.48±0.10	0.52±0.09	PAL	Autumn 2010	Moderate	12	3.29±0.52	0.56±0.04^{*Msc6}	0.52±0.03
POR	Spring 2013	Moderate	16	5.17±1.01	0.48±0.14^{*Msc2,6}	0.49±0.11	POR	Autumn 2011	Moderate	25	4.00±0.49	0.49±0.06^{*Msc5}	0.51±0.04
POR	Autumn 2013	Moderate	15	4.83±0.79	0.52±0.12^{*Msc7}	0.50±0.10	POR	Spring 2013	Moderate	32	3.71±0.64	0.52±0.08	0.50±0.04
UL	Spring 2011	Moderate	11	5.00±1.00	0.64±0.14^{*Msc7}	0.57±0.10	POR	Autumn 2013	Moderate	33	4.00±0.90	0.50±0.03^{*Msc5}	0.53±0.03
							UL	Spring 2011	Moderate	20	4.00±0.69	0.56±0.03	0.55±0.04
EST	Autumn 2010	High	21	5.67±1.09	0.47±0.10^{*Msc2}	0.55±0.08	AGD	Autumn 2010	High	8	3.29±0.36	0.61±0.08^{*Msc3}	0.50±0.06
EST	Spring 2011	High	25	6.00±1.15	0.47±0.12^{*Msc2,6}	0.53±0.11	AGD	Spring 2011	High	25	3.71±0.52	0.51±0.06	0.54±0.03
EST	Spring 2013	High	26	5.67±1.33	0.49±0.12^{*Msc2}	0.50±0.09	AGD	Autumn 2011	High	31	4.00±0.69	0.59±0.04^{*Msc4,6}	0.55±0.04
EST	Autumn 2013	High	36	5.83±1.14	0.49±0.13^{*Msc2,7}	0.52±0.11	AGD	Autumn 2013	High	30	4.14±0.80	0.52±0.07^{*Msc3}	0.54±0.04
							CEL	Autumn 2010	High	25	3.57±0.57	0.53±0.03	0.55±0.02
							CEL	Autumn 2011	High	27	3.57±0.53	0.50±0.05	0.53±0.03
							CEL	Spring 2013	High	6	3.00±0.44	0.52±0.08	0.52±0.04
							CEL	Autumn 2013	High	20	3.71±0.57	0.48±0.05^{*Msc6}	0.54±0.03
							EST	Autumn 2011	High	28	3.71±0.61	0.55±0.02	0.56±0.03
							EST	Autumn 2013	High	8	3.29±0.36	0.65±0.04	0.58±0.03
							SAN	Winter 2012	High	29	3.86±0.67	0.52±0.06^{*Msc6}	0.50±0.05
Overall *C. piger* mean				5.34±0.27	0.49±0.03	0.52±0.03	Overall *C. riparius* mean				3.72±0.12	0.53±0.01	0.53±0.01

3.5. Genetic substructure within *C. riparius* and *C. piger* in the study area

AMOVA revealed a weak but significant genetic structure within both species with 1.05% (F_{ST} = 0.010, p < 0.001) and 1.93% (F_{ST} = 0.019, p < 0.001) of the total variation being attributed to differences among sampling surveys in *C. riparius* and *C. piger*, respectively (Supplementary Material S5).

Population pairwise estimates of F_{ST} varied from 0.000 to 0.079 in *C. riparius* and were significant for different pairwise comparisons. However, after Holm's Bonferroni correction, only the pairwise comparison SMA (autumn 2011) vs CHA (spring 2011) remained significant (Supplementary Material S6). In *C. piger*, pairwise estimates of F_{ST} ranged from 0.000 to 0.097. After Holm's Bonferroni correction, the survey of ALG (spring 2011) remained significantly different from all but two other sampling surveys while ALB (spring 2011) and ARG (autumn 2010) remained significantly different from 5 surveys (Supplementary Material S7).

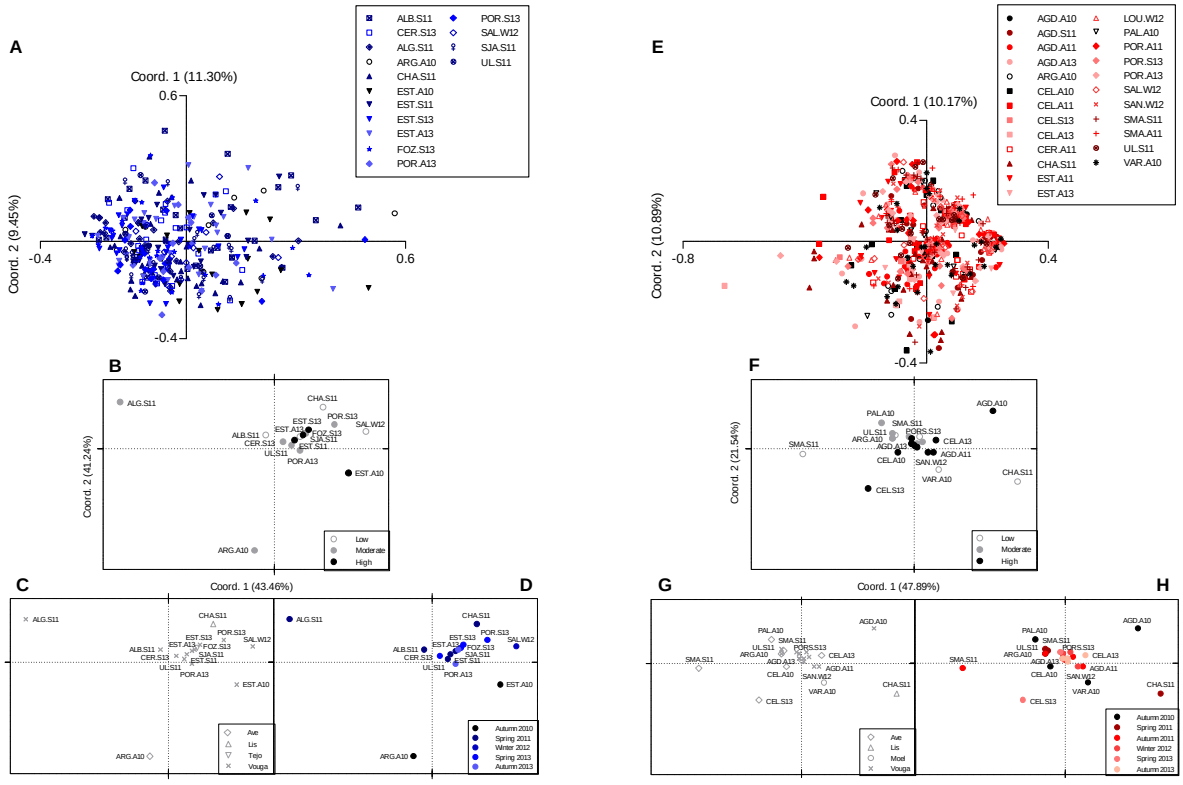

Figure 3 – Results of the Principal Coordinate Analysis (PCoA) of microsatellite data for *C. piger* (A – D) and *C. riparius* (E – H). PCoAs A and E show variation among individuals of *C. piger* (n=336) and *C. riparius* (n=562), respectively, based on Nei's genetic distance. PCoAs B – D show variation among *C. piger* sampling surveys (n=15) and PCoAs F – H show variation among *C. riparius* sampling surveys (n=24), based on pairwise F_{ST} values, according to metal contamination (B and F), river basin (C and G) and sampling season of the sites (D and H).

These results were consistent with the PCoA and STRUCTURE analyses. The PCoAs of *C. riparius* and *C. piger* showed considerable overlapping between sampling surveys and no clear ordination with metal contamination, river basin or sampling season (Figure 3). Similarly, the Bayesian STRUCTURE clustering revealed no substructure within *C. riparius* or *C. piger*, independently of the applied model or the number of chosen clusters *k* (data not shown).

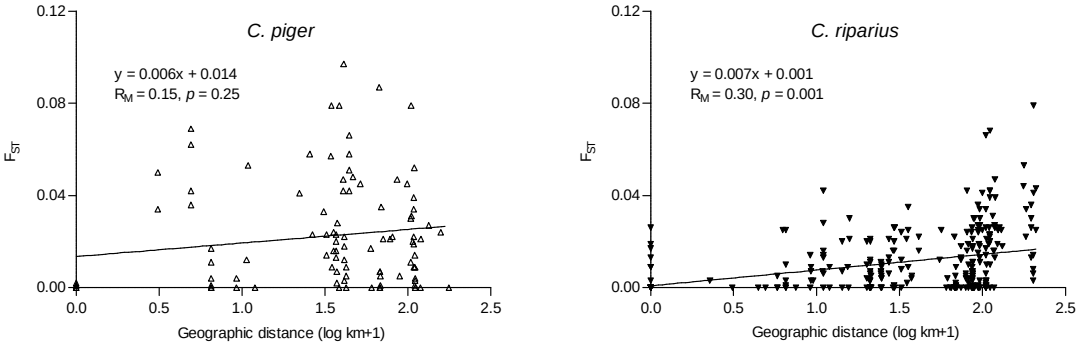

Figure 4 – Genetic isolation by distance in *C. piger* and *C. riparius*. Mantel correlation (r_M) and significance level (*p*) are shown for the correlation between pairwise F_{ST} and the geographical distance (log transformed) based on 9999 permutations of the datasets.

A weak but significant pattern of isolation-by-distance was revealed for *C. riparius* across the study area (Mantel test: $r_M = 0.30$, $p = 0.001$), but not for *C. piger* (Mantel test: $r_M = 0.15$, $p = 0.25$) (Figure 4).

3.6. Impact of metal contamination on species'abundance and genetic variation

ANOVA revealed no significant differences in the abundance and genetic diversity (as N_A, H_O or H_E) of *C. riparius* and *C. piger* populations inhabiting sites of low, moderate and high metal contamination ($p > 0.05$ for all one-way ANOVA tests). *Chironomus piger* abundance was, nonetheless, low in three out of the four highly contaminated sites surveyed (i.e. sites AGD, CEL and SAN) and no microsatellite analysis could be performed in these sites while, in turn, *C. riparius* abundance was generally high across highly contaminated sites (Figure 5).

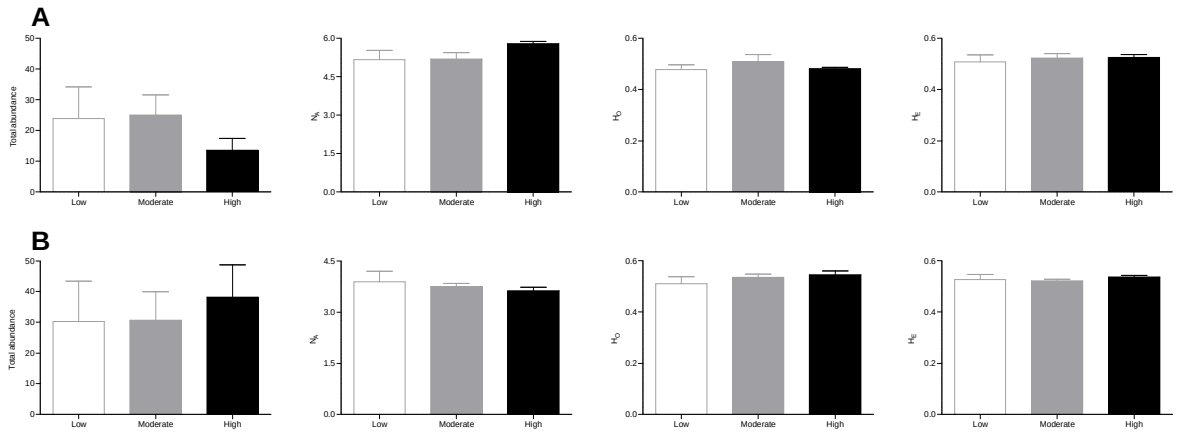

Figure 5 – Mean values (±SEM) of abundance (N), mean number of alleles (N_A), observed heterozygosity (H_O) and expected heterozygosity (H_E) in *C. piger* (A) and *C. riparius* (B) across sites of low, moderate and high levels of metal contamination.

Overall, correlations between the metal concentration of sediments (as metal-by-metal and total concentration) and the abundance and genetic diversity were weak for both species. For *C. riparius*, significant positive correlations were found between abundance and Cr concentration and between H_O and Hg concentration. For *C. piger*, negative correlations were found between total abundance and Cu and Ni concentrations (Supplementary Material S8 – S11). However, after Holm's Bonferroni correction, no significant differences were retained. Mantel tests revealed no significant genetic substructure with metal contamination in *C. riparius* and *C. piger* (Supplementary Material S12).

3.7. Genetic substructure in *C. riparius* and *C. piger* populations monitored along time

AMOVA did not detect any genetic structure along time in *C. riparius* populations SMA (spring vs autumn 2011) and POR (autumn 2011 vs spring 2013 vs autumn 2013) as well as in *C. riparius* populations surveyed from the highly contaminated sites AGD (autumn 2010 vs spring 2011 vs autumn 2011 vs autumn 2013), CEL (autumn 2010 vs autumn 2011 vs spring 2013 vs autumn 2013) and EST (autumn 2011 vs autumn 2013) ($p > 0.05$ for all AMOVA tests). Similarly, no genetic structure along time was found in the *C. piger* population of site POR (spring 2013 vs autumn 2013) ($F_{ST} = 0.000$, $p = 0.643$). However, there was a significant genetic structure along time in the *C. piger*

population surveyed from the highly metal contaminated site EST (autumn 2010 vs spring 2011 vs spring 2013 vs autumn 2013; F_{ST} = 0.012, p = 0.004).

3.8. Evidence for bottlenecks and loci under selection

No evidence for recent bottleneck events was found in either *C. riparius* or *C. piger* populations across the study area (p > 0.05 for all one-tailed Wilcoxon tests).

Test for loci under selection showed “decisive” signatures of balancing selection in all seven microsatellite loci in *C. riparius* (Bayes factor of 10^{1000}). In *C. piger*, locus Msc6 showed “strong” evidence for balancing selection (Bayes factor > 15) while the other microsatellite loci showed “decisive” signatures of balancing selection (Bayes factor of 10^{1000}).

4. Discussion

Here, we present the first study in which patterns of genetic variation of the ecotoxicological model species *C. riparius* and its cryptic sister species *C. piger* are investigated in their natural environments according to gradients of metal contamination and spatiotemporal factors.

Chironomus riparius and *C. piger* co-occurred together in most of the sampling sites which supports their sympatric distribution (Pfenninger and Nowak, 2008). However, a significant negative correlation between the abundance of the two species was also found across the study area. Despite environmental conditions (apart from metal contamination) were not investigated at the light of the present study, it is likely that the prevalence of one species over the other at different sites might be related to the different ecological niches occupied by *C. riparius* and *C. piger* (Pfenninger and Nowak, 2008; Schmidt et al., 2013). Previous field and laboratory research indicated that *C. riparius* is favored in freshwater systems with higher organic matter content and more anaerobic conditions while, in turn, *C. piger* is favored in freshwater systems with higher maximum water temperatures as well as with higher salinity, nitrite and calcium concentrations (Nemec et al., 2012; Pfenninger and Nowak, 2008; Schmidt et al., 2013).

A major goal of the present study was to investigate the influence of metal contamination in the patterns of genetic variation of *C. riparius* and *C. piger*. Based on the sediment quality guidelines, some of the sampling sites located nearby industrial

areas (namely sites AGD, CEL, EST and SAN) were considered highly contaminated and contained metals at concentrations that are expected to cause adverse toxic effects on sediment-dwelling organisms (MacDonald et al., 2000). However, no signs of genetic erosion or genetic substructure with metal contamination were found for *C. riparius* or *C. piger* populations, which contrasts with a number of studies showing genetic erosion on populations inhabiting environmentally contaminated areas (Benton et al., 2002; Krane et al., 1999; Paris et al., 2015) but agrees with many other studies reporting little impacts of contaminants on the overall patterns of genetic variation of exposed populations (Giska et al., 2015; Martins et al., 2009; Miller et al., 2012). We need, nonetheless, to stress that patterns of genetic variation were measured at a limited number of microsatellite markers. While even lower marker numbers have been successfully used to show fine-scale changes in pollutant-induced genetic variability of *C. riparius* under laboratory conditions (Nowak et al., 2009; Vogt et al., 2007), there is clear evidence that larger marker numbers or genomic approaches are needed to ensure sound representations of genome-wide variability. Recently, genomic resources have been made available for *Chironomus* (Oppold et al., 2016b), which will allow for the development of advanced genomic tools enabling more in-depth studies in this field and to detangle neutral and selective effects of pollutant exposure on natural *Chironomus* populations.

