THE EVOLUTIONARY ECOLOGY OF ADAPTATION TO POLLUTED ENVIRONMENTS

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To my family.

To my dad who fostered my love of animals and plants,

to my mom who supported me in all possible ways,

to my brothers and sister for always believing in me, and

to my beloved Mother Nature for giving me life and showing me every day the beauty of all things.

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Abstract

Human actions have resulted in multiple changes on a global scale that often drive contemporary population extirpation and extinctions. Making decisive steps in managing the biodiversity crisis requires an understanding of the causes and consequences of biodiversity loss and a knowledge on eco-evolutionary dynamics. In this thesis, I contribute to our understanding of the eco-evolutionary dynamics that shape the response of biodiversity to aquatic chemical pollution. In Chapter 1, I assess current research strategies and trends (phenotypic, molecular genetics, and demographic approaches) and provide a comprehensive synthesis of our current knowledge on micro-evolutionary responses of algae, plants, invertebrates, and vertebrates to pollution and their ability to persist in polluted environments. I also conduct a meta-analysis to calculate the magnitude of phenotypic change in invertebrates in response to metal pollution, the most studied system. I found that the majority of studies were focused on phenotypic responses at the individual level. Most of the studies that included demographic estimates found that the detrimental effects of contaminants were exacerbated over multiple generations. The meta-analysis did not reveal a significant relationship between metal pollution intensity and changes in the traits studied. The complexity of eco-evolutionary responses to contamination, and their difficult interpretation in the context of taxonomic, and methodological biases made it arduous to make broad statements about adaptation to pollution. The review highlighted the need for long-term monitoring programs on exposed populations that link demography to phenotypic, genetic, and selection assays. In Chapter 2, I focus my attention on the evolutionary and demographic effects of pollution at the population level, using the microcrustacean *Daphnia*. I tested the contribution of genetic variation to population persistence by exposing *Daphnia* populations to copper. A higher level of genetic variation favoured a longer population persistence comparing to a monoclonal composition, however, all populations became extinct. Genetic variation was depleted over time especially in the exposed populations. Considering the rapidity of species loss that we are witnessing, the development and validation of new approaches for the inventory of biodiversity is critical. In Chapter 3, I extend my investigation on the effects of aquatic pollution to zooplankton communities. I assess whether environmental DNA-based metabarcoding can be used to assess rapid diversity changes during herbicide

(glyphosate) contamination. I compare estimates of diversity and taxa turnover obtained with metabarcoding and microscopy and I test whether common ecological trends can be detected. I also provide insights on the effects of glyphosate on the response of intra-specific genetic variation. Despite an under-estimation of some taxa, a similar ecological signal could be observed with both methods in mesocosms under glyphosate pulses. Glyphosate concentration was a strong driver for the estimated diversity trends. Intraspecific genetic variation was negatively affected by glyphosate. Overall, this thesis is characterized by novel approaches and empirical contributions to our knowledge on the dynamic of eco-evolutionary responses to various forms of pollution across different levels of biological organization, from genes to ecosystems.

Résumé

Les activités humaines ont causé de multiples changements à l'échelle mondiale qui entraînent souvent la disparition contemporaine de populations et extinctions. Gérer efficacement la crise de la biodiversité nécessite une compréhension des causes et des conséquences de la perte de biodiversité et une connaissance des dynamique éco-évolutives. Dans cette thèse, je contribue à notre compréhension des dynamiques éco-évolutives qui façonnent la réponse de la biodiversité à la pollution chimique aquatique. Dans le Chapitre 1, j'évalue les stratégies et les tendances de recherche actuelles (phénotypique, génétique moléculaire et approches démographiques) et je présente une synthèse complète de nos connaissances actuelles sur les réponses micro-évolutives des algues, des plantes, des invertébrés, et des vertébrés à la pollution et leur capacité à persister dans des environnements pollués. J'effectue également une méta-analyse pour calculer l'ampleur du changement phénotypique chez les invertébrés en réponse à la pollution par les métaux, le système le plus étudié. J'ai trouvé que la majorité des études étaient axées sur les réponses phénotypiques au niveau individuel. La plupart des études qui présentaient des estimations démographiques ont révélé que les effets néfastes des contaminants s'exacerbaient à travers le temps, sur plusieurs générations. La méta-analyse n'a pas révélé de relation significative entre l'intensité de la pollution par les métaux et les changements dans les traits étudiés. La complexité des réponses éco-évolutives à la contamination, la difficulté liée à leur interprétation dans le contexte des biais taxonomiques et méthodologiques empêchent de facilement faire des déclarations générales sur l'adaptation à la pollution. Les résultats de la revue soulignent la grande nécessité de programmes de surveillance à long terme des populations exposées qui relient la démographie aux tests phénotypiques, génétiques et de sélection. Dans le Chapitre 2, je me concentre sur les effets évolutifs et démographiques de la pollution au niveau de la population, en utilisant la micro-crustacé *Daphnia*. J'ai testé la contribution de la variation génétique à la persistance de la population en exposant les populations de *Daphnia* au cuivre. Un niveau plus élevé de variation génétique favorisait une persistance plus longue de la population par rapport à une composition monoclonale, cependant, toutes les populations se sont éteintes. La variation génétique s'est épuisée au fil du temps, en particulier dans les populations exposées. Compte tenu de la rapidité de la

disparition des espèces que nous observons, le développement et la validation de nouveaux approches pour faire l'inventaire de la biodiversité est critique. Au chapitre 3, j'élargis mon enquête sur les effets de la pollution aquatique sur les communautés de zooplancton. J'évalue si métabarcoding basées sur l'ADN environnemental peut être utilisé pour évaluer les changements rapides de diversité pendant la contamination par les herbicides (glyphosate). Je compare les estimations de la diversité et du renouvellement des taxons obtenues avec des évaluations de métabarcoding et microscopie et je teste si des tendances écologiques communes peuvent être détectées. Je donne également un aperçu des effets du glyphosate sur la réponse de la variation génétique intra-spécifique. Malgré une sous-estimation de certains taxons, un signal écologique similaire a pu être observé avec les deux méthodes dans les mésocosmes sous stress de glyphosate. La concentration de glyphosate a été un puissant moteur des tendances estimées de la diversité. La variation génétique intraspécifique a été affectée négativement par le glyphosate. Dans l'ensemble, cette thèse présente de nouvelles approches et des contributions empiriques à nos connaissances sur la dynamique des réponses éco-évolutives à diverses formes de pollution à différents niveaux d'organisation biologique.

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Preface

Thesis format

This thesis is written in a manuscript-based format. It includes three research chapters, each corresponding to an individual manuscript on which I am the lead author. The manuscripts have been or will be submitted to scientific journals for publication. The document begins with a general introduction explaining key concepts in the field and framing the importance of the study, followed by the chapters. Connecting statements are included between each chapter in order to explain their relationship. A discussion and final conclusion section summarize the findings, describe the scientific contribution of the study, and suggest directions of future research.

Chapter 1 (*published*)

- Loria. A., Cristescu M. E., Gonzalez A., (2019). Mixed evidence for adaptation to environmental pollution. *Evolutionary Applications*, 12 (7), 1259–1273. https://doi.org/10.1111/eva.12782

Chapter 2 (*in preparation*)

- Loria. A., Cristescu M. E., Gonzalez A. Genotype diversity promotes persistence in *Daphnia* populations during copper stress.

Chapter 3 (*in preparation*).

- Loria A., Hébert M. P., Costa N. B., Fugère V., Hleap S. J., Barrett R., Beisner B., Bell G., Shapiro J., Gonzalez A., Cristescu M. E. The performance of eDNA-

based metabarcoding in estimating rapid zooplankton diversity changes during herbicide contamination.

Ethics statement

All research included in this thesis followed safety regulations imposed by McGill University.

Author contributions

I am the lead author of the thesis and of each of its manuscript chapters. I developed the research questions, hypotheses, analyzed the data and wrote the manuscripts. My supervisors, Melania E. Cristescu and Andrew Gonzalez, provided feedback at each step of the research study, edited the thesis, and funded the research.

Chapter 3 was based on a new experimental infrastructure for research on aquatic ecosystems at McGill University's Gault Nature Reserve involving the collaboration of several research groups and multiple co-authors with complementary expertise. Vincent Fugère, Marie-Pier Hébert, Rowan D.H. Barrett, Beatrix E. Beisner, Graham Bell, Jesse B. Shapiro, Melania E. Cristescu and A. Gonzalez designed the LEAP experiment which involved the study of: i) the abundance, biodiversity, and functional composition of plankton; ii) growth and survival of duckweed; iii) the microbial community composition and evolution; iv) primary production and ecosystem respiration. V. Fugère, M-P. Hébert, and N. Costa conducted the microcosm experiment and collected chemical, physical, and biological data. Specifically, V. Fugère focused on the study of phytoplankton communities, M.-P. Hébert focused on the study of zooplankton communities using traditional biodiversity assessments (abundance and density through

microscopy analysis) and provided the microscopy data used here. N. B. Costa studied the bacterial community, collected the eDNA samples used for metabarcoding and extracted the DNA. S. J. Hleap helped with the bioinformatic analysis. M. E. Cristescu and A. Gonzalez provided feedbacks, edited the manuscript, and funded the metabarcoding analysis. A.L. analyzed data, made the figures, and drafted the manuscript. All co-authors have given me the permission to include the chapters associated with their names in my thesis.

Statement of originality

All content of the present thesis is original. The three manuscripts are novel contributions in the fields of evolutionary ecology and ecological genomics. The thesis crosses all levels of biodiversity and explores different components of pollution, one among the most concerning threats to biodiversity.

Chapter 1 represents a broad literature review providing a contemporary and critical perspective on adaptation to different type of pollutants. It identified several research gaps like the scarcity of long-term studies at the population level embracing approaches that integrate demography, ecology and evolution. It also emphasizes the need for standardized protocols across studies, especially for similar taxa and approaches combining field and laboratory studies.

Chapter 2 fills important gaps identified by the literature review. Testing the ability of *Daphnia* populations to persist in a metal contaminated environment not only provides useful insights on the applicability of novel evolutionary theories such as evolutionary

rescue on aquatic invertebrates but also advances our understanding on the adaptability to copper pollution of a keystone species in freshwater habitats.