It was nevertheless interesting to note that *C. piger* was consistently found at low frequencies in three out of the four most highly contaminated sites of our study area (i.e. sites AGD, CEL and SAN) and negative correlations were obtained (before Holm's Bonferroni correction) between *C. piger* abundance and the Cu and Ni concentrations in sediments. On the contrary, *C. riparius* were generally found at much larger frequencies in all highly contaminated sites and significant positive correlations were obtained (again before Holm's Bonferroni correction) between *C. riparius* abundance and Cr concentration and between H_O and Hg concentration. Finally, in the only *C. piger* population from a highly contaminated site in which it was possible to carry out the microsatellite analysis (i.e. site EST), there was a weak but significant genetic differentiation along time which contrasted with no significant genetic differentiation in none of the other populations that were monitored along time, including those *C. riparius* populations collected from highly contaminated sites. Despite no ecotoxicological information is available in the literature for *C. piger,* these findings may be indicative of different tolerance to metals between the two species and, thus, this deserves further investigation. Alternatively, the higher prevalence of *C. riparius* in highly contaminated sites may be related to other ecological disturbances that are often associated with metal

contamination, such as the accumulation of large amounts of organic matter and reduced dissolved oxygen (Cormier et al., 2002; Grimalt et al., 1999) that favor *C. riparius* (Pfenninger and Nowak, 2008; Schmidt et al., 2013).

Regional (i.e. river basins) and seasonal variations did not seem to influence the genetic structure of both species. However, a signature for isolation-by-distance (IBD) was observed in *C. riparius* indicating that individuals living in closer geographic proximity are genetically more related than those further apart. Although the IBD signature was weak, this suggests that genetic structure within the study area was shaped, at least in part, by limited dispersal capability and genetic drift with increasing geographic distance (Nemec et al., 2013). For *C. piger*, no statistical evidence for IBD was retrieved. However, IBD was estimated based on a lower number of sites that were located within a smaller geographic area.

Taken together, the overall results of the microsatellite analysis revealed high levels of genetic diversity, weak genetic structure and high genetic connectivity between *C. riparius* populations across the entire study area. A similar pattern was observed also for *C. piger* suggesting that even if seasonal variations occurred in the metal concentrations in local sediments, they did not greatly influence the overall genetic patterns of the species. Therefore, our results confirm the high population dynamics of these two chironomid species on the regional scale (Pfenninger and Nowak, 2008).

While the weak genetic structure might be indicative of a single large population covering the entire study area, deviations from HW equilibrium in different surveys at one or more loci rather suggest an unstable, highly dynamic population structure with potential local extinction and recolonization events, as previously described as a general trait of common chironomid species (Thienemann, 1954). However, the successful colonization by a large number of individuals likely prevents the detection of recent bottleneck events across the sampling sites (Peery et al., 2012). Indeed, although *Chironomus* imagoes possess relatively weak flying abilities and can only actively disperse for some hundreds of meters, they can be carried out by wind over longer distances (Armitage et al., 2012). Furthermore, larval drift in river currents constitutes also an important dispersal mechanism in chironomids (Groenendijk et al., 1998). Therefore, high aerial and aquatic mobility coupled to multivoltine life-cycles and rapid colonization of new environments will likely enhance the rate of gene flow between populations and limit genetic structure.

A remarkable variation in *Chironomus* abundance was also registered in some sites over time. For example, *C. riparius* was regularly found in sites AGD and POR at

relatively large frequencies in different seasons. However, in other sites, such as SMA or CER, the abundance of *C. riparius* fluctuated largely and no larvae could be collected in some seasons (data not shown). Since Iberian streams are characterized by a pronounced seasonality and hydrological instability (Feio et al., 2010; Graça et al., 2004), such variations in *Chironomus* abundance might be related to the hydromorphological features of the sampling sites (Bazzanti et al., 1997; Puntí et al., 2007). *Chironomus riparius* and *C. piger* typically inhabit the sedimentation areas of the streams in which water flow is low and organic matter accumulates (Armitage et al., 2012), allowing chironomids to burrow their cages, feed on sediment-deposited detritus and reach large population sizes (Armitage et al., 2012; Groenendijk et al., 1998). However, the persistence of stable populations over time depends largely upon the stability of their habitats (Syrovátka et al., 2009; Xuehua et al., 2009). Unstable sedimentation areas may become isolated (or even dry) during drought periods and suddenly disrupted following floods (Feio et al., 2015). Consequently, considerable spatial rearrangement of sediments and organic matter may occur, affecting resident populations directly through drift in the stream current and indirectly through habitat instabilities or availability of food resources (Jowett, 2003; Syrovátka et al., 2009). Thus, while stable sedimentation areas will likely allow the persistence of chironomid populations along large periods of time, populations inhabiting unstable sedimentation areas may undergo frequent local extinction and recolonization processes and their persistence over time will depend on whether local extinctions are compensated by effective recolonization processes (Thienemann, 1954).

It seems plausible that those high grades of spatiotemporal population dynamics and gene flow will prevent any genetic erosion effects and contribute to maintain the allelic variation within the two species across the study area as suggested by the strong signature of balancing selection. In addition to this, it is obvious that *Chironomus* exhibits a certain tolerance towards ecological disturbances, and particularly *C. riparius* might even have a selective advantage in these environments (De Haas et al., 2006), which will prevent any reduction in effective population size leading to genetic erosion. Indeed, we have tested some populations collected in the frame of the present study in parallel laboratory ecotoxicology tests and found that populations sampled from highly contaminated sites (AGD, CEL and EST) were generally more tolerant to acute Cd exposures in comparison to populations collected from sites with lower levels of metal contamination (CER, POR and SMA) (Pedrosa et al., *in press*). Moreover, we also found that increased Cd tolerance in the populations from highly contaminated sites was associated to enhanced defense mechanisms, including higher baseline levels of the

major cellular thiols metallothionein and glutathione (Pedrosa et al., 2017b). These findings show that chironomids inhabiting contaminated sites indeed show microevolutionary responses to anthropogenic pollution. However, their very large population sizes and great dynamism (Groenendijk et al., 1998), limit inferences about microevolutionary processes when using population genetic markers such as microsatellites (Hoffmann and Willi, 2008). Consequently, we recommend to focus on functional markers when investigating genetic effects of contaminants on species of this genus or to choose less tolerant species with lower effective population size.

Despite *C. riparius* and *C. piger* being, in general, reproductively isolated across the study area, analysis of the microsatellite data allowed us to report a rare episode of field hybridization between these two species. All hybrids were identified as *C. piger* according to their mtDNA CO I sequences, showing that the hybridization must have involved *C. piger* females. While NewHybrids failed to provide clear assignments to a single hybrid category, most intermediate genotypes seem to be backcrosses with *C. piger* females. Earlier laboratory studies found that in the *C. riparius* (♀) x *C. piger* (♂) crosses, the Rud syndrome originates sterility close to 100% and lethal egg sizes in the F-2 generation (Hägele, 1984, 1999). In the reciprocal cross *C. piger* (♀) x *C. riparius* (♂), the hatchability of the egg ropes is strongly reduced and external malformations and chromosome aberrations are visible in the hybrids. Only nearly 10% reach the adult stage but hybrids show normal developed gonads and are fertile in both sexes (Hägele, 1984, 1999). Thus, our finding that all hybrids carry mitochondrial *C. piger* haplotypes and are thus likely different hybrid categories of interspecific crosses of *C. piger* females with *C. riparius* males (or the respective hybrids) are in line with these studies. Furthermore, the fact that all hybrids were recorded in only a single site and in a single sampling survey shows that hybridization between both species might in fact be a rare event and does not severely impact the genetic composition of the two species. As hybridization could only be detected at a moderately contaminated site, there is no reason to assume that metal contamination might erode species barriers between *C. riparius* and its sister species *C. piger*. Future studies using more advanced genomic tools will certainly shed more light on this interesting case of hybridization between closely related sympatric sister species.

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Supplementary material

Table S1 – Sampling locations, geographic position and date of collection of chironomids (by season and year). Shown are also concentrations (in µg·g^{-1}) of each element analysed (As, Cd, Cr, Cu, Hg, Ni, Pb, and Zn) as well as the total metal concentration (TMC) of the field sediments.

Site	Location	Geo. Coordinate		Stream	Basin	Date of collection	As	Cd	Cr	Cu	Hg	Ni	Pb	Zn	TMC
		Longitude	Latitude												
AGD	Águeda	-8.453	40.575	Vale da Erva	Vouga	A10, S11, A11, A13	BDL	0.18	30.16	60.04	0.00	**69.09**	19.49	74.44	253.41
ALB	Alb. da Serra	-8.275	40.867	Caima	Vouga	S11	5.40	0.17	13.00	6.40	0.00	5.70	9.20	64.00	103.87
ALG	Algeriz	-8.369	40.417	Serra	Vouga	S11	16.00	BDL	29.00	24.00	0.00	36.00	14.00	56.00	175.00
ARG	Argemil	-8.498	41.352	Sanguinhedo	Ave	A10	31.40	0.31	31.28	32.70	0.05	11.39	44.97	128.11	280.22
CEL	Celeirós	-8.435	41.522	Este	Ave	A10, A11. S13, A13	6.90	0.99	22.00	**349.00**	0.09	14.00	97.00	**860.00**	1349.98
CER	Cercosa	-8.220	40.682	Alfusqueiro	Vouga	A11, S13	BDL	1.20	5.90	BDL	0.00	BDL	22.00	62.00	91.10
CHA	Chãs	-8.809	39.783	Milagres	Lis	S11	2.25	0.28	7.22	BDL	0.01	2.36	12.95	25.15	50.21
EST	Estarreja	-8.577	40.750	S. Filipe Ditch	Vouga	A10, S11, A11, S13, A13	**101.00**	0.64	6.60	52.00	**17.40**	BDL	87.00	174.00	438.64
FOZ	Foz do Ribeiro	-7.882	40.073	Unhais	Tejo	S13	13.00	BDL	23.00	25.00	0.00	32.00	17.00	77.00	187.00
LOU	Loureiro	-8.510	40.781	Antuã	Vouga	W12	29.00	0.31	18.00	19.00	0.00	11.00	22.00	99.00	198.31
PAL	Palmeira	-8.503	41.362	Pele	Ave	A10	8.46	0.32	21.63	29.77	0.02	8.71	40.45	136.19	245.55
POR	Porto de Baixo	-8.528	40.741	Jardim	Vouga	A11, S13, A13	17.00	0.73	77.00	22.00	0.01	34.00	19.00	118.00	287.74
SAL	Salreu	-8.546	40.734	Salreu	Vouga	W12	5.80	0.11	5.50	6.60	0.00	4.10	8.90	49.00	80.01
SAN	Sant. Riba-Ul	-8.498	40.858	Antuã	Vouga	W12	**109.50**	0.39	31.94	22.64	0.08	12.29	34.48	132.10	343.44
SJA	S. João do Monte	-8.236	40.596	Águeda	Vouga	S11	3.20	BDL	3.80	3.30	0.00	2.20	6.90	55.00	74.41
SMA	S. Mamede Este	-8.360	41.572	Este	Ave	S11, A11	2.70	BDL	7.70	6.30	0.00	3.30	7.10	39.00	66.10
UL	Ul	-8.498	40.810	Ínsua	Vouga	S11	11.00	0.10	16.00	12.00	0.00	10.30	18.00	81.00	148.40
VAR	Várzea	-8.936	39.755	Várzea	Moel	A10	6.49	0.25	21.54	6.59	0.01	6.27	21.79	38.11	101.06

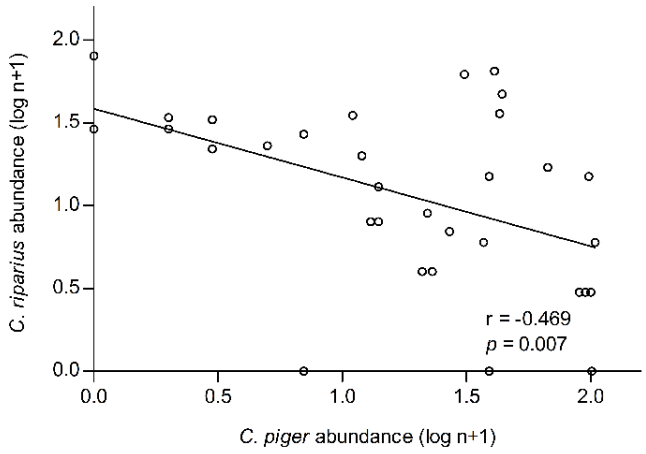
Sampling collection: A10 – autumn 2010, S11 – spring 2011, A11 – autumn 2011, W12 – winter 2012, S13 – spring 2013, A13 – autumn 2013.
Detection limits: As: 2 µg·g^{-1}, Cd 0.08 µg·g^{-1}, Cu 6 µg·g^{-1}, Ni 2 µg·g^{-1}.

Table S2 – Repeat motif, primer sequences, size range (in base pairs) and GenBank accession number of the microsatellite loci used to estimate the genetic diversity of *C. riparius* populations.

Locus	Motif	Primer sequence	Size (bp)	Bibliography	Accession no.
Msc1	CA_9	F: CATCATCCTTAACAACCCAC R: CTAGCTTTGCAGGCGAGTGC	95-103	(Nowak et al. 2006)	DQ408105
Msc2	$(TAA)_9,T_{10}$	F: AGACTAATGACCAGACTTGC R: CTTGTGATGCGAAAAGCCTG	114-141	(Nowak et al. 2006)	DQ408106
Msc3	$(GT)_{14}$, T_9, T_6	F: ACTACGCGTGCCTCAACAGC R: AGCTAATTCTCATGTTGGTC	168–176	(Nowak et al. 2006)	DQ408107
Msc4	$(TCA)_6$	F: TGACTGAACTTCCGCAATGGG R: CCGAGAATGCTGCGATCCAG	211–216	(Nowak et al. 2006)	DQ408108
Msc5	$(CA)_{11}$, A_9	F: AACATTTGAACGCACATCG R: ATTTGATTGTATGTCCTG	264–278	(Nowak et al. 2006)	DQ408109
Msc6	GA	F: TATCCCACCATATCGGCATC R: CACCCGCAAATGATATACACAA	168–229	(Soeter et al. 2010)	-
Msc7	GT	F: GCTGAATCGTGTGATGTGCT R: TGCTGCTTCTGTCGTATGCT	235–245	(Soeter et al. 2010)	-

Supplementary Material S3 – Total abundance of *C. riparius* and *C. piger* across the sampling sites of the study area.