Chapter 3 provides insights on the response of zooplankton communities to eutrophication and herbicide contamination and their effects on diversity through the analysis of environmental DNA combined with high-throughput sequencing (metabarcoding). Metabarcoding represents a promising approach to the assessment of complex biological aquatic assemblages. This chapter greatly contributes to the validation of its application in eco-evolutionary studies.

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General introduction

Biodiversity is represented by a nested hierarchy that encompasses multiple levels of organization including ecosystem, community, population, and genetic diversity (Franklin *et al.* 1981; Noss 1990). These components are closely interlinked and alterations at each of these levels can have direct and indirect impact on the others (Bickham *et al.* 2000; Fig. I). Human-induced environmental changes are causing biodiversity losses at an extraordinary rate (Vinebrooke *et al.* 2004; Brook *et al.* 2008; Ceballos *et al.* 2015). Habitat loss and degradation, climate change, invasive species, eutrophication, and over-exploitation are the most concerning pressures on biodiversity (Tilman *et al.* 2017; Secretariat of the Convention on Biological Diversity, 2010). Pollution is a widespread form of habitat degradation and its long-term ecological effects on the sustainability of ecosystems have increasingly drawn attention from the scientific community and regulatory agencies (Bickham *et al.* 2000; Palumbi 2001; de Vries & Hanley 2016).

One of the most pressing questions in evolutionary biology and ecology is whether and how natural populations respond to chemical pollution. When dispersal is not possible and the level of contamination is persistently high, adaptive changes, with a genetically inherited increase in tolerance, become necessary to avoid extirpation. The adaptive success of a population depends on the degree of contamination but also relies on the presence of genetic variability (standing genetic variation and de novo mutations) in a population's gene pool which provides variable traits upon which selection can act (Blows & Hoffmann 2005; Hoffmann *et al.* 2017). When adaptation occurs quickly enough to prevent extinction due to maladaptation to a new environment, a population might undergo a process called evolutionary rescue (ER; Lynch *et al.* 1991; Burger & Lynch 1995; Gomulkiewicz & Holt 1995; Bell & Collins 2008; Gonzalez *et al.* 2013). ER theory integrates the study of genetic and demographic dynamics in order to understand not only the evolutionary fate of populations but also the ecological consequences of micro-evolutionary responses by many species, communities and ecosystems. In the light of ER theory, it has become clearer that research aiming to mitigate environmental impacts should focus on studying the effects of changing genetic variation due to mutations, population

bottlenecks, and selection caused directly or indirectly by pollution (Bickham *et al.* 2000; Mimura *et al.* 2017), and the combinations of genetic and demographic conditions that promote persistence (Gomulkiewicz & Holt 1995; Bell 2013; Lowe *et al.* 2017).

For my PhD thesis, I explore the ecological, evolutionary and genetic consequences of pollution on different levels of biodiversity (Fig. II). In Chapter 1, I first assess, with a literature review, the evidence for adaptation to pollution through a broad synthesis of strategies and trends in evolutionary toxicology research encompassing multiple levels (e.g. taxonomic, methodological, molecular, demographic). I, then, identify research gaps that deserve further attention. In Chapter 2, I report an experiment on the eco-evolutionary response of *Daphnia pulex* populations to chemical pollution. In Chapter 3, I use environmental DNA to evaluate the response of plankton communities to contamination by glyphosate (Roundup®) in the context of a large mesocosm experiment. Throughout this thesis, I will refer to resistance (or tolerance) as the ability of an individual to survive in a contaminated habitat (Forbes & Forbes 1994), and to persistence as the ability of a population to persist in a contaminated habitat maintaining a positive growth rate.

Biodiversity and its role in eco-evolutionary dynamics

The term resilience was first defined by the ecologist C. S. Holling (1973) as a measure of the disturbance that an ecosystem can face without any change in processes and structures. In 1991, S. Pimm defined resilience as the time a system takes to return to an equilibrium after a perturbance. Following a perturbance, in the absence of resilience, an ecosystem's variable may be more or less resistant. According to S. Pimm (1991), if there is low resistance, many other variables may change as a consequence of a permanent change in an ecosystem variable. Biodiversity contributes to community and ecosystem stability in the face of environmental change (environmental fluctuation that impact performance and/or fitness; Koehn & Bayne 1989) and fluctuations (Cottingham et al. 2001; Balvanera et al. 2006; Gonzalez & Loreau 2009; Isbell et al. 2017; Wang et al. 2019) promoting the maintenance of ecosystem services which support human well-being (Cardinale *et al.* 2012; Hooper *et al.* 2012; Naeem *et al.* 2016; Brondizio *et al.* 2019).

When resilience and stability (return to equilibrium after perturbance) have a margin for change, in ecology, we refer to an adaptive capacity (Gunderson 2000). In evolution, ecological resistance (a permanent change in an ecosystem variable followed by limited ecological consequences) and adaptive capacity, are equivalent to evolutionary resilience (Sgrò *et al.* 2011). An adaptive response to environmental change at the species level relies on the presence of phenotypic diversity. Phenotypic diversity underlined by genotypic diversity, can have both an ecological and evolutionary role in ecosystem and community stability (Fussmann *et al.* 2007; Bassar *et al.* 2010; Becks *et al.* 2010). Not only does genetic diversity represent the raw material for evolution by natural selection (Fisher 1930) but it is also fundamental for population persistence (the ability of a population to persist in a contaminated habitat maintaining a positive growth rate; Newman & Pilson 1997; Vilas *et al.* 2006; Rizvanovic *et al.* 2019); colonization success (Gamfeldt *et al.* 2005; Hovick & Whitney 2019), growth (Pelletier *et al.* 2007; Tito de Morais *et al.* 2019), productivity (Bell 1991; Smithson & Lenne 1996; Zeng *et al.* 2017), and resistance to pathogens (Pearman & Garner 2005). Despite their role in the persistence of populations and in community ecology and ecosystem function, evolutionary processes and genetic diversity are still largely ignored in conservation policy (Pressey *et al.* 2007; Mace & Purvis, 2008).

The integration of ecology and evolution is indispensable for advancing our knowledge on the processes that shape and preserve biodiversity (Ezard *et al.* 2009; Pelletier *et al.* 2009). The role of ecology in evolutionary changes has been recognized since Darwin's research studies in the 1859 (Darwin 1859) but evolutionary changes were considered slow and this view was sustained for decades. Presently we know that evolution at the species and population level (micro-evolution) could occur in as little as few generations (Hendry & Kinnison 1999; Messer *et al.* 2016). The interlinked nature of ecology and evolution often called "eco-evolutionary-dynamics" (Fussmann *et al.* 2007; Alberti 2015; Hendry 2016), however, have been only recently considered.

Threats to biodiversity and their potential consequences

We have good evidence for a positive relationship between biodiversity and ecosystem processes in the face of environmental change (Balvanera *et al.* 2006; Hughes *et al.* 2008;

Campbell *et al.* 2011; Cardinale *et al.* 2012; Loreau & De Mazancourt 2013; Cusson *et al.* 2015), and the potential for rapid micro-evolution at the population and species level (Reznick & Ghalambor 2001). However, we are uncertain about whether rapid evolution will be sufficient to counter human-caused environmental stress (environmental change that affect survival and reproduction (Bijlsma & Loeschcke 2005; Pires *et al.* 2014).

The most ubiquitous and powerful human-generated drivers of biodiversity change are climate change, habitat loss and degradation, land use changes, overexploitation, and species invasions (Vitousek *et al.* 1997; Chapin Iii *et al.* 2000; Board 2005; Benayas *et al.* 2009; Butchart *et al.* 2010; Tilman *et al.* 2017; Brondizio *et al.* 2019). Human-driven global changes may lead to biodiversity declines such as functional extinctions, local extinction of species and populations, and loss of ecosystems at local, regional, ad continental scales (Fig. I; Vinebrooke *et al.* 2004; Brook *et al.* 2008; Barnosky *et al.* 2012; Sadava *et al.* 2014; Ceballos *et al.* 2015).

Global, local, and functional extinctions are generally anticipated by a reduction of genetic diversity within populations (Wiens 2016). Changes in genetic diversity and allele frequencies of populations might be due to induced mutations (due to a genotoxic and/or mutagenic effect), population bottlenecks, increased or decreased migration patterns, and selection for inherited resistance (Ribeiro & Lopes 2013). When sensitive individuals are replaced by more resistant ones, the overall tolerance of a population increases (Lopes *et al.* 2004b). This happens when organisms either acclimate through phenotypic plasticity (e.g. increased enzyme activities, production of metal binding proteins) without shifts in the genetic structure of the population or via adaptation to the new environment, with a subsequent changes to the genetic structure of the population (Lopes *et al.* 2004b; Barrett & Hendry 2012).

To safeguard biodiversity and to limit future losses there is an urgent need to understand whether microevolution can occur rapidly enough to promote persistence in populations that are facing threatening environmental stressors. This is the central question in the framework of ER which involves a "genetic adaptation that allow a population to recover from demographic effects initiated by environmental change that would otherwise cause extirpation" (Gonzalez *et al.* 2013). This phenomenon often characterized by U-shaped

demographic time series consists of three stages: decline, stabilization, and recovery (with a corresponding increase in the allelic frequency of particular phenotype with advantageous traits; Gomulkiewicz & Holt 1995; Holt & Gomulkiewicz 2004). The intensity of environmental change, the strength of selection, the initial population size, the presence of standing genetic variation, and dispersal are all key factors affecting the probability of ER (Gonzalez *et al.* 2013).

Community evolutionary rescue (CER; Fussmann & Gonzalez 2013) can also occur if multiple species undergo evolutionary rescue in response to a stressor. When they do, a co-evolutionary response to the stress allows the recovery of a viable community that was not able to survive at the initial levels of stress. A handful of theoretical (Fussmann & Gonzalez 2013; Kovach-Orr & Fussmann 2013; Osmond & de Mazancourt 2013; Mellard *et al.* 2015) and empirical (Low-Décarie *et al.* 2015; Bell *et al.* 2019; Fugère *et al.* 2020) studies on CER have shown that trophic interactions can interact with evolutionary responses in the context of environmental stress.