Site	Season	Contamination	Total abundance	
			C. riparius	*C. piger*
AGD	Autumn 2010	High	13	7
AGD	Spring 2011	High	26	6
AGD	Autumn 2011	High	103	5
AGD	Autumn 2013	High	94	2
ALB	Spring 2011	Low	0	79
ALG	Spring 2011	Moderate	1	28
ARG	Autumn 2010	Moderate	38	14
CEL	Autumn 2010	**High**	**20**	**3**
CEL	Autumn 2011	**High**	**97**	**14**
CEL	Spring 2013	**High**	**6**	**0**
CEL	Autumn 2013	**High**	**22**	**3**
CER	Autumn 2011	Moderate	40	64
CER	Spring 2013	Moderate	1	33
CHA	Spring 2011	Low	42	35
EST	Autumn 2010	High	4	22
EST	Spring 2011	High	6	26
EST	Autumn 2011	High	43	46
EST	Spring 2013	High	2	21
EST	Autumn 2013	High	10	34
FOZ	Spring 2013	Moderate	2	32
LOU	Winter 2012	Moderate	38	0
PAL	Autumn 2010	Moderate	12	7
POR	Autumn 2011	Moderate	99	2
POR	Spring 2013	Moderate	30	61 (+11 hybrids)
POR	Autumn 2013	Moderate	66	16
SAL	Winter 2012	Low	13	12
SAN	Winter 2012	High	89	2
SJA	Spring 2011	Low	0	28
SMA	Spring 2011	Low	21	8
SMA	Autumn 2011	Low	100	0
UL	Spring 2011	Moderate	11	19
VAR	Autumn 2010	Low	36	5



Supplementary Material S4 – Pearson correlation test between the total abundance (log transformed) of *C. riparius* and *C. piger* across the study area.

Supplementary Material S5 – Analysis of molecular variance in *C. riparius* and *C. piger* across the study area based on 9999 permutations of the datasets. Shown are the degrees of freedom (df), sum of squares (SS), percentage of variation, overall genetic structure among sampling surveys (F_{ST}) and significance level (*p*-value).

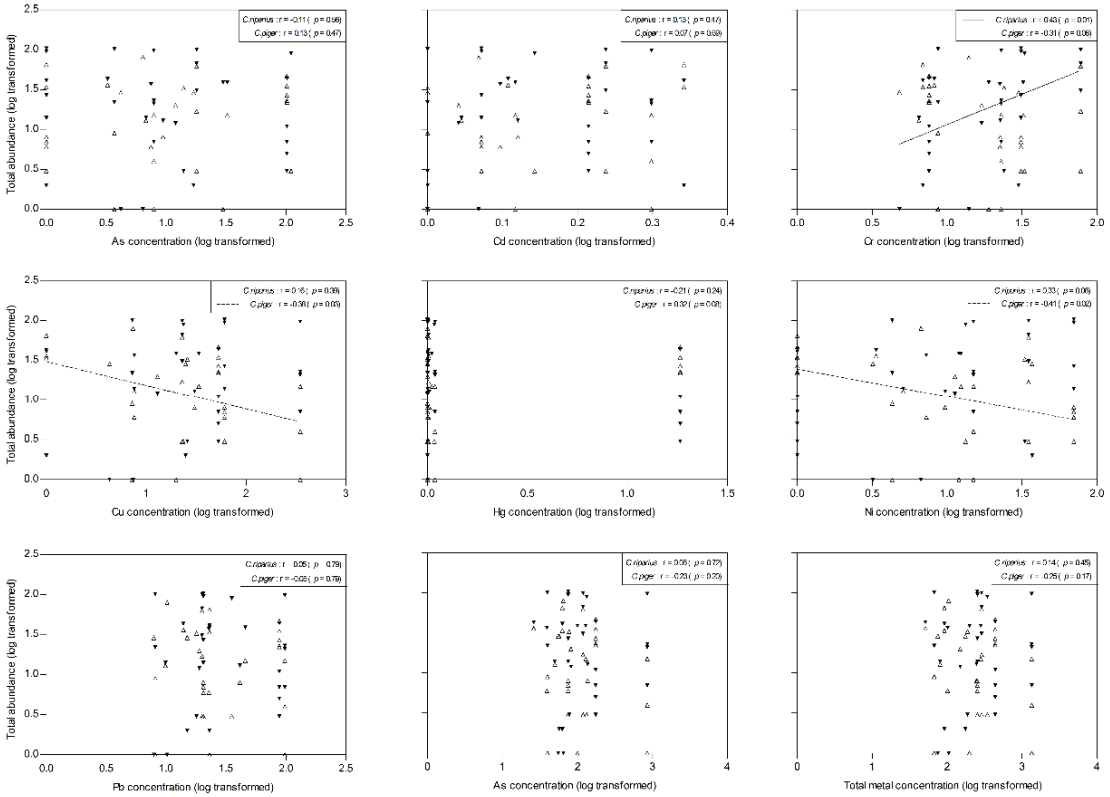
Source	df	SS	% variation	F_{ST}	*p*-value
1. *Chironomus riparius*					
Among populations	23	66.03	1.05	0.010	0.0001
Within populations	1110	2114.47	98.47		
Total	1123	2180.50	100.00		
2. *Chironomus piger*					
Among populations	14	43.41	1.93	0.019	0.0001
Within populations	657	1086.29	98.07		
Total	671	1129.70	100.00		

Supplementary Material S6 – Genetic differentiation estimates for *C. riparius*. Pairwise F_{ST} values among the 24 sampling surveys are displayed below the diagonal based on 9999 permutations of the datasets. Significance levels are indicated as * for $p < 0.05$ and ** for significant differences after Holm's Bonferroni correction.

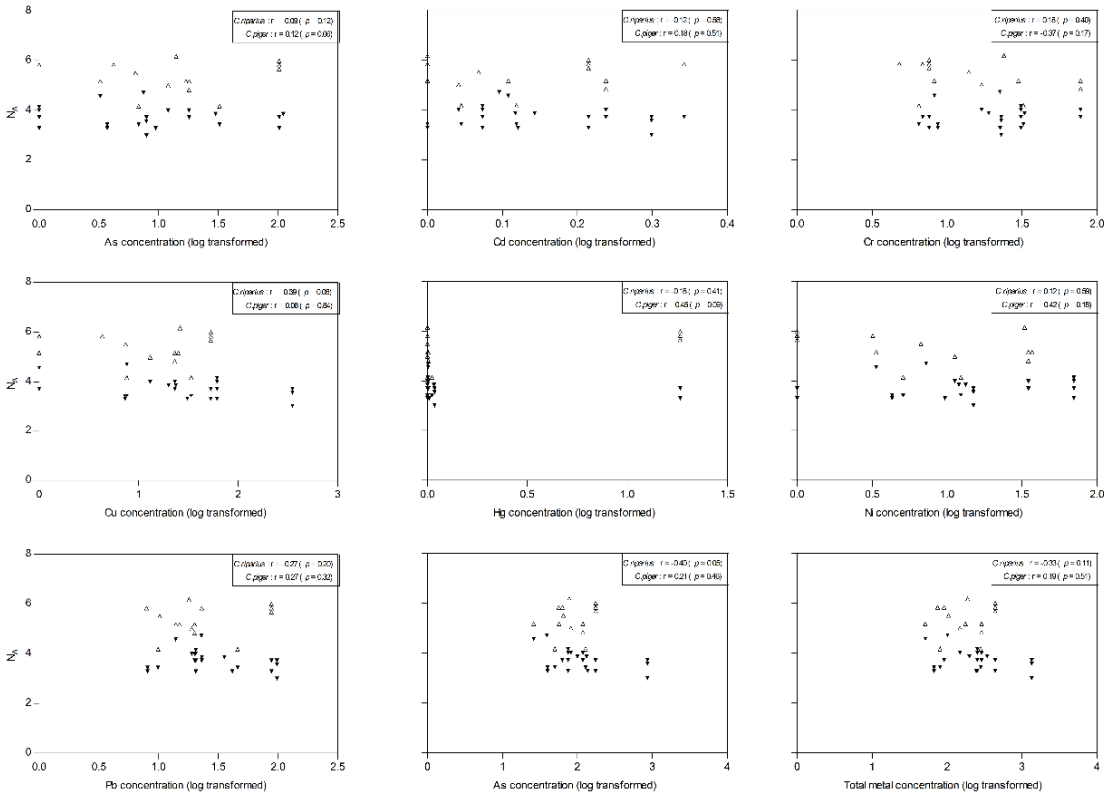
AGD A10	AGD S11	AGD A11	AGD A13	ARG A10	CEL A10	CEL A11	CEL S13	CEL A13	CER A11	CHA S11	EST A11	EST A13	LOU W12	PAL A10	POR A11	POR S13	POR A13	SAL W12	SAN W12	SMA S11	SMA A11	UL S11	VAR A10	
-																								AGD.A10
0.017	-																							AGD.S11
0.013	0.009	-																						AGD.A11
0.013	0.000	0.003	-																					AGD.A13
0.026	0.009	0.007	0.007	-																				ARG.A10
0.034	0.000	0.001	0.003	0.003	-																			CEL.A10
0.014	0.000	0.000	0.000	0.000	0.000	-																		CEL.A11
0.066*	0.026	0.000	0.017	0.000	0.000	0.000	-																	CEL.S13
0.000	0.006	0.000	0.002	0.003	0.009	0.000	0.026	-																CEL.A13
0.026	0.001	0.016	**0.020***	**0.024***	0.013	0.003	0.034	**0.018***	-															CER.A11
0.000	**0.030***	0.011	**0.027***	**0.044***	**0.030***	**0.026***	**0.036***	0.013	**0.039***	-														CHA.S11
0.010	0.004	0.000	0.005	0.000	0.000	0.000	0.000	0.000	0.016	**0.026***	-													EST.A11
0.009	0.000	0.004	0.000	0.022	0.005	0.003	0.017	0.000	0.022	0.023	0.000	-												EST.A13
0.011	0.000	0.006	0.007	0.000	0.002	0.000	0.010	0.004	0.002	**0.031***	0.000	0.014	-											LOU.W12
0.018	0.000	0.021	0.011	0.003	0.000	0.004	0.020	0.010	0.019	**0.053***	0.000	0.005	0.000	-										PAL.A10
0.020	0.012	0.011	0.000	0.000	0.006	0.005	0.001	0.004	**0.035***	**0.042***	0.000	0.010	0.005	0.001	-									POR.A11
0.021	0.003	0.007	0.000	0.000	0.011	0.000	0.018	0.007	**0.025***	**0.039***	0.001	0.025	0.000	0.009	0.000	-								POR.S13
0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.006	0.000	0.009	**0.022***	0.000	0.003	0.000	0.000	0.000	0.000	-							POR.A13
0.000	0.000	0.005	0.000	0.002	0.000	0.000	0.030	0.000	0.005	0.019	0.000	0.000	0.000	0.000	0.000	0.000	0.000	-						SAL.W12
0.024	0.000	0.012	0.008	0.012	0.012	0.000	0.018	0.013	0.004	**0.025***	**0.021***	0.030	0.003	0.025	**0.020***	0.007	0.000	0.000	-					SAN.W12
0.020	0.000	0.010	0.000	0.000	0.006	0.000	0.013	0.000	**0.025***	**0.041***	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	**0.016***	-				SMA.S11
0.068*	**0.023***	**0.042***	**0.023***	**0.026***	0.014	**0.028***	0.016	**0.042***	**0.041***	**0.079****	**0.024***	0.025	**0.030***	0.016	**0.015***	**0.036***	**0.027***	0.019	**0.042***	**0.019***	-			SMA.A11
0.021	0.006	0.011	0.013	0.000	0.004	0.000	0.006	0.005	**0.021***	**0.047***	0.000	0.009	0.000	0.004	0.009	0.007	0.003	0.007	**0.025***	0.000	**0.024***	-		UL.S11
0.021	0.010	0.003	0.001	**0.022***	0.006	0.008	0.003	0.014	**0.025***	0.009	**0.016***	0.000	**0.019***	**0.034***	**0.027***	**0.026***	**0.013***	0.016	**0.018***	**0.025***	**0.043***	**0.026***	-	VAR.A10

Supplementary Material S7 – Genetic differentiation estimates for *C. piger*. Pairwise F_{ST} values among the 15 sampling surveys are displayed below the diagonal based on 9999 permutations of the datasets. Significance levels are indicated as * for $p < 0.05$ and ** for significant differences after Holm's Bonferroni correction.

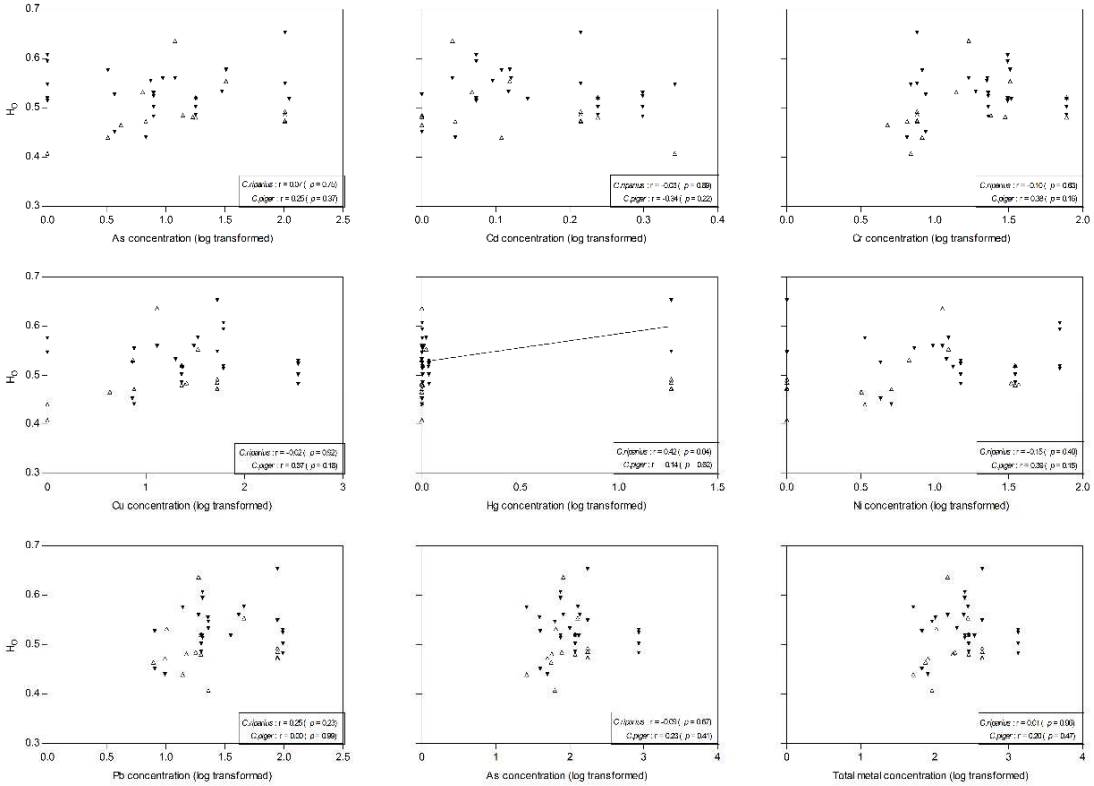
ALB S11	ALG S11	ARG A10	CER S13	CHA S11	EST A10	EST S11	EST S13	EST A13	FOZ S13	POR S13	POR A13	SAL W12	SJA S11	UL S11	
-															ALB.S11
0.045**	-														ALG.S11
0.057**	**0.097****	-													ARG.A10
0.017*	**0.031***	**0.035***	-												CER.S13
0.033**	**0.058****	**0.079****	0.005	-											CHA.S11
0.041**	**0.079****	**0.028***	**0.022***	0.012	-										EST.A10
0.016*	**0.042****	**0.042***	0.000	0.005	**0.018***	-									EST.S11
0.020*	**0.058****	**0.062****	0.007	0.009	**0.022***	0.001	-								EST.S13
0.027**	**0.047****	**0.052***	0.000	0.003	**0.021***	0.001	**0.014***	-							EST.A13
0.023*	**0.048****	**0.053***	0.000	0.000	0.009	0.000	0.000	0.000	-						FOZ.S13
0.023*	**0.066****	**0.069***	0.007	0.000	0.012	0.000	0.000	0.009	0.000	-					POR.S13
0.013	**0.051****	**0.036***	0.000	0.000	0.003	0.000	0.001	0.009	0.000	0.000	-				POR.A13
0.045*	**0.087****	**0.079****	**0.024***	**0.021***	**0.021***	0.019	**0.039***	0.009	0.020	0.022	**0.034***	-			SAL.W12
0.023*	**0.047****	**0.050***	0.005	0.002	**0.016***	0.000	0.001	0.004	0.000	0.000	0.001	**0.030***	-		SJA.S11
0.014	**0.042***	**0.034***	0.001	0.007	**0.024***	0.004	0.017	0.000	0.004	0.011	0.017	0.011	0.002	-	UL.S11