Despite growing empirical evidence of ER and CER, their occurrences in nature may be challenged by limits to adaptation such as environmental and genetic constrains (Blows & Hoffmann 2005; Futuyma 2010; Bell 2013). Small populations can easily undergo extinction due to demographic and environmental stochasticity (Bell & Gonzalez 2009). If population decline is very fast, the recovery through genetic adaptation and the fixation of the advantageous alleles is unlikely, even in the presence of high genetic and phenotypic variation (Haldane 1957). Moreover, genetic variation is inversely proportional to the rate and severity of environmental stress (Bell 2013) and is low in small populations, or when eroded by selection of resistant genotypes (Hoffmann & Willi 2008). Even when ER and CER take place and prevent extirpations, they can exert long-term effects on the future adaptive potential of populations and communities due to a lag response in the recovery of population size and genetic diversity (Belfiore & Anderson 2001; Morgan *et al.* 2007; Ribeiro *et al.* 2012).

Evidence for ER and CER is growing rapidly (Carlson *et al.* 2014; Bell 2017) with an important goal of attaining a general theory of rescue applicable to multiple levels of biological organization (Low-Décarie *et al.* 2015; Fugère *et al.* 2020). However, in order

to apply it to the conservation of biodiversity at population, community and ecosystem levels, we need further theoretical and experimental insights focused on understanding trans-generational genetic and demographic effects caused directly or indirectly by different human-derived environmental stressors. Progress has been made via literature reviews and meta-analyses to assess the ability of populations and communities to evolve in the face of changing environments (Hendry 2019).

Pollution as a driver of biodiversity and micro-evolutionary change

Pollution is a widespread form of habitat degradation and represents one of the strongest human-induced drivers of biodiversity and micro-evolutionary changes (Kettlewell 1956; Antonovics *et al.* 1971; Williams & Oleksiak 2008). It is represented by the introduction of contaminants into natural environments (air, water bodies, sediments, and soil), that cause detrimental effects to ecosystems and human health (Hill 2010; Mathew *et al.* 2017). Detrimental effects of pollution can be detected at all levels of biological organization (Bickham & Smolen 1994) from damages to enzymes, nucleic acids, cell membranes to changes at the individual and population level. Detrimental effects at the population level could involve alterations in sex ratios, age structure, reduction in fitness, inbreeding, genetic structure and diversity changes, and population declines. At the community level, common effects involve shifts in species composition while at the ecosystem level detrimental effects involve bioaccumulation and biomagnification of contaminants (Woodwell 1970; Peralta-Videa *et al.* 2009; Mussali-Galante *et al.* 2013). Moreover, additive and synergic effects are common in the presence of multiple pollutants and other environmental stressors (Côté *et al.* 2016; Niinemets *et al.* 2017).

Water pollution is one of the most significant types of pollution (Inyinbor Adejumoke *et al.* 2018). Its ecological effects may vary depending on the nature and source of contamination. Heavy metals, pesticides, nutrients, organic pollutants, industrial waste can reach lakes, ponds, rivers, groundwater, and oceans through storm drain, wastewater treatment plants, or simply through surface runoff. Moreover, the toxicity of most aquatic pollutants (but this applies also on soil and sediment contaminants) is difficult to determine because it depends on a variety of chemical and physical factors characterizing the contaminated habitat (temperature, pH, oxygen content, chemical composition, and

other abiotic and biotic factors; Hamelink *et al.* 1994; Mountouris *et al.* 2002; Oziolor *et al.* 2016).

Experimental approaches for studying the effects of aquatic pollution

Freshwater ecosystems are one of the most impacted habitats by anthropogenic stressors (Angeler *et al.* 2014; Reid *et al.* 2019) and, paradoxically, they also offer the ideal settings to study eco-evolutionary responses to pollution (De Meester *et al.* 2005; De Meester & Pantel 2014). Their outlined borders allow an easy determination of populations, and their connectivity (e.g. through rivers). Moreover, ponds can be mimicked in tanks and mesocosm settings where different conditions can be rigorously manipulated to study evolution and its impact on multiple trophic levels within replicated experiments (Logue *et al.* 2011; Matthews *et al.* 2011; Spivak *et al.* 2011; Verreydt *et al.* 2012). Mesocosms can facilitate the study of small-scale consequences of large-scale global drivers of biodiversity such as climate change and pollution (Stewart *et al.* 2013) by allowing rigorous testing of demographic traits (e.g., survival, population growth) and community assemblages (e.g., species composition, biotic interactions) under a range of future scenarios. These findings could support the development of models aiming to determine extinction risks, biodiversity losses and predict the consequences of environmental stress on biodiversity. For this purpose, it is important to consider a wide range of stressors including synergistic combinations (Fordham 2015).

Canada, where 9% of the territory is represented by freshwater ecosystems, is home to two field infrastructures conceived to perform large and highly replicated experiments on aquatic ecosystems. The first, the Experimental Lake Area (IISD-ELA), was realized in Ontario in 1968, consists of 58 lakes, and hosted over 50 ecosystem experiments on anthropogenic stressors (eutrophication, acidification, metal contamination, etc.) leading to more than 1000 peer reviewed scientific publications (Emmerton 2015). The second, the Large Experimental Array of Ponds (LEAP) was created in the research area of the Gault Nature Reserve, Mont Saint Hilaire (Quebec) in 2015. It represents a state-of-the-art field infrastructure specifically designed to allow highly replicated experiments that integrate evolution, ecology and metagenomics and focused on how complex aquatic communities evolve in response to environmental stressors.

Daphnia as a model organism in eco-evolutionary studies

Aquatic organisms are good candidates for eco-evolutionary studies. Their ease of culture, short generation times, small body sizes, and fast reproduction are all advantageous traits for laboratory studies (De Meester & Pantel 2014). One of the most studied freshwater organisms that shows all these characteristics is the microcrustacean *Daphnia* (Miner *et al.* 2012). Commonly referred as water fleas, daphniids are filter feeders, ingesting mainly micro-algae and organic detritus including bacteria and protists – occupying a key role in aquatic ecosystems (Lampert 2006). *Daphnia* has a cyclical parthenogenetic life cycle in which asexual reproduction alternates sexual reproduction (Fig. III). Its life cycle and short life span represent great advantages for studies in laboratory settings allowing to create replicate populations relatively easy and fast. Moreover, *Daphnia* represents an indicator genus for water quality and toxicity studies because of its sensitivity and rapid response to environmental changes (Miner *et al.* 2012). Its use in standard acute and chronic tests has been widespread with over 7000 references in the literature and about 500,000 records in the ECOTOX database (Shaw *et al.* 2008).

Daphnia longispina, *Daphnia magna*, and *Daphnia pulex* are the most studied species. *D. longispina* has been largely studied mainly for the effects of acid mine drainage ([AMD] a metal-rich outflow of acidic water from a mining site) on life-cycle traits (Lopes *et al.* 2004a; Lopes *et al.* 2005, 2006), and variations in polymorphic enzymes (Martins *et al.* 2007). *D. magna* has been studied through life-table experiments, quantitative genetic analysis, and metal body burden estimates, for the development of resistance to cadmium (Muyssen & Janssen 2004; Ward & Robinson 2005; Messiaen *et al.* 2012), zinc (Barata et al. 2002; Muyssen *et al.* 2002), copper (Bossuyt & Janssen 2003, 2004, 2005), and a mixture of them (LeBlanc 1982; Barata *et al.* 2002). *Daphnia pulex* has been studied for the effect of copper (Koivisto & Ketola 1995) and nickel (Kozlova *et al.* 2009) on life-history traits, and for its coping mechanisms in the response to copper stress (Chain *et al.* 2019). Tolerance to metal contamination was found in most studies, however, it was also accompanied by genetic erosion (Ward *et al.* 1995; Lopes *et al.* 2004a; Messiaen *et al.* 2012; Ribeiro *et al.* 2012), and fitness costs (Agra *et al.* 2010, 2011).

In many of the above studies, acclimation through physiological adjustments was considered the main responsible of the increased tolerance. *Daphnia* shows, indeed, a remarkable phenotypic plasticity in response to environmental stress (Dodson 1989; Ward & Robinson 2005). Distinguishing acclimation from adaptation is key for predicting the fate of populations facing rapidly changing levels of heavy metal pollution. Experimental evolution studies covering multiple generations that include the use of molecular tests to track genetic change are desirable. For example, Stoddard & Hochmuth (2007) exposed *D. magna* to copper for six months and carried out acute tests with neonates at every generation. They did not find an increase in tolerance to the metal over the course of the experiment. However, Hochmuth *et al.* (2015) conducted a microevolution experiment where they studied the effects of copper and zinc on *D. magna*, for about three months and observed a higher metal tolerance in pre-exposed populations.

Contrasting results and a scarcity of similar studies on more sensitive species of the genus (Koivisto *et al.* 1992; Koivisto 1995; Shaw *et al.* 2006) highlights the need for studies that can help clarify whether, and to what extent, freshwater biodiversity, including key stones species like *Daphnia*, will be able to cope with rapidly changing environmental stress such as chemical contamination. The application of ER theoretical framework to these systems can shed some light on the relative extent of genetic erosion caused by direct toxic effects, genetic drift, and selection of resistant phenotypes and whether demographic traits can allow recovery through genetic adaptation, despite genetic erosion.

DNA metabarcoding and its potential use in eco-evolutionary studies

The stability of communities and ecosystems is linked to biological diversity (Ives & Carpenter 2008). To limit the consequences of human-induced environmental change on ecosystem functions allegedly require the preservation of biodiversity and the ability of species to evolve to changing conditions (Sgro *et al.* 2011; Isbell *et al.* 2015). Biodiversity monitoring is essential for tracking change in biodiversity. Monitoring in this context is the process of assessing the status and following trends in living organisms and their environment (Lamb *et al.* 2009). By providing a reliable evaluation of the presence, absence, distribution of species, and patterns of diversity, biodiversity

monitoring allows the evaluation of the integrity of ecosystems, the consequences of environmental change, and the results of conservation actions and recovery measures (Larigauderie *et al.* 2012; Thomsen & Willerslev 2015).

Traditionally, biodiversity monitoring was performed through morphological identification of species for presence/absence by visual survey and assessment of abundances. However, traditional surveys have appeared to be time-consuming, labor-intensive, expensive, invasive, and sometimes even destructive (Jones 1992; Baldwin *et al.* 1996; Wheeler *et al.* 2004). Moreover, they can be imprecise due to difficulties in identifying larvae and juvenile life stages of closely related species, detecting cryptic species (Thomsen & Willerslev 2015), and species with very low abundance (Darling & Mahon 2011). The urgent need to limit the global biodiversity loss and assess current biodiversity trends, demands alternatives. There is, therefore, interest in innovative, effective, integrative strategies, and technologies for monitoring (Beumer & Martens 2013; Valentini *et al.* 2016).

Molecular identification systems, through the use of small segments of the genome to discriminate biological diversity, represent a valid alternative to overcome many of these challenges (Hebert *et al.* 2003; Schwartz *et al.* 2007). In the last years, it arose the possibility to use environmental DNA (eDNA) to identify species (Ficetola *et al.* 2008) and identify the composition of entire ecosystems (Thomsen *et al.* 2012; Bohmann *et al.* 2014). Environmental DNA is the "genetic material obtained directly from environmental samples (soil, sediment, water, etc.) without any obvious signs of biological source material" (Thomsen & Willerslev 2015). Methods based on eDNA have shown a higher detection capability and cost-effectiveness compared to traditional methods (Darling & Mahon 2011; Dejean *et al.* 2012). Moreover, they are non-invasive (no living organism is caught and/or killed during monitoring) and can decrease the probability of unintentional introduction of alien species and/or diseases (Valentini *et al.* 2016).

The use of eDNA can be coupled with other laboratory techniques (e.g., quantitative PCR (qPCR; Lacoursière-Roussel *et al.* 2016), droplet digital PCR (ddPCR), metagenomics, droplet digital PCR, and high-throughput sequencing. eDNA metabarcoding is a methodology similar to DNA barcoding (Hebert *et al.* 2003) that allows for concomitant

identification of several taxa within the same environmental sample (Taberlet *et al.* 2012). Following the selection of a marker, a primer is designed and used to amplify the collected eDNA sample and, following high-throughput sequencing (Shokralla *et al.* 2012), it can probe the presence of communities of organisms with high sensitivity and efficiency (Beja-Pereira *et al.* 2009; Blaalid *et al.* 2012; Thomsen *et al.* 2012; Clare 2014; Biggs *et al.* 2015). One of the main limitations of eDNA-based metabarcoding, is that it requires a reference library through the analysis of specimens belonging to the targeted taxonomic groups (Hajibabaei *et al.* 2007). Moreover, metabarcoding is a relatively new technique that still requires careful validation and interpretation (Bohmann *et al.* 2014; Cristescu 2014; Roussel *et al.* 2015). The chances of reporting false positives and negatives can be high without scrupulous calibrations. Thoughtful attention needs to be put in the choice of the specific methods used (in the field and laboratory and in bioinformatics), and the influences of spatial, temporal, and ecological factors on the final results (Cristescu & Hebert 2018).

The use of metabarcoding in conservation studies is increasing rapidly and more and more studies are applying metabarcoding to monitor species composition of natural communities (Pont *et al.* 2018; Valdez-Moreno *et al.* 2019), detect endangered and hard-to-sample species in freshwater (Rees *et al.* 2014) and terrestrial environments (Ishige *et al.* 2017); detect invasive species (Brown *et al.* 2016; Chain *et al.* 2016; Holman *et al.* 2018), etc. In the last few years, metabarcoding has been used also in population genetics where it gains within-species population data (Sigsgaard *et al.* 2017; Baker *et al.* 2018). This allowed the identification of known haplotypes (Baker *et al.* 2018) and "de novo" within-species variation (Parsons *et al.* 2018). Moreover, the validation of advanced computational techniques coupled with metabarcoding have allowed the detection of intraspecific genetic variation within multiple species simultaneously (Callahan *et al.* 2016; Stat *et al.* 2017; Elbrecht *et al.* 2018; Tsuji *et al.* 2019; Turon *et al.* 2019). This is opening the door to biodiversity monitoring at the population level and holds great potential for its integration with eco-evolutionary studies.

With this rapid development of DNA-based methods, the possibility to apply them to unveil biodiversity trends and to study the effects of pollution and other anthropogenic

stressors on populations, communities and ecosystems is becoming concrete (Barnes & Turner 2016; Deiner *et al.* 2017; Cristescu & Hebert 2018). However, a lot less is known about whether multi-taxa haplotypic diversity though metabarcoding could be used to contribute to the design of an eco-evolutionary framework based on eDNA-based-approaches in the context of designed experiments (but see Rudman *et al.* 2018; Adams *et al.* 2019).

Research gaps and thesis objectives

Human-induced alterations of eco-evolutionary dynamics may have a substantial impact on the ability of ecosystems to maintain their stability, especially in aquatic environments (Alberti 2015). The preservation of biodiversity and the limitation of future losses require an integrative research approach that integrates: i) comprehensive literature reviews and meta-analyses; ii) empirical investigations (ideally in mesocosm systems) of ecological, evolutionary, and demographic responses of biodiversity to different anthropogenic stressors; and iv) the use of molecular and genomic tools to monitor biodiversity with a particular emphasis of intra-specific genetic variation.

For my PhD's thesis, I explore the ecological, evolutionary and genetic consequences of pollution on different levels of biodiversity (Fig. II). In Chapter 1, I review the literature (258 research articles) and evaluate the evidence for micro-evolutionary responses following exposure to a wide range of pollutants. I assess the evidence for adaptation across multiple taxonomic groups and conduct a formal meta-analysis to calculate the magnitude of phenotypic change in response to metal pollution in invertebrates. Several literature reviews have been drawn up on the effects of pollution on biodiversity since (Kettlewell 1956) study of the evolutionary responses of peppered moths to industrial pollution and since the first observations made by Prat (1934) and Bradshaw (1952) on the ability of plants to grow in heavy metal contaminated soils (Prat 1934; Bradshaw 1952). Researchers have summarized studies on the ability of organisms to tolerate pollutants (Weis 2002; Wirgin & Waldman 2004; Klerks *et al.* 2011), on the genetic effects of pollution (Gillespie & Guttman 1999; Hoffmann & Daborn 2007; DiBattista 2008; Bijlsma & Loeschcke 2012), on genetic resistance to pollution (Roelofs *et al.* 2010; Whitehead *et al.* 2010), on micro-evolutionary effects of chemical stressors (De Coninck

et al. 2014; Oziolor *et al.* 2016), and on evolutionary limits of adaptive changes (Shaw 1999; Blows & Hoffmann 2005; Willi *et al.* 2006; Bell 2013). However, what is missing is a comprehensive synthesis across multiple taxonomic groups of the evidence of adaptation to different types of pollution in its all facets: methodological, molecular, and demographic. I draw attention to the importance of considering the response to pollution and its effects at different levels of biological organization within the same study system: genetic (genes); phenotypically (individual) and demographically (population), focusing also on long-term trends. Moreover, with the meta-analysis, I test the variety of data provided by the literature to determine the presence of common trends in response to pollution.

In Chapter 2, I use *Daphnia* populations to study genetic diversity and its interplay with metal pollution. Specifically, I study whether populations with high genetic variation have a longer persistence and higher probability of survival in a copper polluted environment than clonal populations. I also study the effect of copper on genetic variation and clonal composition patterns in *Daphnia* populations. The lack of genetic variation in traits under selection is one of the most common reason for the absence of micro-evolutionary responses; however, it is also one of the most underestimated explanations (Blows & Hoffmann 2005; Bell 2013). A handful of studies have looked at the role of intraspecific genetic variation in the evolutionary rescue theory framework. Agashe (2009) and Agashe *et al.* (2011) looked at the response of the flour beetle *Tribolium castaneum* to different food resources, Ramsayer *et al.* (2013) tested the effect of the antibiotic streptomycin on the Gram-negative *Pseudomonas fluorescens*, and Lachapelle & Bell (2012) used *Chlamydomonas reinhardtii* to study ER in the presence of salt. However, experimental evidence of ER in more realistic experimental settings is missing.

In Chapter 3, I test the feasibility of using eDNA-based techniques to detect zooplankton taxa and follow turnover and trends in their diversity and genetic variation in a mesocosm experiment. Understanding the ecological responses of communities to environmental stressors has never been an easy task. Researchers need to monitor every populations belonging to the community, estimate species richness and other ecological indexes, track trends in intraspecific variation (if possible) and link these processes in the framework of

eco-evolutionary dynamics. The use of eDNA gives the possibility to overcome some of these challenges and estimate species composition on a global scale in short time frames (Cristescu & Hebert 2018). However, the method presents some limitation for its use in the context of conservation and eco-evolutionary investigations due to the difficulty in estimating abundance, and the relatively long eDNA turnover rate that may not fully correspond to the species turnover rate overall a short temporal scale. This is why more studies are needed to explore to which extent these limitations affect its performance and future application in eco-evolutionary studies.

Overall, this thesis addresses key research gaps in eco-evolutionary biology, ecological genomics, and biodiversity science. It provides a broad understanding of the current knowledge of the response of different taxa to pollution and it highlights current research needs. It advances our knowledge of the effects of water pollution on aquatic invertebrates by crossing multiple levels of biodiversity and applying recent molecular methods to understand biodiversity change.