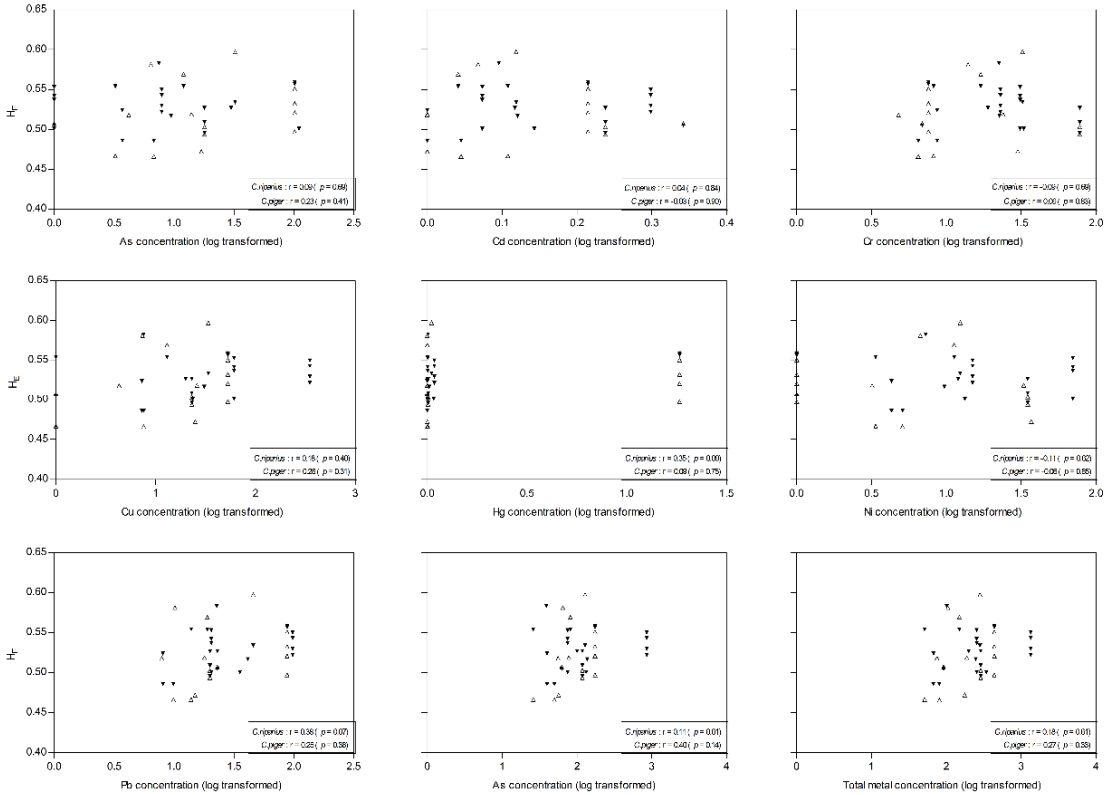
Supplementary Material S8 – Pearson's correlation test between the metal contamination of the sites (as total and metal-by-metal log transformed concentrations) and the total abundance of *C. piger* (open triangles) and *C. riparius* (filled triangles) across the study area. Shown are Pearson correlation (r) and significance levels (*p*) for all tests.



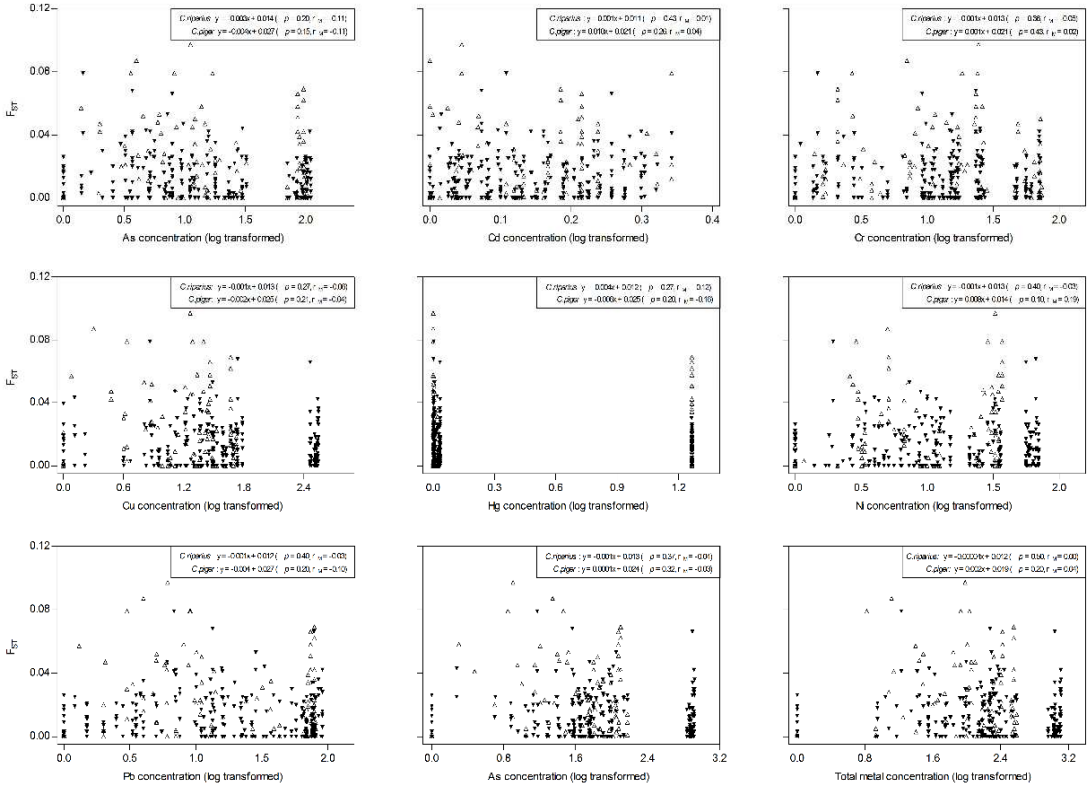
Supplementary Material S9 – Pearson's correlation test between the metal contamination of the sites (as total and metal-by-metal log transformed concentrations) and the mean number of alleles (N_A) of *C. piger* (open triangles) and *C. riparius* (filled triangles) across the study area. Shown are Pearson correlation (r) and significance levels (*p*) for all tests.



Supplementary Material S10 – Pearson's correlation test between the metal contamination of the sites (as total and metal-by-metal log transformed concentrations) and the observed heterozygosity (H_O) of *C. piger* (open triangles) and *C. riparius* (filled triangles) across the study area. Shown are Pearson correlation (r) and significance levels (*p*) for all tests.



Supplementary Material S11 – Pearson's correlation test between the metal contamination of the sites (as total and metal-by-metal log transformed concentrations) and the expected heterozygosity (H_E) of *C. piger* (open triangles) and *C. riparius* (filled triangles) across the study area. Shown are Pearson correlation (r) and significance levels (*p*) for all tests.



Supplementary Material S12 – Genetic structure in *C. piger* (open triangles) and *C. riparius* (filled triangles) with metal contamination of the sites. Mantel correlation (r_M) and significance levels (*p*) are shown for the association between pairwise F_{ST} values and metal contamination of the sites (total and metal-by-metal log transformed concentrations) based on 9999 permutations of the datasets.

CHAPTER VI:

ASSESSING THE SUITABILITY OF GENETIC DIVERSITY OF *CHIRONOMUS RIPARIUS* (MEIGEN) AS AN INDICATOR OF ENVIRONMENTAL POLLUTION

Published in the Journal Ecological Indicators.

Abstract

Reliable and cost-effective indicators are needed to reduce uncertainty in ecological risk assessments of chemicals so that their long-term and evolutionary impacts in the dynamics of stressed biological communities can be accounted for.

In the present study we investigate the suitability of genetic diversity as a previously proposed indicator of ecological disturbance of freshwater ecosystems. For that, we selected three freshwater systems with well-known histories of metal contamination and three nearby-located reference sites located in Northern Portugal and the effects of metal contamination were assessed both in the levels of genetic diversity of the non-biting midge *Chironomus riparius* Meigen (at microsatellite markers) and in the diversity and composition of the benthic macroinvertebrate communities.

Results showed a remarkable impoverishment in the abundance and diversity of macroinvertebrate communities of metal polluted sites in which most sensitive taxa were eliminated and replaced by more opportunistic ones belonging mainly to Oligochaeta and Diptera. In contrast, no evidence for pollution-induced genetic erosion was found in *C. riparius* populations in the studied area with genetic diversity of the species being indeed higher in sites with impoverished macroinvertebrate communities and poor habitat quality.

Our findings suggest that there are important limitations to the use of measures of genetic diversity of *C. riparius per se* to unravel ecological disturbance. Field studies aiming at quantifying pollutant-driven genetic erosion need to carefully consider the ecological and demographic aspects of potential target species.

Keywords: Benthic communities, biomonitoring, ecological risk assessment, freshwater, genetic diversity, metal contamination, microsatellites, neutral markers.

1. Introduction

Traditional ecological risk assessments (ERAs) derived from standard ecotoxicological laboratory tests that predict the impacts of contaminants based on a limited number of life-history alterations of short-lived, fast-growing model species are no longer considered a solution to understand the complicated relationships between contaminants and natural biota (Artigas et al., 2012; Beketov and Liess, 2012). Although being undoubtedly useful for criteria settings and to estimate the potential intrinsic hazard of thousands of chemicals, the reductionist nature of these assessments hamper the extrapolation to natural conditions (Breitholtz et al., 2006; Fischer et al., 2013; Janssen et al., 2000). On top of the highly dynamic and complex nature of natural systems, indicators that can reliably reflect the potential long-term and evolutionary consequences of contaminants over timescales that exceed the generation time of the standard laboratory tests are also needed to be accounted for in ERA approaches (De Coninck et al., 2014).

The search for more robust and context-driven bio-indicators that add ecological relevance and can quickly and inexpensively assess the effects of contaminants has led to an increased interest on the topic of genetic diversity that is expected to yield unique insights to the routine ERA approaches (De Coninck et al., 2014; van Straalen and Timmermans, 2002). Genetic diversity constitutes the foundation for all biological diversity on Earth providing the raw material on which selection pressures act. Therefore, preserving high levels of genetic diversity has been argued to be of utmost importance for the long-term viability and sustainability of populations and, ultimately, species (Frankham et al., 2002). In this sense, environmental contaminants may interfere with the natural dynamics of populations and constitute highly disruptive selection pressures, affecting the fitness and/or the reproductive success of individuals. Consequently, exposed populations may experience dramatic reductions in their effective population sizes that result in significant reductions of genetic variability (Nowak et al., 2009). This so-called process of genetic erosion has been predicted to eventually diminish the ability of populations to evolve and adapt to future, unpredictable environmental change and, thus, accelerates the extinction risk of populations and species (Bijlsma and Loeschcke, 2012; Ribeiro and Lopes, 2013; van Straalen and Timmermans, 2002).

Evidences from laboratory experimentation have illustrated that chemical exposure may rapidly depress the original gene pool of populations within only few generations (Athrey et al., 2007; Nowak et al., 2009). Also good evidence has accumulated showing that reduced genetic diversity increases population's vulnerability towards environmental

stress (Nemec et al., 2013; Nowak et al., 2007; Nowak et al., 2012). However, inferences obtained from laboratory studies still need to be confirmed by empirical evidence under natural conditions. In fact, while some studies have reported losses of genetic diversity in populations inhabiting impacted areas (Bourret et al., 2008; Paris et al., 2015), many others have failed to show genetic erosion in natural populations of invertebrates (Costa et al., 2012; Martins et al., 2009), fishes (Miller et al., 2012; Whitehead et al., 2003) or mammals (Berckmoes et al., 2005; Wirgin et al., 2015). Therefore, further studies are necessary to gain knowledge on the effects that environmental contaminants may produce on the genetic diversity of natural populations.

Furthermore, it is also necessary to understand in which manner contaminant-driven genetic erosion is reflected at higher and ecologically more relevant levels of biological organization (Hughes et al., 2008; Pauls et al., 2014). Measures of composition and diversity of the benthic macroinvertebrate communities have been largely used to monitor ecological disturbances in freshwater systems (Clements et al., 2000; Hering et al., 2006). The application of such measures is based on the assumption that more complex and heterogeneous habitats allow the persistence of more diverse taxa that are gradually replaced by more tolerant ones as the conditions become more adverse (Gerhardt et al., 2004; Smolders et al., 2003). The diversity of EPT (Ephemeroptera, Plecoptera and Trichoptera) taxa, for instance, has been argued to be a sensitive indicator of stream's biological quality and declines in these taxonomic groups entail important impairments in fundamental processes of the ecosystems such as decomposition of organic matter, release of plant nutrients or secondary productivity (Clements et al., 2013; Moore and Palmer, 2005; Wallace et al., 1996). However, and although measures at the community level are advantageous monitoring tools that represent exposure at the collection site, they can only detect relatively strong ecological disturbances that originate the elimination of taxonomic groups (Gerhardt et al., 2004; Smolders et al., 2003). Therefore, their utility to monitor and predict subtle cumulative effects of contaminants is limited and important questions related, for instance, with reproductive isolation and future ecological condition of exposed biota cannot be addressed.

The essence of including measures of genetic diversity in ERAs is, therefore, to make inferences about the impacts that environmental pollution may produce on the long-term viability and sustainability of exposed biota in such a way that they allow linkages with the ecological integrity of natural ecosystems (Hoffmann and Willi, 2008; van Straalen and Timmermans, 2002). From an ecotoxicological perspective, the use of measures of genetic diversity should rely, at a first instance, on well-established model

ecotoxicological species, such as the non-biting midge *Chironomus riparius* Meigen (Diptera: Chironomidae) that has been extensively used for biomonitoring the effects of thousands of contaminants in freshwater systems (Campos et al., 2016; Rodrigues et al., 2015). Larvae of *C. riparius* are tube-dwelling deposit feeders that live closely associated to the sediments where they burrow their “cases” and feed on particulate matter. Because several environmental contaminants tend to accumulate in the sediments, effects observed in chironomids are expected to reflect sediment contamination. Furthermore, and apart from its ecotoxicological relevance, *C. riparius* is able to colonize virtually all freshwater habitats throughout the Holarctic, (Armitage et al., 2012; De Haas et al., 2005; Groenendijk et al., 1998) which makes the species ideal to investigate the potential role of contaminants as mediators of genetic erosion in natural populations.

The main goal of the present work was thus to test the suitability and robustness of measures of genetic variation of *C. riparius* natural populations as indicators of ecological disturbance of freshwater ecosystems. As laboratory studies have confirmed that contaminant exposure can reduce genetic diversity in this species (Müller et al., 2012; Nowak et al., 2012; Nowak et al., 2009; Vogt et al., 2007a), leading to reduced fitness and tolerance towards additional chemical exposure and other environmental stressors (Nowak et al., 2007; Vogt et al., 2010; Vogt et al., 2007b), we hypothesized that *C. riparius* populations inhabiting metal contaminated sites would comprise lowered levels of genetic diversity compared to uncontaminated sites which may threaten their long-term survival. For that, three freshwater systems with well-known histories of metal contamination and three nearby-located reference sites were selected and effects on the levels of genetic diversity of *C. riparius* were assessed and compared with those effects occurring at the community level. Genetic diversity and structure of *C. riparius* populations was estimated based on the variation of seven microsatellite loci while community-level effects were assessed based on benthic macroinvertebrate community composition and diversity metrics.