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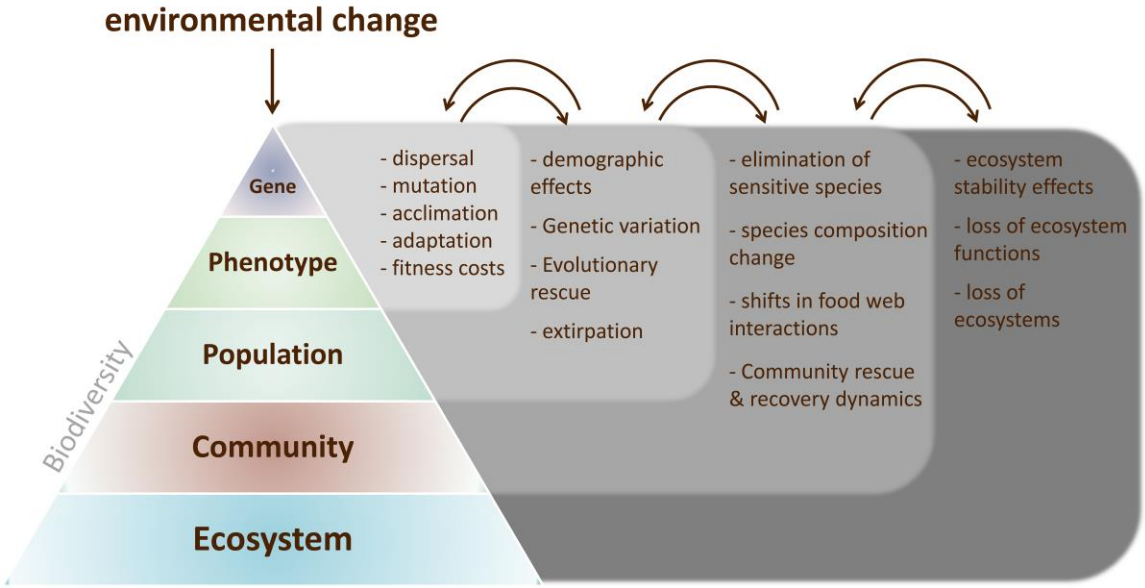

Fig. I. Environmental change can affect several aspects of biodiversity at all of its levels of organization. Depending on the rate and degree of environmental change, we can observe many effects at the ecosystem, community, population and individual level. The effects on the latter can influence directly and indirectly the fate of the population which can influence species composition in the community and also recovery dynamics. The effects occurring at each level are interlinked and together they influence the occurrence of other effects at other lower or higher levels of biological organization.

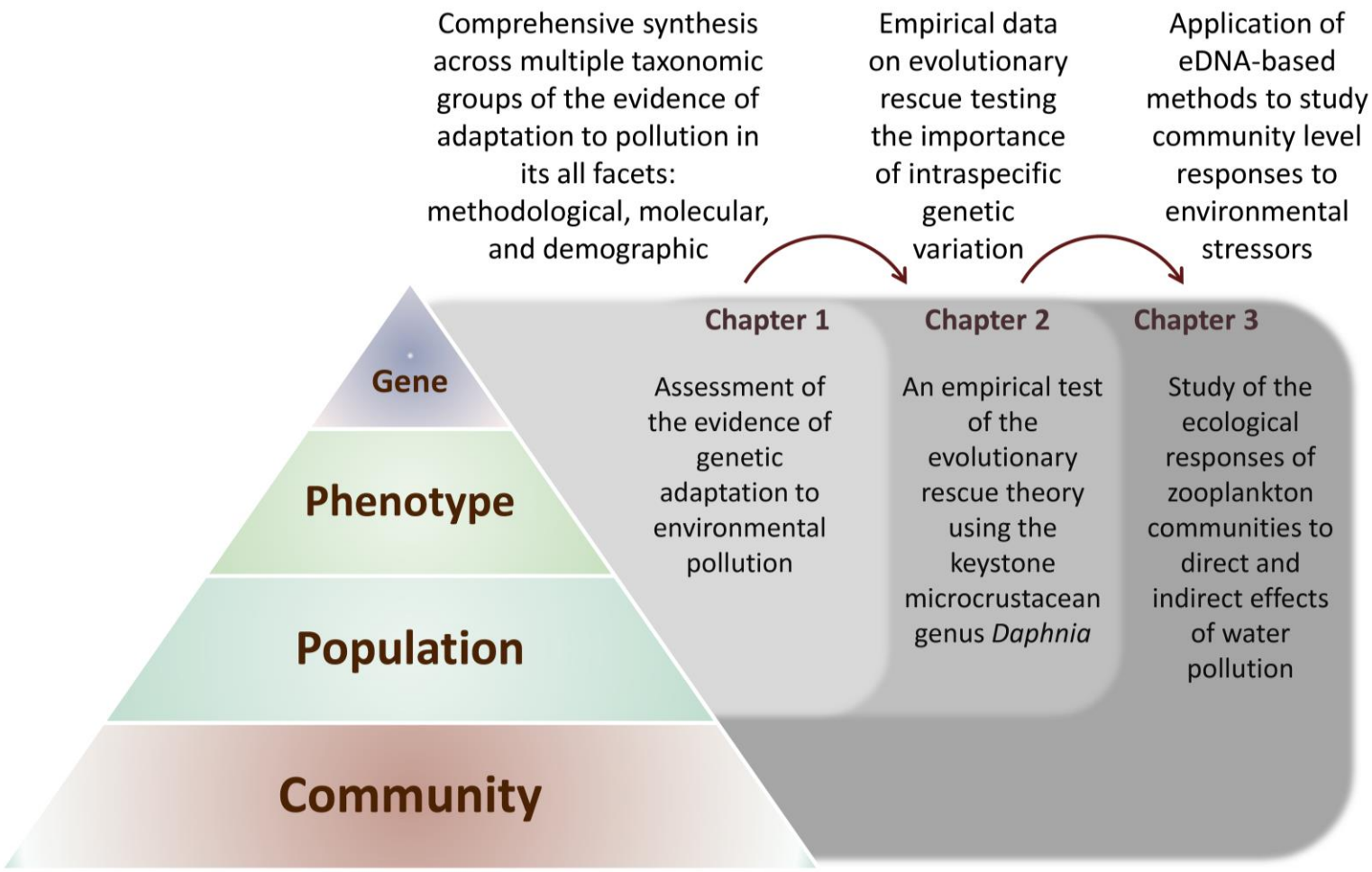

Figure II. Conceptual diagram of the thesis chapters. The gray areas point to the level of biodiversity that is considered in each chapter. The contribution of each part is summarized on top.

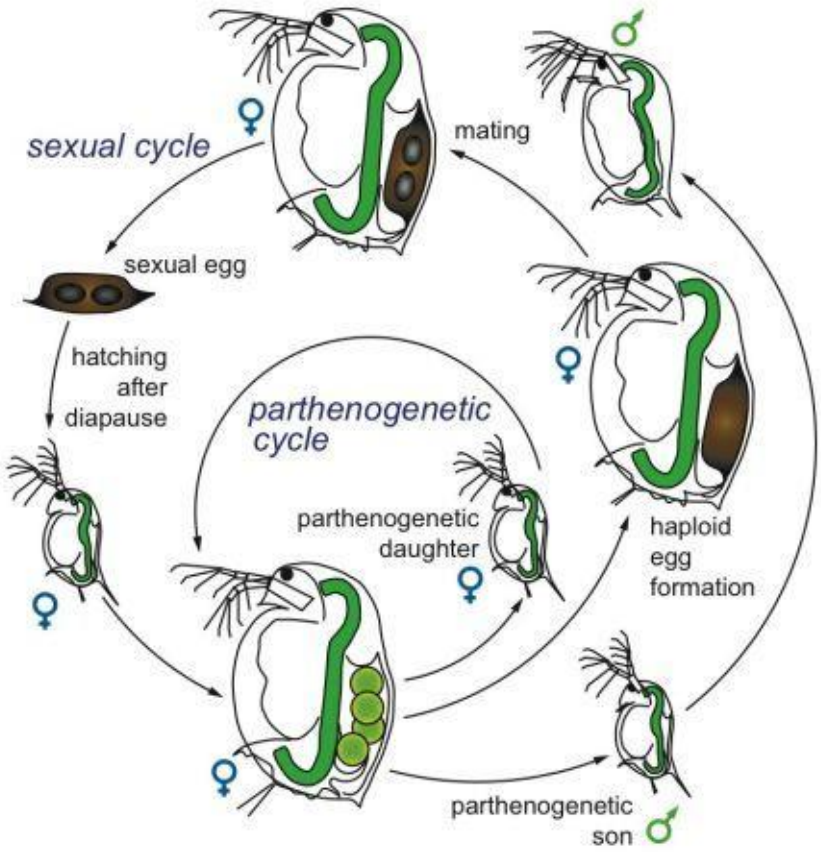

Figure III. Diagram of *Daphnia* life cycle. During the sexual cycle, female *Daphnia* develop haploid eggs that are fertilized by males and hatch after a period of dormancy. During the asexual cycle, female *Daphnia* form diploid eggs that develop in the brood pouch into parthenogenetic daughters and are released. Drawn by Dita B. Vizoso, Fribourg University (Ebert, 2005).

Chapter 1

Mixed evidence of adaptation to environmental pollution

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1.1 Abstract

Adaptation to pollution has been studied since the first observations of heavy metal tolerance in plants decades ago. To document micro-evolutionary responses to pollution, researchers have used phenotypic, molecular genetics, and demographic approaches. We reviewed 258 articles and evaluated the evidence for adaptive responses following exposure to a wide range of pollutants, across multiple taxonomic groups. We also conducted a meta-analysis to calculate the magnitude of phenotypic change in invertebrates in response to metal pollution. The majority of studies that reported differences in responses to pollution were focused on phenotypic responses at the individual level. Most of the studies that used demographic assays in their investigations found that negative effects induced by pollution often worsened over multiple generations. Our meta-analysis did not reveal a significant relationship between metal pollution intensity and changes in the traits studied, and this was probably due to differences in coping responses among different species, the broad array of abiotic and biotic factors, and the weak statistical power of the analysis. We found it difficult to make broad statements about how likely or how common adaptation is in the presence of environmental contamination. Ecological and evolutionary responses to contamination are complex, and difficult to interpret in the context of taxonomic, and methodological biases, and the inconsistent set of approaches that have been used to study adaptation to pollution in the laboratory and in the field. This review emphasizes the need for: (a) long-term monitoring programs on exposed populations that link demography to phenotypic, genetic, and selection assays; (b) the use of standardized protocols across studies especially for similar taxa. Approaches that combine field and laboratory studies offer the greatest opportunity to reveal the complex eco-evolutionary feedback that can occur under selection imposed by pollution.

1.2 Introduction

Humans have been described as the world's greatest evolutionary force with pollution as one of the most potent forces of ecological and evolutionary change (Palumbi 2001). However, how often evolution can result in an adaptive response to contaminants remains largely unknown (Brady *et al.* 2017). Fossil fuel combustion, the application of synthetic fertilizers and pesticides in agriculture, and the increasing use of complex chemicals are considered the main causes of pollution. For example, the number of complex chemicals is rapidly increasing. In Europe alone, more than 100,000 substances have been recorded in the market (Massey *et al.* 2013). Over the last 40 years, the long-term effects of pollutants on the sustainability of ecosystem processes have become a significant concern of the scientific community and regulatory agencies (Bickham *et al.* 2000).