2. Material and Methods

2.1. Study area

This study was carried out in the Northern Portugal in June (Spring) and October (Autumn) of 2013. Three historically metal contaminated sites (sites C_1, C_2, C_3) and three closely located reference sites (sites R_1, R_2, R_3) were selected (Figure 1, Table 1). The

occurrence of *Chironomus riparius* natural populations on the selected sites had also been previously confirmed (Pedrosa et al., submitted for publication).

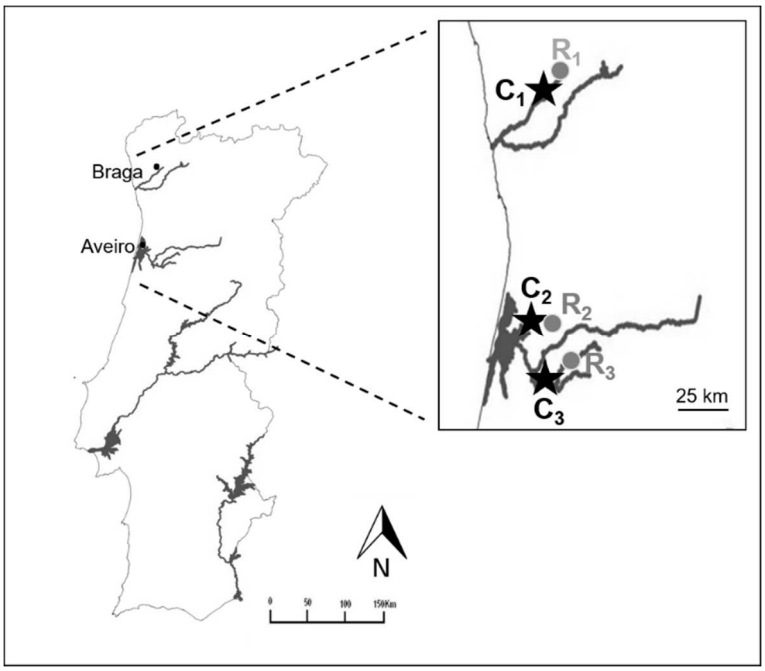


Figure 1 – Location of the metal contaminated (C_1, C_2, C_3) and reference (R_1, R_2, R_3) sites chosen for the present study.

Shortly, site C_1 located in the Este River downstream the city of Braga, and site C_2 located in a manmade ditch constructed to carry effluent loads from the industrial complex of Estarreja, are recognized examples of grossly metal contaminated freshwater systems (Reis et al., 2009; Soares et al., 1999). The third contaminated site (site C_3), located in Vale da Erva stream, receives the discharge loads from the industrial complex of Águeda and has also been shown to be impacted by several metals (Reis et al., 2005). For the best of our knowledge, no episodes of metal pollution were previously reported for any of the chosen reference sites.

Table 1 – Geographical location and identification of metal contaminated and references sites of the study.

Site	Coordinate	Location	Watercourse	Subbasin	Basin
Contaminated 1 (C_1)	41.522 N -8.435 W	Celeirós	Este river	Este	Ave
Contaminated 2 (C_2)	40.750 N -8.577 W	Estarreja	S. Filipe Ditch	Antuã	Vouga

Contaminated 3 (C_3)	40.575 N -8.453 W	Águeda	Vale da Erva Stream	Águeda	Vouga
Reference 1 (R_1)	41.572 N -8.360 W	S. Mamede de Este	Este river	Este	Ave
Reference 2 (R_2)	40.741 N -8.528 W	Porto de Baixo	Jardim river	Antuã	Vouga
Reference 3 (R_3)	40.682 N -8.220 W	Cercosa	Alfusqueiro river	Águeda	Vouga

2.2. Metal contamination of sediments

Sediment samples of the upper 5 cm surface layer were collected from each site and analyzed for total content of arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), nickel (Ni), lead (Pb) and zinc (Zn). Total Hg was measured by thermal decomposition atomic absorption spectrometry with gold malgamation (LECO® model AMA-254) with no previous digestion of the material. For the analysis of the remaining chemical elements, sediments were acid digested according to standard procedure Method 3052B for Acid Digestion of sediments (USEPA, 1996). Total As, Cd, Cr, Cu, Ni and Pb was analyzed by Graphite Furnace AA (GFAA model Thermo X Series, Peltier Nebulizing Camera, Burgener Nebulizer; CETAC AS510 auto-sampler), whereas Zn was analyzed by Flame Atomic Absorption Spectrometry (FLAA model Jobin Yvon Activa M). All samples were homogenized, dried and sieved through a 1-mm mesh prior to chemical analyses. Total metal and As content was determined in triplicate and results are expressed as mean values. Minimum 80-120% of recovery percentages, blanks and certified reference material (NRC MESS-3) were used for analytical accuracy of laboratory measurements.

2.3. Habitat quality

Sites were characterized in terms of general physico-chemical water quality parameters as well as descriptors of stream channel and riparian vegetation using a much-simplified version of the River Habitat Survey (Raven et al., 1998).

Physico-chemical water parameters were determined *in situ* with portable devices and included measures of temperature (ºC), pH, conductivity ($\mu S \cdot cm^{-1}$) and dissolved oxygen (in mg/L). Stream channel was characterized according to the following variables: channel width and depth (m), river bank height (m), substrate size (categories: 8 for

bedrock, 7 for boulder, 6 for cobble, 5 for pebble, 4 for gravel; 3 for sand, 2 for silt/mud and 1 for clay), flow type (categories: 7 for freefall waters, 6 for chute, 5 for broken water, 4 for unbroken water, 3 for rippled, 2 for smooth and 1 for no flow) and cover of in-stream moss, macrophytes and algae (in percentage). Riparian vegetation was characterized according to the complexity at water face and at 1 and 5 meters from the stream channel (categories: 1 for bare, 2 for uniform, 3 for simple and 4 for complex). The percentage of herbaceous and tree shading of the channel was also assessed.

2.4. Macroinvertebrate community survey

Benthic macroinvertebrate communities were surveyed using a 500 µm mesh net and kick and sweep procedures, according to a multi-habitat kick-sampling, covering a river stretch of ca. 100 m. Sampling effort was divided between riffles (2 minute), pools (1 minute) and water column substrates like wood, tree roots and/or macrophytes (1 minute). All macroinvertebrates collected were preserved in 70% ethanol and subsequently identified to Family level, except Annelida and Turbellaria that were identified at the Class level.

2.5. Genetic diversity and structure of *C. riparius* popuations

Due to taxonomic difficulties in species identification related with the cryptic nature of *Chironomus* larvae, each individual larva was genetically identified using a DNA barcoding approach adapted for chironomids (Pfenninger et al., 2007).

Briefly, DNA was extracted according to DNA hotSHOT method (Montero-Pau et al., 2008). Tissue was homogenized in 30 µL alkaline lysis buffer (25 mM NaOH, 0.2 mM Na_2EDTA, pH 8.00) during 30 min at 95 ºC followed by 5 min at 4 ºC. After thermal treatment, 30 µL neutralizing solution (40 mM Tris-HCl, pH 5.00) was added and samples were stored at 4 ºC for posterior use.

Mitochondrial cytochrome oxidase I (mtDNA CO I) fragments were amplified in a T-Gradient thermocycler (Biometra, Göttingen, Germany) in a total volume of 13 µL of reaction mixture and 2 µL of DNA sample according to the cycling conditions: 1 min 92 ºC, 1 min 55 ºC and 1 min 72 ºC repeated 36 times. Reaction mixture contained 3 mM $MgCl_2$, 1x reaction buffer (20 mM Tris-HCl, 50 mM KCl), 0.2 mM dNTP, 0.3 µM of each *Chironomus* specific primer (forward: 5' TCGAGCAGAATTAGGACGACC, reverse: 5' AGGATCACCCCCACCAGCAGG) and 1 U *Taq* DNA polymerase. Sequencing was performed in an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

CO I sequences were, then, aligned in sequence scanner software 2 (Applied Biosystems, Foster City, CA, USA) and Bioedit software version 7.0.5.2 (Ibis Therapeutics, Carlsbad, CA, USA) and identified according to reference GenBank sequences (accession numbers DQ910547-DQ910729; (Nowak et al., 2006)).

Genetically identified larvae of *C. riparius* were afterwards used to estimate the allelic variation at 7 polymorphic microsatellites (Table 2), according to the following cycling conditions: 36 x: 30 sec 94 ºC, 30 sec 55 ºC and 40 sec 72 ºC; and 1 x 5 min 72 ºC. Reaction mixture contained 2.4 mM $MgCl_2$, 0.25 mM dNTP, 0.2 µM of each specific primer and 0.5 U *Taq* DNA polymerase in a total volume of 8 µL of reaction mixture per microsatellite plus 2 µL of DNA sample. Microsatellite data were automatically scored using software Genemarker© version 2.6.3 (Softgenetics, State College, PA) and corrected by eye whenever necessary.

Table 2 – Repeat motif, primer sequences and size range of microsatellite alleles used for assessing genetic diversity of *C. riparius* populations

Locus	Motif	Primer sequence	Size (bp)	Bibliography
Msc1	CA_9	F: CATCATCCTTAACAACCCAC R: CTAGCTTTGCAGGCGAGTGC	95-103	Nowak, Hankeln et al. 2006
Msc2	$(TAA)_9,T_{10}$	F: AGACTAATGACCAGACTTGC R: CTTGTGATGCGAAAAGCCTG	114-141	Nowak, Hankeln et al. 2006
Msc3	$(GT)_{14}$, T_9, T_6	F: ACTACGCGTGCCTCAACAGC R: AGCTAATTCTCATGTTGGTC	168–176	Nowak, Hankeln et al. 2006
Msc4	$(TCA)_6$	F: TGACTGAACTTCCGCAATGGG R: CCGAGAATGCTGCGATCCAG	211–216	Nowak, Hankeln et al. 2006
Msc5	$(CA)_{11}$, A_9	F: AACATTTGAACGCACATCG R: ATTTGATTGTATGTCCTG	264–278	Nowak, Hankeln et al. 2006
Msc6	GA	F: TATCCCACCATATCGGCATC R: CACCCGCAAATGATATACACAA	168–229	Soeter, Bakker et al. 2010
Msc7	GT	F: GCTGAATCGTGTGATGTGCT R: TGCTGCTTCTGTCGTATGCT	235–245	Soeter, Bakker et al. 2010

2.6. Data analysis

Habitat quality: Two-way analysis of variance (ANOVA) was used to determine the influence of the factors “metal contamination” and “season” in the water quality parameters conductivity, temperature, pH and dissolved oxygen. A log-transformation was applied to conductivity and dissolved oxygen datasets to meet the assumptions of normality and homogeneity of variances. Additionally, two Principal Component Analyses (PCA) were performed independently to represent the gradient of channel size

(width, depth and bank weight) and riparian vegetation (complexity and shadow) of the streams of the study area. The resulting site scores were afterwards used as new variables describing the channel size and the riparian vegetation to be related with biological data.

Macroinvertebrate community: The influence of "metal contamination" and "season" on the structure of the benthic macroinvertebrate communities was assessed using permutational multivariate analysis of variance, PERMANOVA (Anderson, 2001). PERMANOVA was undertaken on log-transformed data using Bray-Curtis dissimilarity distance and tested using Monte Carlo permutation test with 999 permutations. Complementary to PERMANOVA, non-metric multidimensional scaling (NMDS) was used to graphically visualize similarity patterns between the different macroinvertebrate communities surveyed (also based in Bray-Curtis dissimilarity of log-transformed data). Vector-fitting analysis was used to represent the association between environmental variables (metal content, water quality characteristics and stream features) and the ordination of biological data provided by the NMDS analysis. Finally, two-way ANOVAs were performed to determine the effect of "metal contamination" and "season" on the community-level metrics: "total macroinvertebrate abundance" (i.e. total number of invertebrates recorded), "total family richness" (i.e. number of different macroinvertebrate families recorded), "EPT family richness" (i.e. number of different families belonging to Ephemeroptera, Plecoptera and Trichoptera) and "% Diptera + Oligochaeta" (i.e. percentage of total macroinvertebrates recorded that corresponded to Diptera and Oligochaeta taxa). The community-levels metrics datasets were tested for normality and homogeneity of variance using formal Kolmogorov-Smirnov test and Levene's test, respectively. "Total macroinvertebrate abundance" data was log-transformed to meet the assumptions of normality and homogeneity of variances. Two-way ANOVAs were carried out using the software package GraphPad Prism® (version 6.0, GraphPad Software, San Diego, CA, USA). PERMANOVA, NMDS, PCAs and vector fitting were performed on R software (version 3.2.0, R Foundation for Statistical Computing, Vienna, Austria) using "vegan" package (Oksanen et al., 2013). All formal statistical hypothesis tests were considered to be statistically significant at $p < 0.05$.

Genetic diversity and structure of C. riparius: Genetic diversity of populations was estimated based on the mean number of alleles (N_A) as well as observed (H_O) and expected heterozygosity (H_E). Departure from Hardy-Weinberg equilibrium (HWE) was tested for all loci using Chi-squared tests ($p < 0.05$). Hierarchical analysis of molecular variance (AMOVA) was also performed to estimate genetic structure of populations as Φ_{PT}. In addition, "isolation-by-distance" was assessed by simple Mantel test in which geographical distance was correlated to genetic distance. Both AMOVA and Mantel test

were based on 999 permutations. All genetic parameters were calculated in GenAlEx 6.501 (Peakall and Smouse, 2006).

3. Results

3.1. Metal contamination of sediments

Chemical analysis showed that the total metal content was higher in the sediments of the three metal contaminated sites compared to their respective references (Table 3).

Table 3 – Average metal and metalloid concentration (µg/g) of metal contaminated (C) and reference (R) sites. Threshold Effect Concentrations (TEC) and Probable Effect Concentrations (PEC) of sediment quality guidelines of MacDonald et al. (2000) are shown below.

Site	As	Cd	Cr	Cu	Hg	Ni	Pb	Zn	TMC
C_1	6.90	0.99	22.00	**349.00**	0.09	14.00	97.00	860.00	1349.98
C_2	**101.00**	0.64	6.60	52.00	**17.40**	<2.00	87.00	174.00	438.64
C_3	<2.00	0.18	30.16	60.04	0.00	**69.09**	19.49	74.44	253.41
R_1	2.70	<0.08	7.70	6.30	0.00	3.30	7.10	39.00	66.10
R_2	17.00	0.73	77.00	22.00	0.00	34.00	19.00	118.00	287.74
R_3	<2.00	1.20	5.90	<5.00	0.00	<2.00	22.00	62.00	91.10
TEC	9.79	0.99	43.4	31.60	0.18	22.70	35.80	121.00	
PEC	33.00	4.98	111.00	149.00	1.06	48.60	128.00	459.00	

Furthermore, and according to the sediment quality guidelines of MacDonald et al. (2000), probable effect concentrations (PECs) above which severe adverse effects for benthic invertebrates are expected to occur were exceeded in all three contaminated sites (MacDonald et al., 2000). Site C_1 exceeded PECs for Cu and Zn, site C_2 had As and Hg above PECs and site C_3 had Ni above PEC. In turn, reference sites did not exceed PEC values for none of the elements analyzed but only the conservative threshold effect concentrations (TECs) that establish the lower limit concentrations below which no adverse effects are likely to occur, were exceeded in sites R_2 and R_3 (Table 3). Therefore, less anthropogenic impact was expected to occur in the three reference sites compared to the metal contaminated sites located downstream metal pollution sources.

3.2. Habitat quality

Water quality parameters of conductivity, temperature, pH and dissolved oxygen (DO) are depicted in Table 4. Results of the two-way ANOVAs showed a significant effect of "metal contamination" in conductivity ($F_{1,8}$ = 17.65, p = 0.003) that was higher in metal contaminated sites than in references. A significant effect of "season" was observed for the water temperature ($F_{1,8}$ = 4.96, p = 0.057) that was higher in July than in October. In contrast, no significant effect of either "metal contamination" or "season" was observed for pH or for DO. However, DO was clearly low in metal contaminated site C_2 (DO = 0.99 – 2.90 mg/L). No significant interaction between "metal contamination" and "season" was found for any of the assessed water quality parameters (p > 0.350).