The intensity, extent, and duration of pollution are important factors in determining whether a population can survive in the short term or persist and evolve in the long term. In the presence of reachable alternative habitats, dispersal can enable population persistence. However, when dispersal is limited or suitable habitats are not available, escaping stressful conditions is often not possible. In the short-term and in the presence of weak levels of pollution, organisms can adjust their phenotypes (e.g., physiology, behaviour) by means of plastic responses without changes in genetic composition (Gienapp *et al.* 2008). Moreover, when the level of pollution is persistently elevated and mortality is high, populations can become maladapted because of the presence of phenotypes lacking advantageous traits; standing phenotypes might be so maladapted that the loss of absolute fitness (W_{abs}) results in population decline (maladaptation in the strict sense; Hendry & Gonzalez 2008; Brandon 2014). In many cases, the population will be extirpated; however, in some cases individuals with advantageous traits and genetically inherited resistance to pollution may arise, recovering the absolute fitness ($W_{abs} > 1$) and resulting in population recovery through the process of evolutionary rescue (Gonzalez *et al.* 2013a; Fig. 1.1). The lack of functionally advantageous variation affecting traits such as survival, reproduction, and other life-history traits is perhaps one of the most common constraints to evolution in polluted habitats (Fisher 1930; Bradshaw 1991; Blows & Hoffmann 2005). However, the selection of resistant phenotypes alone does not

guarantee that a population will persist through adaptation. Small populations may undergo rapid extinction due to demographic and environmental stochasticity before they can recover (Lande 1988; Lynch & Lande 1993; Gomulkiewicz & Holt 1995; Lande 1999; Bell & Gonzalez 2009, 2011; Gonzalez *et al.* 2013b). Moreover, the effects of induced mutations caused by chronic exposures to mutagens can be exacerbated in small or declining populations, leading to "mutational meltdown," a process similar to a chain reaction in which the decrease in fitness due to mutations leads to further reduction in population size (Lynch *et al.* 1995).

The assessment of adaptive responses in natural populations should ideally involve field studies focused on phenotypic traits and/or molecular markers, and population monitoring over time (Fig. 1.1). However, this is often challenging, particularly for species with long generation time. As a result, many studies are restricted to comparing populations living under contrasting environmental conditions (Hansen *et al.* 2012). This approach, however, gives rise to problems concerning the unknown genetic history of the populations studied and does not take into account the fact that sensitive populations may disappear before investigations are conducted. Artificial selection experiments, and studies of the evolutionary potential in naïve populations, represent another approach to evaluate micro-evolutionary effect of pollutants (De Coninck *et al.* 2014). Such studies can provide accurate measurements of heritability, and fitness, including population growth rates, which are required to pinpoint the reasons for population persistence (Klerks *et al.* 2011; Oziolor *et al.* 2016; Fig. 1.1). Regardless of the main approach used, studies aiming to demonstrate adaptive evolutionary change should satisfy certain criteria (Hansen *et al.* 2012).

To document micro-evolutionary changes and to demonstrate the genetic basis of adaptation to pollution studies should ideally: (a) identify a trait(s) that can provide a fitness advantage in dealing with the stressor, (b) assess the presence of suitable genetic variation for the particular trait(s); (c) show that selection (as opposed to genetic drift) has taken place; (d) assess the contribution of the advantageous trait(s) to the population fitness by estimating population growth rate (Gomulkiewicz & Holt 1995; Bickham *et al.* 2000; Klerks *et al.* 2011; Bell 2013; Hansen *et al.* 2012; Merilä & Hendry 2014).

Although collectively these criteria are quite stringent, many of them can be satisfied by either focusing research efforts on quantitative trait analysis or testing for selection at candidate molecular markers (Hansen *et al.* 2012).

Several literature reviews have summarized studies focused on the ability of organisms to tolerate pollutants (Weis 2002; Wirgin & Waldman 2004; Klerks *et al.* 2011), on the genetic effects of pollution (Gillespie & Guttman 1999; Hoffmann & Daborn 2007; DiBattista 2008; Bijlsma & Loeschcke 2012), on genetic resistance to pollution (Roelofs *et al.* 2010; Whitehead *et al.* 2010), on micro-evolutionary effects of chemical stressors (De Coninck *et al.* 2014; Oziolor *et al.* 2016), and on evolutionary limits of adaptive changes (Shaw 1999; Blows & Hoffmann 2005; Willi *et al.* 2006; Bell 2013). However, a broad synthesis of strategies and trends in evolutionary toxicology research encompassing multiple levels (e.g., taxonomic, methodological, molecular, demographic) is still lacking.

To get a better understanding of what is currently known about adaptation to pollution, we conducted a literature review that encompasses multiple levels or organization (genetic, individual, and population level), taxonomic groups (algae, plants, invertebrates, and vertebrates), methods (field and laboratory studies), and pollutants (metals, acidification, PAHs, PCBs, etc.). We performed a quantitative meta-analysis with a subset of the data to evaluate the effect of metal pollution on the magnitude of phenotypic response (e.g., weight, number of offspring, and metal body content) in invertebrates. We also evaluated how shifts in methodological approaches have changed our understanding of micro-evolutionary responses to pollution. In particular, we assessed molecular evidence for adaptation and the candidate genes potentially involved in the pollution-induced evolutionary processes (Appendix A).

1.3 Methods

In this study, we reviewed articles published since 1992 found by searching on Google Scholar. We used the keywords “genetic adaptation,” “adaptation,” “micro-evolution” in combination with “pollution” or “pollutants” or “contaminants.” These search terms reduced bias to a particular approach or method. We also searched with “identification of candidate genes AND pollution/pollutants/contaminants” and “genomics OR

transcriptomics AND pollution/pollutants/contaminants" to collect papers focused on the identification of candidate genes involved in resistance to pollution for our descriptive compilation (Appendix A). We also reviewed the articles listed in the bibliographies of the retrieved papers and reviews with titles that pertained to adaptation to pollution. Studies focused only on toxic effects (e.g., deleterious mutations) were excluded. Our search returned a total of 258 papers corresponding to 278 studies (complete references list in Appendix A). The vast majority of these articles investigated only one species, another twelve articles investigated two species, and one article assessed three species. The number of studies indicated in our figures assumes each species as a different study. The articles were classified based on the type of pollution, the species studied, source populations (e.g., from contaminated and reference sites or laboratory cultures), genetic methods, type of study (field vs. laboratory study), and type of response (Appendix A, Tables A.1 and A.2). We considered studies on algae, plants, invertebrates, and vertebrates that focused on the effects of metals, acidification (terrestrial and ocean acidification caused by CO_2 increase), polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and various other chemical pollutants (identified as "other"; Appendix A, Fig. A.1) on several phenotypic traits and genetics at both individual and population levels. Some of the articles included investigations on more than one pollutant (Appendix A, Fig. A.2). We did not consider thermal, visual, and noise pollution. We also excluded studies on simplified agricultural systems where agrochemicals were intentionally applied in the environment.

We identified a set of response variables that are measured in laboratory and field experiments to study the evolution of resistance through molecular markers and/or quantitative traits. Studies were then assessed and classified according to the approach(es) used: (a) analysis of phenotypic responses to pollution leading to resistance ("phenotypic assays"); (b) characterization of the genetic basis/underlying genetic variation of the advantageous phenotypic traits ("genetic assays"); (c) tests for evidence of selection against random genetic drift and gene flow ("selection assays"); (d) assessment of population growth rate ("demographic assays"; Table 1.1, Appendix A, Table A.2; Vasemägi & Primmer 2005; Hansen *et al.* 2012; Merilä & Hendry 2014). Phenotypic assays consisted of laboratory and field phenotypic surveys and were not

limited to studies that ruled out plasticity. Genetic assays consisted of laboratory and field common garden experiments as well as molecular assays aiming to explore the genetic basis of advantageous traits, decompose total variation into its components (disentangling genetic and environmental bases for trait variation), and identify and analyse functional DNA polymorphisms. However, heritability estimates of single traits, extrapolated within common garden experiments, may not accurately predict the trait's selection response and the ability to evolve resistance (Klerks *et al.* 2011). Selection assays inferred the adaptive basis of trait change by studying how changing trait values reflected patterns of selection (i.e., animal model analyses, methods that compared differentiations for quantitative traits to those for neutral genetic markers) and changes in allele frequencies (i.e., FST- based outlier tests). In these cases, random genetic drift was ruled out. Since it is common in evolution studies to substitute time for space and use geographic variation in resistance as an alternative for temporal changes (the clean site represents the state of the contaminated site prior to contamination; Byars *et al.* 2007; Klerks *et al.* 2011), these types of investigations were included in the selection category. Several experimental designs and analyses included more than one assay category. For example, field and laboratory common garden experiments were represented by both phenotypic and genetic assays since the study of phenotypic trait responses is usually followed by estimates of heritability. Phenotypic selection estimates were represented by both phenotypic and selection assays; genotypic selection estimates by genetic and selection assays. We noted whether a phenotypic response was documented in multiple studies (focused on the same population) and whether (following evidence for selection) further tests were performed to ensure that selection was due to pollution and not to other confounding factors. We grouped studies conducted on the same population(s) and considered them as one composite study while aggregating their methods and outcomes (Appendix A, Table A.2).