Table 4 – General physico-chemical water parameters at the different sampling sites.

	Site C_1		Site C_2		Site C_3		Site R_1		Site R_2		Site R_3	
Parameter	**July**	**Oct**	**July**	**Oct**	**July**	**Oct**	**July**	**Oct**	**July**	**Oct**	**July**	**Oct**
Conductivity	188.5	178.9	577.0	554.0	555.	411.0	95.7	91.9	150.7	165.0	51.9	80.5
Temperature	20.8	15.9	23.6	16.0	23.1	18.2	16.4	16.4	18.5	14.6	21.9	16.0
pH	6.97	6.82	6.43	7.90	7.07	7.26	6.56	7.07	7.12	7.85	6.57	6.84
DO	8.17	7.53	0.99	2.90	6.76	7.20	9.62	9.50	9.01	8.70	7.58	8.60

The Principal Component Analyses (PCAs) performed for the channel and riparian vegetation showed the natural gradient in channel size as well as the complexity of riparian vegetation in the chosen streams of the present study and did not clearly separate metal contaminated from reference sites. Regarding the channel size, 92.4% of the PCA variation was explained by the two primary axes and described a gradient from wide and deep streams with high banks to narrower and shallow streams with low banks (Supplementary Material S1). In the PCA of the riparian vegetation, 85.8% of the global variation was explained by the two primary axes and described a gradient from less towards more complex vegetation followed by higher percentage of shadow cover (Supplementary Material S2).

3.3. Community-level endpoints

Results of the PERMANOVA analysis revealed a significant effect of "metal contamination" ($F_{1,8}$ = 6.14, p = 0.002) but not "season" ($F_{1,8}$ = 0.96, p = 0.420) or the interaction between factors ($F_{1,8}$ = 0.45, p = 0.514) in the structure of the benthic macroinvertebrate communities. This is in agreement with those results of the non-metric multidimensional scaling ordination that clearly separated the benthic macroinvertebrate communities of metal contaminated from reference sites, independently of the season (Figure 2). Additionally, vector fitting analysis indicated that macroinvertebrate communities of metal contaminated sites were more related with higher conductivity and higher metal content whereas assemblages of reference sites related more with larger substrate size, higher cover percentage of in-stream moss and higher levels of DO. The complexity of riparian vegetation influenced the macroinvertebrate communities of both metal contaminated and reference sites (Figure 2).

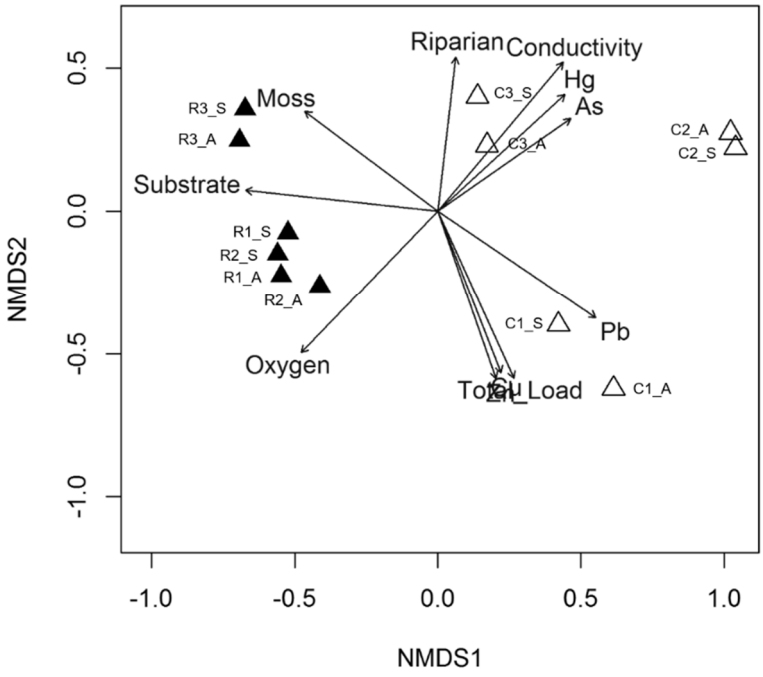


Figure 2 – Ordination plot produced by non-metric multidimensional scaling (NMDS) of the macroinvertebrate community surveys of Autumn (A) and Spring (S) of 2013 for reference sites R_1, R_2 and R_3 (closed triangles) and metal contaminated sites C_1, C_2 and C_3 (open triangles). Significant environmental features resulting from the vector fitting analysis were superimposed on the top of the NMDS graph.

Total macroinvertebrate abundance varied considerably among sampling surveys and, thus, no significant effects of "metal contamination" ($F_{1,8}$ = 4.40, p = 0.069), "season" ($F_{1,8}$ = 0.53, p = 0.488) or interaction between factors ($F_{1,8}$ = 0.69 ,p = 0.431) was

revealed by the two-way ANOVA analysis. In contrast, a highly significant effect of "metal contamination" ($p < 0.001$), but not "season" or interaction "contamination x season" ($p > 0.350$), was revealed by the two-way ANOVA tests for the community-level metrics: "total family richness", "EPT family richness" and "% Diptera + Oligochaeta" (Table 5).

Table 5 – Two-way ANOVA results for the community-level metrics "total abundance", "total family richness", "EPT family richness" and "% Diptera + Oligochaeta". Shown are F-values, significance level (*p*) and percentage of variation explained by the factors "metal contamination" (cont) and "season" (season) and the interaction between factors (cont x season).

Endpoint	Source	F	P	% variation
1. Total abundance	Cont	4.40	0.069	32.31
	Season	0.53	0.488	3.88
	Cont x season	0.69	0.431	5.05
2. Total Family Richness	Cont	46.73	< 0.001	85.15
	Season	0.12	0.743	0.21
	Cont x season	0.04	0.855	0.06
3. EPT family richness	Cont	132.80	< 0.001	93.73
	Season	0.44	0.526	0.31
	Cont x season	0.44	0.526	0.31
4. % Diptera+Oligochaeta	Cont	97.57	< 0.001	91.36
	Season	1.03	0.340	0.96
	Cont x season	0.19	0.672	0.18

A total of 8 to 30 different taxonomic groups were identified in metal contaminated sites whereas in reference sites, the number of taxonomic groups was considerably higher, varying between 46 and 52 (Figure 3). Concerning EPTs, results showed they were almost or completely absent from the surveys of metal contaminated sites. Indeed, no single Plecoptera or Trichoptera larva was recorded in any of the three metal contaminated sites while the family richness of Ephemeroptera varied between 0 (site C_2) and 1 (sites C_1 and C_3). In turn, EPT family richness recorded in reference sites varied between 12 and 20 (Figure 3).

Finally, dipterans and oligochaetes together, were highly predominant in metal-impacted sites accounting for 77.9 – 98.3% of the total macroinvertebrates sampled in

these sites. Oppositely, the percentage of dipterans and oligochaetes was clearly lower in reference sites corresponding to 17.6 – 44.8% of the total sampled benthic macroinvertebrates (Figure 3).

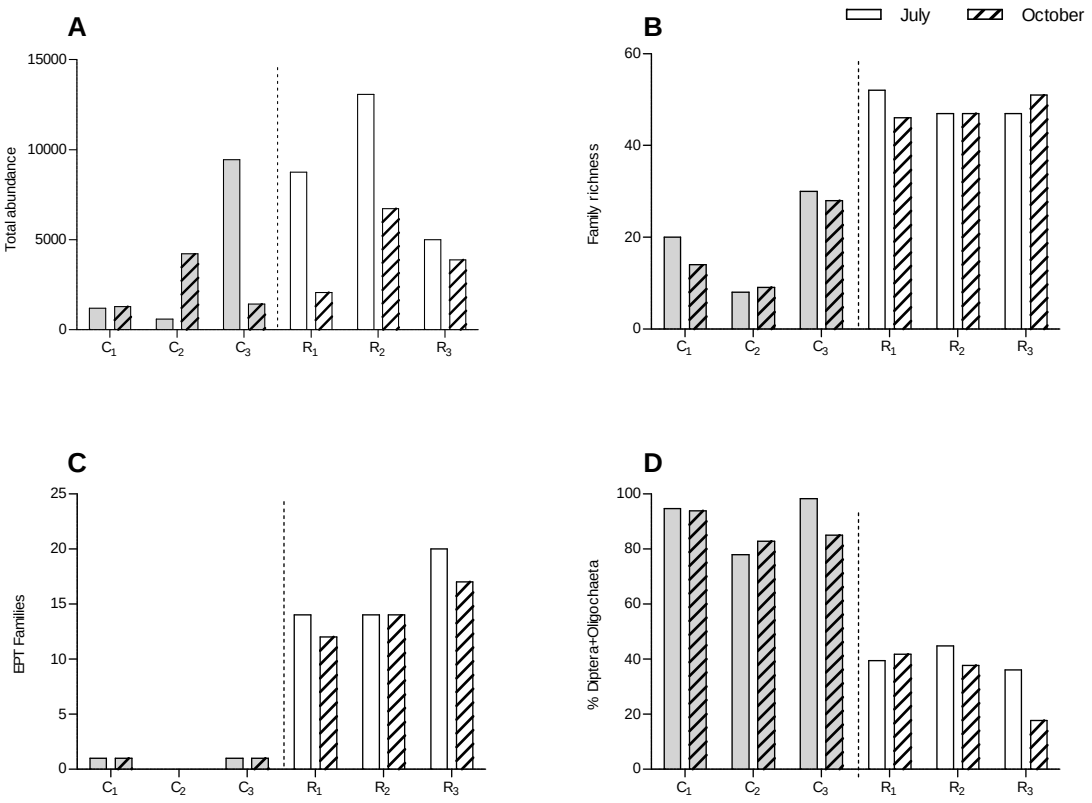


Figure 3 – Community-level response to metal pollution: (A) total abundance, (B) total family richness, (C) EPT family richness and (D) percentage of Diptera and Oligochaeta in respect to total invertebrate abundance. Shown are benthic macroinvertebrate communities of metal contaminated (grey bars) and reference (white bars) sites.

3.4. Genetic diversity and structure of *C. riparius*

A total of 146 larvae were genetically identified as *C. riparius* according to their mtDNA sequences (Table 6). Given the low number of larvae at most sites, estimations of genetic variation were performed by assembling individuals from the surveys of July and October. Unfortunately, a single *C. riparius* larva was recorded in sites R_1 and R_3. Thereby, these sites were excluded from subsequent microsatellite analyses and genetic diversity of *C. riparius* populations from metal contaminated sites could be compared only with reference population R_2 (Table 6).

Table 6 – Summary of genetic diversity of tested *C. riparius* populations. Shown are the number of larvae in the sites (n), mean number of alleles (N_A), observed heterozygosity (H_O) and expected heterozygosity (H_E). Asterisks denote significant deviations from Hardy-Weinberg equilibrium at microsatellite loci.

Site	n	N_A	H_O	H_E
C_1	26	4.14	0.50*(MSC2)	0.56
C_2	16	4.00	0.60*(MSC5,6)	0.55
C_3	32	4.00	0.53*(MSC3)	0.54
R_1	1	ND	ND	ND
R_2	70	3.86	0.50*(MSC6)	0.51
R_3	1	ND	ND	ND

ND – not determined

In general, results showed that the mean number of alleles (N_A) and expected heterozygosity (H_E) were lowest in the reference population R_2 and highest in population from metal contaminated site C_1, with N_A ranging between 3.9 (R_2) and 4.1 (C_1) and H_E ranging from 0.51 (R_2) to 0.56 (C_1). Observed heterozygosity (H_O) varied between 0.50 (C_1 and R_2) and 0.60 (C_2). Significant Hardy-Weinberg disequilibria were denoted in all four *C. riparius* populations at different microsatellite loci (Table 6).

No significant differentiation among the four *C. riparius* populations was revealed by the AMOVA analysis (Φ_{PT}=0.003, p = 0.246). Similarly, no significant isolation-by-distance was registered (r_M=0.571, p = 0.169).

4. Discussion

For the best of our knowledge, we present the first study in which ecological disturbance of freshwater ecosystems is evaluated by combining measures of the structure of benthic macroinvertebrate communities with measures of the genetic diversity harbored by populations of the model ecotoxicological species *Chironomus riparius*. Overall, results showed remarkable changes in the composition and diversity of macroinvertebrate communities inhabiting metal contaminated sites in which most sensitive taxa were eliminated and replaced by more opportunistic ones belonging mainly to Oligochaeta and Diptera. However, no evidence of contaminant-driven genetic erosion was found in any of the three *C. riparius* populations collected from the metal contaminated sites of our study area.

Despite some variation in the metal composition of the collection sites, chemical analysis confirmed that the total metal content of sediments was higher in the selected

contaminated sites compared to their closely located references. Besides this, only the sediments from metal contaminated sites exceeded the probable effect concentrations for some of the chemical elements above which harmful effects on benthic macroinvertebrates are expected to occur (MacDonald et al., 2000).

Based on the results of the community-level assessment, historical metal contamination did not produce clear effects in the total abundance of benthic macroinvertebrates as the number of individuals recorded varied substantially among sites and also among seasons. However, clear and profound differences in the taxonomic composition and diversity of the benthic macroinvertebrate communities inhabiting metal contaminated and reference sites were observed independently of the sampling season. It was observed a strong impoverishment of the total number of taxonomic groups recorded in metal contaminated sites. Additionally, the environmental conditions of metal contaminated sites were found to be particularly adverse for the sensitive group of EPTs, especially for Plecoptera and Trichoptera, since no single larva belonging to these taxa was found in metal contaminated sites. On the contrary, there was an increased predominance of more opportunistic invertebrates belonging to the taxonomic groups of Oligochaeta and Diptera (that includes *Chironomus riparius*) that accounted for 78–98% of the total number of macroinvertebrates of metal contaminated sites. Together, the observed differences at the community level were consistent with those described in other studies that have investigated the impacts of metal contamination in the structure of benthic macroinvertebrate communities (Loayza-Muro et al., 2010; Smolders et al., 2003; Svitok et al., 2014).

The association of the benthic macroinvertebrate communities of the metal contaminated sites with the increase of the metal content of sediments suggests that metals played an important role in structuring the benthic macroinvertebrate communities. However, several other habitat features such as conductivity, dissolved oxygen, sediment granulometry and cover percentage of in-stream moss were also associated with the observed macroinvertebrate community patterns indicating, therefore, that changes in the composition and diversity of the macroinvertebrate communities of metal contaminated sites were also due to the highly selective habitat requirements of metal contaminated sites. Indeed, metals may structure faunal assemblages through direct toxic effects in the survival and/or reproduction of stream invertebrates but also due to a number of interrelated contaminant-induced indirect effects that alter the habitat quality and availability of the sites (Cadmus et al., 2016; Loayza-Muro et al., 2014). High conductivity combined to low dissolved oxygen concentrations is indicative of nutrient enrichment and an excessive microbial activity in

metal contaminated sites that depletes dissolved oxygen and both factors have been considered major causes of invertebrate community declines (Cook et al., 2015; Cormier et al., 2013; Justus et al., 2014; Starr et al., 2014). In this context, these factors might have been particularly important in structuring the invertebrate community of site C_2 as the hypoxic or near-hypoxic conditions found in this site (< 3 mg/L DO) allow the persistence of only a few invertebrate species (Cook et al., 2015). Furthermore, the lower substrate grain size of metal contaminated sites compared to references likely reflects the accumulation of fine sediments associated to the anthropic inputs in the local stream beds which has also been shown to affect benthic invertebrates by a number of different ways. Indeed, fine sediment deposition directly affects invertebrates through physical abrasion and smothering. Moreover, by affecting light penetration and clogging the interstitial substrate space, fine sediment deposition may limit primary production of the streams (including in-stream moss production) while simultaneously reducing habitat heterogeneity and complexity necessary to support more diverse benthic macroinvertebrate communities (Jones et al., 2012; Jowett, 2003).