We obtained a subset of 108 articles on invertebrates that focused on metals specifically cadmium, copper, lead, and zinc. We subjected these to a formal meta-analysis to evaluate the magnitude of the phenotypic response (change in the weight, number of offspring, and metal body content) to different metal concentrations. We focused on the taxonomic groups, pollutants, and response variables that are commonly reported in the

literature. Following methods from Collins (1992) and Mondol *et al.* (2016), we were able to convert length traits into weight measures, which added three more studies (Haimi *et al.* 2006; Venier *et al.* 2006; Yap *et al.* 2013) and 15 additional datapoints. We recorded the metal concentration, the response of treatments and controls at each concentration, the total sample size, and the SD. If the study provided only the SE, the SD was calculated by multiplying the SE by the square root of sample size; when only confidence intervals were provided and when the sample size was >60, we applied the formula SD = √sample size (upper limit − lower limit)/3.92. When the sample size was <60, we replaced 3.92 with values obtained from tables of the t distribution with degree of freedom equal to the group sample size minus 1. We converted all metal concentrations to parts per million (ppm) and created a numerical metric for the analyses: the natural logarithm of the metal concentration divided by the threshold concentration specific for each metal and habitat type determined by the Canadian Council of Ministers of the Environment (Appendix A, Table A.3). When data were only provided in a graphical format, we used Getdata Graph Digitizer 2.26 (http://getdata-graph-digitizer.com) to estimate the displayed values. When sample size was given as a range, we calculated the average. The dataset included a large variety of experimental designs that were mainly classified as either "field" or "experiment" (Appendix A, Table A.3). In some studies, the organisms were exposed to more than one metal under two scenarios. In the first scenario, populations were naïve to the particular metal tested, but they had been exposed to other stressors in their original habitats, while in the second case, the populations were sampled from sites contaminated by multiple metals and measured directly for their traits (e.g., size). When the contaminated sites contained more than one metal, we considered the metal with the highest concentration relative to the environmental quality guidelines. For studies involving more than one control and/or treatments, we used all possible combinations for the calculations of effect sizes. Calculation of the effect sizes and variances was performed in MetaWin Version 2 (Rosenberg *et al.* 2000) using Hedges' d. Effect sizes were then plotted against metal concentrations to assess the distribution of the data. Originally, we had 63 datapoints for weight, 89 for the number of offspring, and 260 for body metal content. However, because most of the studies reported data for more than one treatment (two or more

distinct populations impacted by pollution, both sexes, subsequent generations) we had to compute a summary effect for the impact of pollution for all treatments combined. We formed a composite effects size for each study by performing a fixed-effect meta-analysis on the subgroups of each study with the following: z= Effect size/Variance; w= z/Effect size; Computed mean = Sum of z/Sum of w; Variance= 1/Sum of w.

Studies that had multiple datapoints because different concentrations of metals were tested were kept unchanged. We performed a multivariate mixed-effect meta-analysis using the package 'metafor' (Viechtbauer 2010) with R 3.0.2. (R Team 2014) to assess the relationship between effect sizes and metal concentrations on 10 studies (20 datapoints) for weight, 12 studies (38 datapoints) for the number of neonates and 17 studies (64 datapoints) for metal content in tissues. We used an information-theoretic approach to rank statistical models based on Akaike in- formation criteria (AICc; Burnham 2002). For each trait, we explored the null model, the random model, and then models with one, two, and three fixed terms (metal concentration, metal, habitat, phylum, subclass, presence of other metals, and experiment/field factor) for a total of 20 models. Models were ranked according to decreasing values of AICc (Appendix A, Table A.4). Study ID was always considered a random term, and the random effects for the different metal concentrations tested within the same study were correlated through a multivariate parameterization. The models with the lowest AICc were visually inspected with residual plots to assess deviations from homoscedasticity and normality. We calculated heterogeneity (Tau-squared and residual heterogeneity; Deeks *et al.* 2008) and intraclass correlation coefficients (ρ) to calculate the variance components for the between-study and within-study heterogeneity. We also recorded the number of generations studied in the subset of papers used for the meta-analysis (n = 108). Finally, we mapped all study sites to show where pollutants have been studied around the world. For the meta-analysis, we used the R packages metafor (version 2.1; Viechtbauer 2010), while for the world map, we used the packages rgdal (version 1.3-6; Roger *et al.* 2017), rworldmap (version 1.3-6; South 2011), and ggplot2 (version 3.0; Wickham 2016) in R version 3.0.2 (R Team 2014).

1.4 Overview of the studies

1.4.1 Type of pollution and geographic distribution of species

We found that 63% of the reviewed studies (n = 191) focused on metal pollution (Fig. 1.2a) and more than half of metal studies (n = 108) were on invertebrates, followed by vertebrates (n = 74), plants (n = 60) and algae (n = 17). Specifically, metals were the most studied pollutants for terrestrial arthropods and to a lesser extent for terrestrial annelids, aquatic arthropods, and mollusks. Plants were also studied primarily in relation to metals. The effect of acidification on algae, invertebrates (Echinodermata), and vertebrates was investigated in 8% of the studies. Polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) were the least represented (9%) with a dozen studies almost exclusively conducted on fish. The rest of the studies (~20%) focused on pollutants like pesticides, dioxin-like compounds, radiation, waste heap, tributyltin, and other chemical compounds and were grouped together as "other" (Appendix A, Fig. A.1). These types of pollutants were studied mostly in aquatic arthropods and mollusks but also in vertebrates and plants.

Most studies were situated in Europe and eastern North America, which historically have been the most industrialized areas of the world (Fig. 1.2b). Australia and central China were also relatively well sampled, while regions in South America, Africa, and South China experiencing high levels of pollution showed a paucity of data. In Europe, the most studied areas included Northern France and Poland but also Portugal and England. The geographic distribution of studies is biased to historical centers of industrial activity but does not reflect the current geographic distribution of contamination.

Relatively few species were tested within each phylum (Fig. 1.3, Appendix A, Fig. A.2): 77 species of invertebrates were investigated by 133 articles; 36 species of plant by 54 articles; 25 species of fish by 55 articles, two species of amphibian by five articles, 10 species of microalgae by 12 articles, and two species of macroalgae by two articles. Not surprisingly, the use of model species such as *Fundulus* (fish: Actinopterygii), *Chironomus* (invertebrate: Insect), *Daphnia* (invertebrate: Branchiopoda), *Arabidopsis* (plant: Eudicots), *Orchesella* (invertebrate: Entognatha) was common. These species were so extensively studied that they have been the subject of several important literature reviews. For example, (Weis 2002) assessed what is known about tolerance and

mechanisms of tolerance to several pollutants (mercury, dioxins, PCBs, and PAH) in different populations of killifish; Whitehead *et al.* (2017) reviewed key features of killifish populations and the genetic architecture underlying adaptive responses in these populations. Studies of selection on transcriptional regulation of the collembolan *Orchesella cincta* were reviewed by van Straalen & Roelofs (2005) in relation to cadmium contamination; Pauwels *et al.* (2008) compared molecular genetic results with other approaches (e.g., QTL analysis) and discussed the nature of the genes potentially involved in the adaptation to zinc-polluted soils in plants. Studies with model species have built the foundation of our knowledge on micro-evolutionary responses to pollution. Here, our goal was to synthesize the evidence for adaptive responses across multiple levels (genetic, individual, and population levels), in a range of model and non-model taxa and for a number of pollutants.

Phenotypic assays included the study of survival in different concentrations of pollutants (e.g., LC50), changes in development (e.g., hatchability), growth (e.g., growth rate), morphology (e.g., body size, leaf size), physiology (e.g., feeding rate), and reproductive traits (e.g., age at first brood, number of offspring). Survival and reproductive traits were the most studied in invertebrates, while in plants and vertebrates all traits were more or less equally studied (Fig. 1.3). Genetic and selection assays included both quantitative trait studies and molecular genetic studies (Table 1.1). Genetic assays were encountered in 95 articles and were more prevalent in studies on invertebrates, where they were more common than selection assays and led also to more statistically significant findings than tests for selection. For example, for the invertebrates, there was a widespread use of gene expression techniques followed by heritability estimates. In plants, genetic assays were not very common and these studies were almost absent in algae. Gene expression studies were the most frequent genetic assay (n = 34) followed by studies on trait heritability (n = 16). Selection assays were the second most abundant assays (n = 121) after phenotypic assays. Studies on invertebrates were the most common followed by vertebrates and plants. FST-based outlier tests were the dominant way to find evidence for selection. Together with survival, these tests were the most commonly used to study adaptive responses of organisms in polluted conditions. Among the studies that found evidence of selection, the majority (86.5%) tested also for a link between responses to selection and

the environment: 15.7% found evidence for genotype–environment correlations and another 15.7% for phenotype–environment correlations (Appendix A, Table A.2). A number of studies (n = 31) on invertebrates and microalgae included population growth rate estimates.

1.4.2 Evidence from phenotypic, genetic, selection, and demographic assays

After grouping the results from different articles, we obtained 198 studies (Fig. 1.4), 63% of which found significant differences in responses to pollution. Figure 1.4 shows the breakdown of studies across methodological approaches. Forty-two studies (21%) did not find statistical differences among treatments, regardless of the approach used. Only three studies (Messiaen *et al.* 2010; Messiaen *et al.* 2012; Dutilleul *et al.* 2013; Kelly *et al.* 2013; Messiaen *et al.* 2013; Dutilleul *et al.* 2014; Dutilleul *et al.* 2015) found evidence of a phenotypic response to pollution with an underlying advantageous genetic basis and also found the presence of a response to selection followed by a demographic response. These studies provided the most thorough insights on the adaptive potential of the studied organisms.

Most of the studies finding adaptive responses came from phenotypic assays in invertebrates, particularly studies on survival (n = 47) followed by physiological traits (n = 26) and morphological traits (n = 20; Fig. 1.4b). The evidence for phenotypic responses to pollution was often accompanied by evidence from molecular genetic approaches, making a total of 130 out of 198 studies (Fig. 1.4a). Twenty studies included data from phenotypic, genetic, and selection assays and found statistical evidence for all three. However, only three studies assessed whether the observed resistance was heritable (MacNair *et al.* 1993; Shirley & Sibly 1999; Xie & Klerks 2003) while only four assessed whether the specific trait studied (e.g., larval size, net reproductive rate) was heritable (Sunday *et al.* 2011; Foo *et al.* 2012; Messiaen *et al.* 2012; Kelly *et al.* 2013); in all the cases, the traits were heritable. MacNair *et al.* (1993) studied the heritable variation in the degree of copper tolerance in *Mimulus guttatus* seeds collected from an abandoned copper mine (California). They found that populations from contaminated soil and some populations sampled downstream of the mine had 100% tolerance while populations sampled upstream showed variable tolerance that was related more to geographic location

than copper concentration in the soil. Through the study of life-history traits during experimental selection, they were able to demonstrate that tolerance was heritable and widespread in populations from contaminated soil due to beneficial genetic variation. Shirley and Sibly (1999) conducted a 20-generation selection experiment using *Drosophila*, where they measured fecundity and many other traits during exposure to cadmium. Individuals from contaminated cultures developed resistance and had a higher fitness than the controls, and the evolution of resistance was due to a single sex-linked gene. Xie & Klerks (2003) conducted a selection experiment for six generations to investigate the response to selection by cadmium in *Heterandia formosa*. The authors observed an increased resistance in the selection lines and found a heritability of 0.50. By calculating the heritability and testing the survival of six generations of controls and selected individuals, they provided compelling evidence for the evolution of resistance in a vertebrate population.