Therefore, the combination of high metal contamination with the habitat degradation clearly favoured the persistence of only a few benthic invertebrate taxa belonging mainly to Diptera and Oligochaeta in the three metal contaminated sites. It should be noted, however, that the number of dipterans and oligochaetes did not significantly differ between references and metal contaminated sites (data not shown) indicating that the increased predominance of these invertebrates in metal contaminated sites is more related with the elimination of other taxonomic groups rather than shifts in their total abundance. It was clear thus that other taxonomic groups, particularly those belonging to Ephemeroptera, Plecoptera and Trichoptera, were less abundant or even absent from contaminated sites due to their higher sensitivity to metals and the lack of adaptive strategies to overcome the effects of habitat degradation (Loayza-Muro et al., 2010; Svitok et al., 2014).

In contrast to our expectation that the adverse environmental conditions of metal contamination would cause also a decline in the overall levels of genetic diversity of *Chironomus riparius* populations, no signs of genetic erosion were found in any of the three midge populations inhabiting metal contaminated sites. On the contrary, results of the microsatellite analysis highlighted relatively high levels of genetic diversity in all exposed populations as well as a remarkable genetic homogeneity and connectivity among all populations. Therefore, the sensitivity and robustness of measures of genetic diversity of *C. riparius per se* as indicators of the adverse ecological effects of metal pollution seem to be limited.

Indeed, *C. riparius* larvae were frequently found in the three metal contaminated sites whereas, in turn, they were hardly recorded in two out of the three reference sites. Additionally, the levels of genetic diversity of *C. riparius* populations were comparatively higher in the three metal contaminated sites compared to the reference, a finding that corroborates previous assessments performed in the same sites and in which the three midge populations from metal contaminated sites had also shown higher levels of genetic diversity (as expected heterozygosity) than the three reference populations (Pedrosa et al., submitted). Collectively, our results thus agree with other studies showing that *C. riparius* tends to be more abundant in highly disturbed habitats rather than under conditions generally considered favourable for a wide range of aquatic invertebrates (De Haas et al., 2005; Groenendijk et al., 1998; Planello et al., 2015). Increased prevalence of *C. riparius* larvae in metal contaminated sites likely reflects the habitat preferences of the species for sedimentation areas of organically and nutrient enriched streams (Pfenninger and Nowak, 2008). Moreover, *C. riparius* possesses high haemoglobin content that allows the exploitation of oxygen-poor habitats from where other sensitive invertebrates are excluded and where *C. riparius* can reach large population densities (Armitage et al., 2012; De Haas et al., 2005; Groenendijk et al., 1998).

A reduction in the predation intensity and/or competition for resources in metal contaminated sites likely favoured the persistence of only a few opportunistic species such as *C. riparius* that took competitive advantage from the challenging environmental conditions and became more predominant (De Haas et al., 2005). A general predominance in disturbed, metal contaminated sites, however, does not necessarily indicate that *C. riparius* populations inhabiting these impacted sites were not adversely affected by metal contamination. In fact, our previous work on these same sites suggested microevolutionary responses of *C. riparius* populations to historical metal contamination with populations inhabiting contaminated sites showing a higher heritable tolerance to acute levels of metal exposure as well as improved and energy-costly defense mechanisms that likely allow them to cope better with metal toxicity (Pedrosa et al., submitted). The finding that chemical exposure may cause microevolutionary responses in *C. riparius*, including reduced levels of genetic diversity as well as genetic adaptation to metals has been demonstrated in several laboratory studies (Nowak et al., 2009; Vogt et al., 2010). However, loss of genetic diversity in experimental laboratory studies [e.g., (Nowak et al., 2009)] has been argued to be predominantly due to a reduction of the effective population sizes and, hence, due to increased genetic drift effects.

Because effects on the overall levels of genetic diversity harboured by populations strongly depend on reductions of the effective population sizes and/or the relative genetic isolation of populations (Hoffmann and Willi, 2008; Leffler et al., 2012), the large population densities of *C. riparius* in metal contaminated sites together with the great dispersal capabilities and short generation time of the species will likely mask the adverse ecotoxicological effects of metal contamination. Similar to our findings, Nowak (2008) found no pollution effects on the genetic diversity of natural populations of *C. riparius* and its sister species, *Chironomus piger*. In that study, however, there were overall low levels of environmental contaminants at the chosen study area in central Germany (Nowak, 2008), while the levels of contamination in our study area were severe enough to cause major changes in the diversity and composition of benthic macroinvertebrate communities.

Community and evolutionary dynamics of natural biota have been largely studied in separate and only recently there has been growing interest on understanding their linkages (De Meester and Pantel, 2014; Pauls et al., 2014; Vighi and Villa, 2013). The overall outcomes of the present study aim to contribute for further discussion on this topic. Studies investigating genetic erosion in contaminant-exposed biota have been primarily concerned on evaluating whether different populations of a given species exhibit high or low genetic diversity since the levels of genetic diversity are considered a proxy of the evolutionary potential of populations (and species) to evolve and adapt to future environmental conditions (Nowak et al., 2007; Nowak et al., 2012). However, the results provided by our study showing that higher levels of genetic diversity of *C. riparius* populations were associated with depauperate benthic macroinvertebrate communities provide an example that genetic diversity levels harboured by populations likely depend on multiple factors and should be carefully considered in ecological risk assessment strategies. Therefore, when aiming to assess the impacts of contaminants on the levels of genetic diversity, one needs to take into account the ecological and demographic requirements of the chosen species of interest and, if possible, assess genetic diversity of multiple species within affected communities rather than merely comparing levels of genetic diversity for a single focal species (Becker and Liess, 2015; Moya-Laraño, 2011).

5. Conclusions

Anthropogenic disturbance can significantly affect the chemistry and habitat quality of freshwater ecosystems. The present study showed that historical metal metal contamination dramatically altered the diversity and composition of the local benthic macroinvertebrate communities. Despite this, no evidences of genetic erosion were observed in *C. riparius* populations inhabiting the metal-impacted sites.

Collectively, our results highlight the need for consideration of the ecological and demographic features of the selected test-species whenever genetic diversity of natural populations are used to evaluate ecological disturbance in freshwater ecosystems.

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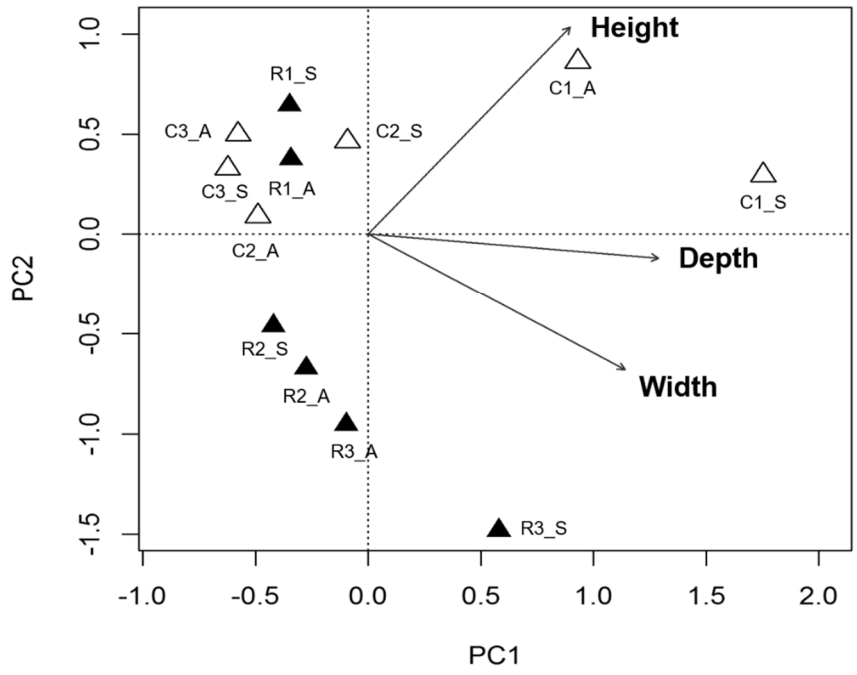
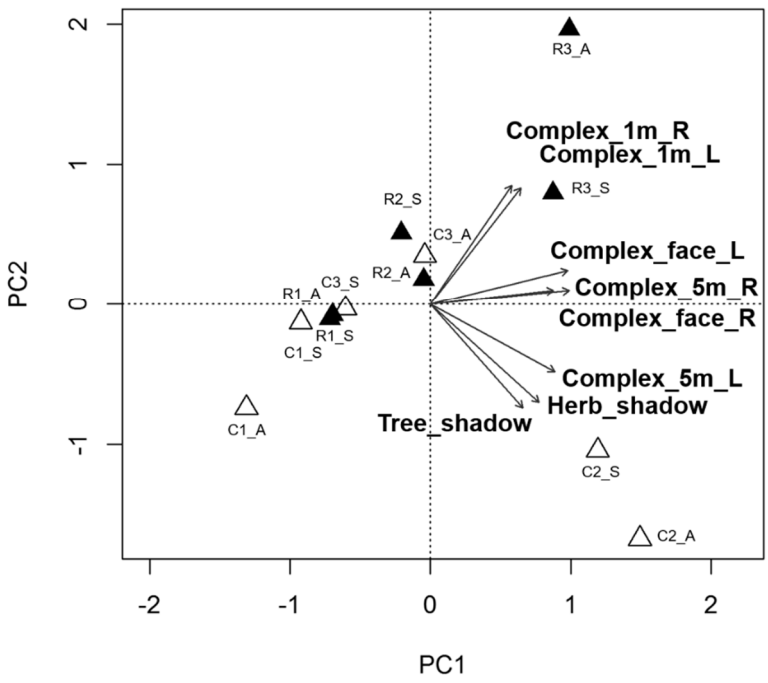
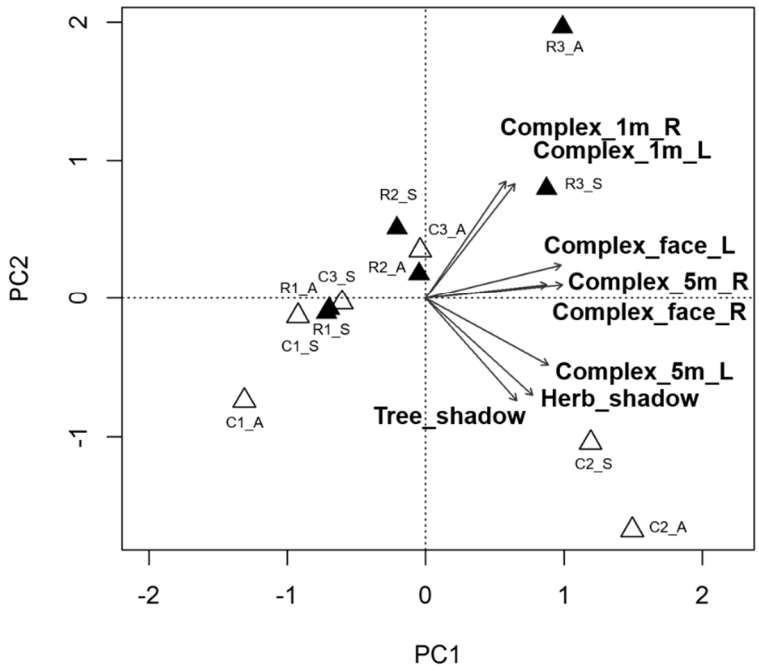
Supplementary Material



Supplementary Material S1 – Principal Component Analysis of the descriptors of channel size: height, depth and width. Reference sites R_1, R_2 and R_3 are indicated with closed triangles and metal contaminated sites C_1, C_2 and C_3 with open triangles for Autumn (A) and Spring (S).



Supplementary Material S2 – Principal Component Analysis of the descriptors of riparian vegetation on the right (R) and left (L) banks of the stream channel: complexity at water face (Complex_face); complexity at 1 meter from the stream channel (Complex_1m); complexity at 5 meters from the stream channel (Comple_5m); percentage of herbaceous shadow (Herb_shadow) and percentage of tree shadow (Tree_shadow). Reference sites R_1, R_2 and R_3 are indicated with closed triangles and metal contaminated sites C_1, C_2 and C_3 with open triangles for Autumn (A) and Spring (S).


R3_A
Complex_1m_R
Complex_1m_L
R3_S
R2_S
C3_A
Complex_face_L
R2_A
C3_S
R1_A
Complex_5m_R
Complex_face_R
R1_S
C1_S
Complex_5m_L
Herb_shadow
C1_A
Tree_shadow
C2_S
C2_A
PC2
PC1
2
1
0
-1
-2
-1
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CHAPTER VII:

GENERAL DISCUSSION

1. General discussion

Metal contamination can impose serious threats to the natural balance of freshwater ecosystems. The overall findings of this thesis documented the importance of such selection pressures in the deterioration of freshwater habitats and the macroinvertebrate communities they support, leading to the elimination of many sensitive species and replacement by more tolerant ones and driving, also, to evolutionary changes in exposed populations.

Recently, ecotoxicological studies have become increasingly concerned in understanding not only the short-term effects of contaminants in terms of life-history alterations such as mortality, growth or reproduction, but also their long-term evolutionary effects that may persist along time even after removal of the toxic stimulus. In this sense, the preservation of genetic diversity of natural populations has emerged as a major issue in ecotoxicology since genetic erosion may significantly diminish the adaptive potential of populations for future environmental change and, so, genetically eroded populations may be at a greater risk of extinction (Ribeiro and Lopes, 2013; van Straalen and Timmermans, 2002). Therefore, the historical metal contamination that has been documented in different Portuguese rivers, caused by decades of continued discharges of industrial and urban effluents (Costa and Jesus-Rydin, 2001; Soares et al., 1999), offered an excellent opportunity to explore how contaminants affected species diversity and which evolutionary consequences they produced in natural populations of the model ecotoxicological species *Chironomus riparius* inhabiting such impacted areas.

The ultimate goal of this thesis was to investigate whether neutral genetic diversity of *C. riparius*, measured through microsatellite variability, could be used as a biomonitoring tool to assess ecological disturbance of freshwater ecosystems. Despite the expectation that genetic drift and directional selection against metal sensitive genotypes would result in a decrease of the overall levels of neutral genetic diversity of populations inhabiting historically metal contaminated sites, no signs of contaminant-driven genetic erosion were registered in any of the metal-exposed *C. riparius* natural populations investigated. Instead, the levels of neutral genetic diversity were relatively high within all populations and a remarkable genetic homogeneity was observed (Chapters 2, 5 and 6). Therefore, these results contrasted with previous studies demonstrating loss of genetic diversity in natural populations inhabiting impacted freshwater systems (Benton et al., 2002; Paris et al., 2015) but add to other studies reporting no genetic erosion in contaminant-exposed invertebrate and vertebrate natural populations (Coors et al., 2009; Knapen et al., 2009; Martins et al., 2009; Santos et al., 2013).

Interestingly, the overall results of the microsatellite markers contrasted with those from the ecotoxicological tests presented in Chapters 2, 3 and 4 of this thesis that, collectively, provided evidences for evolution of metal tolerance in three *C. riparius* populations collected from historically metal contaminated sites compared to three populations from closely-located reference sites.

Results of Chapter 2 showed that populations from metal contaminated sites were generally more tolerant to acute concentrations of cadmium (Cd) than were populations from closely-located reference sites, with significant differences in two out of the three pairwise population comparisons. Similarly, the existence of an elevated acute tolerance to mercury (Hg) was observed in one *C. riparius* population that had been collected from a site historically contaminated by Hg in comparison to the reference population collected from a closely-located Hg-uncontaminated site (Chapter 4). Therefore, despite evolutionary changes caused by metal contamination were suggested by the acute ecotoxicological tests, populations from metal contaminated sites still maintained relatively high levels of neutral genetic diversity in the field.

The apparent mismatch between evolutionary responses to metal contamination and the lack of corresponding loss of neutral genetic diversity in natural populations of *C. riparius* inhabiting metal impacted sites is in agreement with earlier studies on other species. For example, Costa et al. (2012) found higher acute tolerance to cadmium in springtail populations of *Orchesella cincta* collected from metal contaminated sites in comparison to a reference population with no evidences of reduced genetic diversity (Costa et al., 2012). Similarly, no signs of genetic erosion were found in a population of *Daphnia longispina* historically exposed to the acid mine drainage of an abandoned pyrite mine (Martins et al., 2009) despite that metal-sensitive clonal lineages were present solely in reference sites whereas, in turn, metal-tolerant clonal lineages were only present in the impacted site (Agra et al., 2010; Lopes et al., 2004). Finally, no genetic erosion was observed in populations of the killifish *Fundulus heteroclitus* inhabiting areas highly contaminated with polychlorinated biphenyls (PCBs) despite the acute tolerance to PCBs of exposed populations being up to 1000 times higher compared to reference populations (McMillan et al., 2006; Nacci et al., 1999; Nacci et al., 2002).

Natural populations of *C.riparius* are typically found at very large densities reaching up to 70,000 individuals$\cdot m^{-2}$ (Groenendijk et al., 1998). Therefore, a plausible explanation is that even if the metal load of contaminated sites was sufficient to cause microevolutionary effects, the large population sizes in the field in conjunction with the great dispersal capabilities and the multivoltine life-cycles of the species (Armitage et al.,

2012; Groenendijk et al., 1998) will prevent potential declines in the overall levels of neutral genetic diversity.

The results of Chapter 3 suggest that evolution of metal tolerance in *C. riparius* populations may be related, at least in part, with improved and energy-costly defence mechanisms. In this regard, evidences were found suggesting different protein expression profiles under Cd exposure between tolerant populations from metal contaminated sites and sensitive populations from reference sites with an overall higher protein overexpression in tolerant populations. Apart from this, we found also higher constitutive levels of glutathiones and metallothioneins in the two populations from metal contaminated sites experimentally investigated in this chapter in comparison to the lower constitutive levels of these cellular thiols in the two reference populations tested. Since glutathiones and metallothioneins are metal-binding agents that are involved in the sequestration and detoxification of the excess amounts of metal and metalloid ions (Amiard et al., 2006; Lushchak, 2011; Sandbichler and Höckner, 2016), higher constitutive levels of these thiols in tolerant populations may provide better protection against metal induced oxidative damage and, thus, confer competitive advantage under such adverse environmental conditions.

Given that evolution of tolerance may entail detrimental fitness costs that lower the viability of the populations under environmental changing conditions (Ribeiro and Lopes, 2013), this issue was also experimentally investigated in this thesis. Overall, different results were reached for the different metal-tolerant populations under study suggesting that distinct evolutionary trajectories were taken by the populations while adapting to metal contamination. Results of Chapter 3 showed that, in the case of one metal-tolerant population that was collected from the S. Filipe ditch (Vouga basin), evolution of tolerance was associated with high basal energy demands which is in agreement with the evolutionary premise that evolution of tolerance may be energy costly (Ribeiro and Lopes, 2013; Sibly and Calow, 1989; van Straalen and Timmermans, 2002). However, despite theory also predicts that investment in efficient defence mechanisms may result in less energy available for growth and reproduction and, thus, constitute an unnecessary burden in the absence of the toxic stimulus, the overall outcomes of the ecotoxicological tests did not provide any evidence of evolutionary constraints in this population. In fact, no major negative effects in terms of emergence rate, time to development or weight of the imagoes were suggested by the results of the partial life-cycle tests under control clean conditions (Chapters 2 and 4). Similarly, no increased susceptibility under changing environmental conditions could be found when larvae of this population were exposed to acute and chronic levels of salinity stress (Chapter 4). Therefore,

physiological compensatory mechanisms such as altered food intake or assimilation efficiency might interact and counterweigh the high basal energy demands of this population (Sokolova et al., 2012). However, it is noteworthy that ecotoxicological tests did not assess effects directly on reproduction but only indirectly through emergence rates, time to emergence or adult weight. Therefore, additional fitness costs in terms of, for instance, fecundity or egg-rope fertility could not be ruled out. Furthermore, it is also important to consider that the ecotoxicological tests were performed under standardized, optimal laboratory conditions (OECD, 2004) and, thereby, the implications of high energy demands under conditions of, for example, food limitation or starvation were not tested. Finally, it should be also referred that fitness costs of tolerance were investigated only under control clean conditions and under salinity exposures. Therefore, metal tolerance may not express a cost in terms of salinity but, perhaps, in terms of other stressors and, so, additional work would be necessary to fully understand the long-term and evolutionary consequences of metal tolerance in this population.

The results obtained for this population contrasted, however, with those obtained for the two other metal-tolerant *C. riparius* populations collected from metal impacted sites from the Águeda river (Vouga basin) and from the Este river (Ave basin) in which evidences of fitness costs under environmental changing conditions were denoted. Indeed, the population from the Este river that exhibited the highest acute tolerance towards Cd (i.e. highest 48h-LC_{50}), exhibited also poorer fitness performance under control clean conditions as suggested by the significant lower emergence rates, longer developmental times and lighter imagoes compared to its nearby-collected reference population (Chapter 2). In this sense, results of Chapter 3 did not provide clear evidences for increased energy demands under control clean conditions in comparison to the assessed reference populations. Therefore, failure of this population to meet the energy cost hypothesis of tolerance add to the many reported exceptions as fitness costs are, in many cases, explained by negative pleiotropic effects on fitness-related traits (Harper et al., 1997; Shirley and Sibly, 1999). Finally, in the metal-tolerant population collected from the Águeda river, no differences in terms of emergence rates or time to emergence were found under control clean conditions in comparison to its respective reference population (Chapter 2). However, imagoes of metal-tolerant population were significantly lighter which might suggest lower reproductive output (Sibley et al., 2001).

A discrepancy between the acute and chronic tolerance of populations was also observed as the pattern of elevated metal tolerance in populations originating from metal contaminated sites was not always maintained when chronic tolerance was investigated through partial life-cycle tests (Chapters 2 and 4). Such differences between acute and

chronic tolerance are probably due to the fact that the former involves specific mechanisms of tolerance that are controlled by few genes of large effect (probably involving, besides others, glutathione and metallothioneins) whilst the latter involves more general mechanisms of tolerance that are probably regulated by many genes of small effect and, thus, depend more on the physiological condition of the organisms (Barata et al., 2000). In this context, results of Chapter 4 provided evidence that susceptibility of *C. riparius* populations to chronic exposures of environmental stressors is greatly dependent on the levels of neutral genetic diversity. By experimentally investigating the chronic tolerance towards Hg and salinity stress in populations with different levels of neutral genetic diversity, results showed that genetically eroded and inbred *C. riparius* populations were more adversely affected by both stressors than genetically diverse ones. This is likely because of the cumulative deleterious effects of genetic drift and inbreeding that can lead to an increased expression of deleterious alleles and, thereby, cause a decline in the overall fitness of the populations that become more evident under stressful environmental conditions (Fox and Reed, 2011). In this sense, it is important to note that despite these evidences highlighting the negative consequences of genetic impoverishment in the responsiveness to environmental stressful conditions, such reduced levels of neutral genetic diversity were not found under real-field scenarios. However, and because laboratory populations are established from a limited number of organisms, they are reproductively isolated and have comparatively much smaller population sizes which make them particularly prone to losses of genetic diversity caused by chance events (Nowak et al., 2007b).

Finally, results of Chapter 6 provided evidences on the severe effects that historical metal contamination had on the benthic macroinvertebrate communities. Accordingly, a remarkable impoverishment in both abundance and diversity of most benthic macroinvertebrate taxonomic groups was observed in metal contaminated sites that were colonized, mainly, by opportunistic taxa belonging to Oligochaeta and Diptera (in which the species *C. riparius* is included). This loss of invertebrate diversity was found to be related with the high metal content of the sediments as well as with several other physico-chemical features associated to the poor habitat quality of the metal contaminated sites, such as increased conductivity, reduced stream flow, low dissolved oxygen and reduced sediment granulometry. Nonetheless, results suggested that *C. riparius* tended to be favored under metal contaminated sites as larvae of *C. riparius* were commonly found in the surveys of metal contaminated sites while, in turn, they tended to be numerically less abundant in most surveys of reference sites. Moreover, and despite the high levels of neutral genetic diversity observed in all *C. riparius*

populations investigated, we found that genetic diversity of *C. riparius* increased with the impoverishment of the benthic macroinvertebrate communities as well as with metal contamination and decline of habitat quality. Taken together, these results match quite well with the opportunistic nature of the species that takes competitive advantage and becomes more predominant in environments from where other competitors and predators are eliminated (De Haas et al., 2006). Therefore, the fast life-cycles of the species coupled to its ability to exploit the environmental adverse conditions allows chironomids to become numerically more abundant in metal contaminated sites even if microevolutionary changes due to metal contamination were suggested by the results of the previous chapters (i.e. Chapters 2, 3 and 4).

2. Future directions

The overall outcomes of this thesis provided evidences on the evolutionary impacts that contaminants may produce on natural biota and corroborate earlier studies reporting the ability of populations to adapt to different anthropogenic stressors such as pesticides (Coors et al., 2009; Gordon et al., 2015), antibiotics (Czekalski et al., 2015; Finley et al., 2013), persistent organic contaminants (Nacci et al., 2002; Oziolor et al., 2014) or metals (Agra et al., 2010; Lopes et al., 2006). It is, therefore, clear there is a need to include the long-term biological consequences of contaminants in ecological risk assessment approaches since their impacts on natural populations may still persist even after the removal of contaminants from the natural ecosystems. Of particular importance is the case of fitness costs arising from adaptation to contaminants which constitute important indirect effects that are usually neglected when only short-term effects of contamination are considered (De Coninck et al., 2014). In this sense, deciphering adaptation and other evolutionary effects may be critical because the adverse effects of contaminants may be largely amplified by the global climate changes anticipated for the next decades with unpredictable but expectable detrimental consequences to the persistence of natural populations and species (Pauls et al., 2013).

Collectively, the results presented along this thesis lend support to the use of *C. riparius* as a potential model species to be used in evolutionary toxicology studies aiming to address the long-term impacts of contaminants in freshwater ecosystems as the susceptibility of midge populations towards different environmental stressors was found to be influenced by processes of genetic drift and natural selection. Indeed, the use of *C. riparius* seems to be particularly attractive in this field as the species is still able to

persist under heavily contaminated sites from where most invertebrate species are eliminated, which constitutes itself an indispensable prerequisite to evolutionary change.

Nonetheless, results also suggested that levels of genetic diversity of *C. riparius* under natural conditions, assessed through microsatellite markers, did not reflect the underlying microevolutionary effects caused by metal contamination and, thus, measures of neutral genetic diversity do not seem to be particularly advantageous biomonitoring tools of environmental disturbance *per se*. Constraints to its use are likely due to the large population densities (Groenendijk et al., 1998) together with the great dispersal capability of the species both at the larval and adult stage.

Research on evolutionary responses focusing in natural populations of *C. riparius* should, therefore, rely more in high resolution genome-wide markers to trace microevolutionary changes caused by environmental pollution. In the era of next generation sequencing technology, genomic marker systems might provide novel opportunities for gathering genome-scale sequence data and reveal processes of selection and genetic erosion that might be hidden using genetic markers such as microsatellites (Davey et al., 2011; Seeb et al., 2011). The results of this thesis open an avenue for research in this area. The baseline work is now done and multiple *C. riparius* populations inhabiting metal contaminated and uncontaminated sites are identified. Therefore, future studies covering multiple replicate populations of *C. riparius*, should employ techniques such as single nucleotide polymorphisms or even whole genome sequencing to test for differential effects on genome parts and search for loci under selection (Oppold et al., 2016; Schmidt et al., 2013). Population genomic studies can identify putative regions of adaptive relevance and reveal candidate genes that can be associated to the different evolutionary trajectories of midge populations inhabiting metal contaminated and uncontaminated sites.

Several lines of evidence suggest, nonetheless, that the use of microsatellite markers in *C. riparius* may assist in different experimental situations, including: 1) detection of interspecific hybridization; 2) establishment of minimum threshold levels of genetic diversity in *C. riparius* laboratory populations; and, finally, 3) integrative use of measures of neutral genetic diversity in a more community-level context.

First, by analysing the microsatellite profiles of different midges, it was possible to report for the first time a case of field hybridization between *C. riparius* and its morphologically cryptic sister species *Chironomus piger* (Chapter 5). Although all hybrids had been identified as *C. piger* according to their mtDNA sequences, the existence of species-specific microsatellite alleles allowed the identification of hybridization between

the two species. Therefore, genetic identification of organisms from these two cryptic sister species should be done using not only mtDNA sequences as previously suggested in literature (Pfenninger et al., 2007), but also by additional analysis of the microsatellite profiles in order to avoid erroneous species' identifications since mitochondrial DNA is inherited maternally without recombination. Regarding the hybridization episode herein reported (Chapter 5), all hybrids were identified in a single survey and were confined to a single site. Despite microsatellite profiles suggested that the hybrids could be viable for more than one generation, they corresponded to approx. 1% of all genetically identified organisms and, thus, hybridization between the two sister species seems to be very rare in the field. The low interspecific hybridization between these species has been attributed to different swarming behaviors (Miehlbradt and Neumann, 1976) and different ecological niche partitioning (Pfenninger and Nowak, 2008) that act as prezygotic isolation mechanisms, but also to postzygotic isolation mechanisms of dysgenesis syndromes that lower the viability of the progeny (Hägele, 1999).

Second, measures of neutral genetic diversity of *C. riparius*, assessed through microsatellite markers, should also be used to occasionally monitor the levels of genetic diversity of laboratory-reared *C. riparius* populations that are used in ecotoxicological assays. As demonstrated by the results presented in Chapter 4, *C. riparius* populations become genetically eroded and inbred after being maintained under common-garden laboratory conditions for certain periods of time. As already mentioned, while acute tolerance is probably determined by a limited number of loci and, thus, depends heavily on the intrinsic genetic background of the populations, an overall loss of genetic diversity will likely reduce the fitness of the populations. The results presented in this work reinforce the need to frequently refresh laboratory-reared *C. riparius* populations with new genetic input in order to maintain healthy and genetically diverse populations (Nowak et al., 2007a). It is therefore advisable to establish minimum levels of genetic diversity in laboratory cultures of *C. riparius* so as to increase the accuracy and reproducibility of the ecotoxicological tests and in particular the assessment of the sub-lethal effects of contaminants.

Finally, the finding that higher levels of genetic diversity in *C. riparius* were associated with the impoverishment of the benthic macroinvertebrate communities may have major ecological implications and, thus, this issue deserves further research. In general, it is argued that high levels of genetic diversity are required by natural populations to maintain their evolutionary potential and adapt to future environmental change (Ribeiro and Lopes, 2013; van Straalen and Timmermans, 2002). Although the results of Chapter 4 provided lines of evidence about the importance of maintaining high levels of genetic

diversity to better respond to stressful environmental conditions, results of Chapter 6 suggest that this issue is far more complex and that higher levels of genetic diversity in a species such as *C. riparius* may actually reflect important impairments at the community level. Ecological processes such as productivity, interspecific competition, predation or community structure depend, ultimately, on the levels of genetic diversity of their interrelated species (Moya-Laraño, 2011). However, to date, most research on the impacts of environmental contaminants has focused on effects at single species (e.g. (Martins et al., 2009; Paris et al., 2015)). Here, it was observed that many predators and competitors were eliminated from the metal contaminated sites and *C. riparius* increased its abundance which likely prevented any losses of genetic diversity, even if evolutionary changes were observed. Therefore, effects of environmental contaminants should be carefully inferred not only in terms of high/low levels of genetic diversity of a given focal species but should rather be inferred in a more community-level context in which genetic diversity of different species is quantified across the food-web in order to gain broader insights on the detrimental effects of environmental contamination.

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