Three studies provided a complete assessment of the adaptive potential of the aquatic microcrustacean *Daphnia magna* (Messiaen *et al.* 2010; Messiaen *et al.* 2012; Messiaen *et al.* 2013), the free-living soil nematode *Caenorhabditis elegans* (Dutilleul *et al.* 2013; Dutilleul *et al.* 2014; Dutilleul *et al.* 2015), and the sea urchin *Strongylocentrotus purpuratus* (Kelly *et al.* 2013). Messiaen *et al.* (2010) used laboratory cultures of *D. magna* to study the response to cadmium and temperature. Through life-history trait analysis, they found that chemical pollution can affect genetic variation and between-trait correlations. The response to other stressors (e.g., temperature) was also affected by pollution. Moreover, Messiaen *et al.* (2012) estimated additive and nonadditive components of the genetic variability of net reproductive rate during cadmium and temperature stress and uncovered a substantial level of stress, which translated into a decrease in the population mean reproductive rate. Broad-sense heritability and total genetic coefficients of variation suggested a genetic determination of net reproductive rate. Clonal selection on this trait could positively influence population mean fitness. Additionally, they suggested that both asexual and sexual reproduction phases in the life cycle of *Daphnia* could play a role in the long-term adaptive potential of populations to cadmium stress. Finally, Messiaen *et al.* (2013) measured reproductive performances of hundreds of clones from naïve populations and compared them with the laboratory

cultures used by Messiaen *et al.* (2010) and Messiaen *et al*. (2012). They found that although there was no significant difference in the initial tolerance of clones, estimates of broad-sense heritability of cadmium tolerance suggested great variation ranging from not significantly different from 0 to between 0.48 and 0.81. The authors stated that "it's difficult to predict the long-term response to chemical pollution of unstudied populations from tolerance data on a sample of other populations," suggesting that methods for forecasting long-term responses (e.g., predictive models based on population genomic and tolerance time-series data) are needed.

Dutilleul *et al*. (2013), Dutilleul *et al*. (2014), Dutilleul *et al*. (2015) conducted a series of studies on laboratory cultures of the nematode *C. elegans*. In their initial study, Dutilleul *et al.* (2013) studied uranium stress and its effect on phenotypic traits like survival, generation time, brood size, body length, and body bend. They found that at low concentrations of uranium, negative effects were reduced, but at high concentrations, negative effects were amplified across generations. Acclimation was not enough to ensure survival. Subsequently, Dutilleul *et al.* (2015) studied the genetic basis of survival, fecundity, and growth under uranium and salt stress while also estimating the heritability of these traits. Surprisingly, the most heritable traits in the control environment (fecundity and early growth) had a reduced heritability in the uranium-contaminated environment. This reduction in heritability, possibly due to differences in gene expression of tolerance genes (e.g., metallothionein), was not proportional to the decrease in population fitness, and this could have impeded selection from acting on phenotypic traits. The authors concluded that by altering the genetic structure of populations, pollution can influence their potential to adapt to other stressors.

Kelly *et al.*'s (2013) study on the sea urchin *S. purpuratus* was the only individual study that employed all four approaches we advocate for here, albeit indirectly (Fig. 1.4). The effects of acidification were studied using estimates of additive genetic variance for body size under high pCO_2 (partial pressure of carbon dioxide) across populations. The authors used these data to parameterize a model predicting the rate of evolution under changing pCO_2 and the effect of evolutionary change on demographic rates. Their model showed that when selection on body size was weak, there was very little evolutionary change, but

the impact of genetic variation became stronger with increasing selection intensity. When inclusion of population processes to experimental designs is challenging (e.g., due to long generation time), mathematical models can be crucial for strong inferences about the long-term effects of pollution on fitness.

1.4.3 Invertebrates and metals: a meta-analysis

We tested the relationship between metal concentration and effect sizes of weight, number of neonates, and body metal content. We accounted for the phylum and subclass of the studied organism, the habitat, the type of metal, and other factors such as the presence of other metals in the original habitat and the study type (laboratory experiment or field monitoring). We expected that body weight and number of neonates would decrease with increasing metal concentration and that body metal content would increase.

Surprisingly, we did not find a strong effect of metal contamination (Fig. 1.5; Appendix A, Table A.5). For body weight, which was the trait with the smallest dataset (10 studies and 20 datapoints), the best-fit model was the random-effect model (AICc = 32.41), which provided a small and nonsignificant mean effect size (0.12, SE = 0.16; Appendix A, Fig. A.5). For the number of neonates, the best-fit model included the metal concentration (ln[ppm]/threshold), the presence of other metals, and the metal in question (AICc = 118.5). Metal concentration effect was weak (Fig. 1.5), but the presence of other metals suggested a negative effect on the number of neonates produced, with lower numbers in the presence of other metals. Metal type (Cd, Cu, and Zn) had also a negative effect with copper being the strongest. For body metal content, the best-fit model included the metal concentration, the metal type, and the taxonomic subclass (AICc = 175.05), and in this case, the metal concentration effect was slightly statistically significant. However, these results are to be interpreted with caution given the limited size of the datasets. In fact, the interclass correlation coefficients (ρ) were quite large for body weight (0.89; Appendix A, Table A.5), indicating that the effects within studies (different metal concentrations tested) were highly correlated. Residual variabilities were large too, indicating that other unmeasured factors were contributing to the effect sizes.

The fact that we did not find strong relationships between the response variables and metal concentration suggests several issues. First of all, the power of our analysis was

likely small given the limited number of studies and datapoints available. Moreover, the high heterogeneity of methods, factors tested, and types of experiments made comparisons very difficult. This issue was encountered by Oziolor *et al.* (2016) when attempting a meta-analysis of evolutionary events in response to PAHs and PCBs. They found "a complexity and diversity in the academic investigations of population-level ecotoxicological impacts that make it difficult to directly compare across studies" (Oziolor *et al.* 2016). Moreover, the different bioavailability of metals likely played a role in the heterogeneity of results we observed (De Coninck *et al.* 2014). Bioavailability of metals depends on a large variety of chemical, environmental, and biological parameters. Factors such as pH and acid-buffering capacity, temperature, presence of organic matter or minerals, element speciation, concentrations of other substances can all play a role in the availability of a metal. Thus, the processes affecting bioavailability are heavily influenced by the type of habitat and are expected to change over time and among different organisms (John & Leventhal 1995). Another important issue is that different individuals and cohorts within a population might have distinct strategies for coping with pollutants. The difference in effect size that we found across subclasses can be explained by the fact that traits such as weight and number of offspring may change in opposing directions during stress, depending not only on the intensity of the stress, but also on other biotic and abiotic conditions. For example, Amorim *et al.* (2017) measured survival, reproduction, size, and metallothionein gene expression during a 3.5-year selection experiment with *Folsomia candida* exposed to cadmium. They found body size was smaller in animals exposed to EC10 than EC50 concentrations. Body size is a complex trait that changes as a result of metal toxicity, detoxification costs, and shifts in energy allocation (Grześ *et al.* 2015) and is often a compromise of all the above (Kozłowski & Gawelczyk 2002). The number of neonates is predicted to be low during stress, and it is often linked to large egg size as optimality models of life-history theory predict (Sibly & Calow 1986; Lloyd 1987; McGinley *et al.* 1987). Winkler and Wallin (1987) have also demonstrated that these traits are closely correlated. The number of offspring is also an adaptive compromise during stress given that larger eggs ensure a greater chances of survival and faster development (Fox & Czesak 2000) while numerous small eggs ensure higher fecundity (Bernardo 1996). As expected from optimality

models, we found a general decrease in the number of offspring, although this was not statistically significant. The body metal content response showed a slight but not significant positive correlation with metal concentration. However, an observation of low body metal content as described in other studies (Donker *et al.* 1996) might indicate an adaptive response such as increased detoxification ability (Sibly & Calow, 1986) or decreased metal uptake (Harper *et al.* 1997). Another potential reason for the weak effects we found is that effects of metals might be difficult to disentangle from other factors. For example, Kozlov and Zvereva (2011) conducted a meta-analysis on primary producers (bryophytes, vascular plants), primary and secondary consumers (arthropods), and decomposers (fungi, arthropods) with the aim of revealing regional and global pattern from small-scale observational studies. They found that the effect of pollution depended on the pollutant (type, amount, duration of exposure), the organism (life cycle, life history), the level of biotic organization at which the response was measured (organism, population, community), and the environment (biome, climate). Moreover, the effect of one factor was often modified by other factors with many interactions among them. Overall, the magnitude of responses to pollution was weak, and trophic level, type of pollution, and biome explained only 7% of the variation (Kozlov & Zvereva 2011).

Predicting the outcome of adaptive allele dynamics in a changing environment is generally very challenging given fitness × environment interactions, and variable responses mechanisms and rates across taxa (Morgan *et al.* 2007; Milesi *et al.* 2016). There is a clear opportunity to improve and build on the dataset we have assembled here. Future meta-analyses will have the task of accounting for a complex set of predictors and confounding variables.

1.5 Summary

Generally speaking, evidence for adaptive responses to pollution requires the demonstration of increased heritable resistance to relevant environmental pollutants. When assessing both individual and population studies, our review found relatively modest support for long-term adaptive responses to pollution.

A handful of the reviewed studies demonstrated that including measures of population growth rate often reveals how pollution can negatively affect population trends despite

the presence of tolerant phenotypes (Postma & Davids 1995; Haimi *et al.* 2006; Medina *et al.* 2009; Anderson *et al.* 2013; Dutilleul *et al.* 2014). Studies that combined several laboratory approaches (demographic assays with quantitative trait methods and molecular genetics) provided clearer evidence for adaptive responses to pollution. These studies also found that a successful adaptive response to pollution can be altered by another stressor like temperature or increased salinity (Dutilleul *et al.* 2014; Messiaen *et al*. 2012). They also suggest that it is not generally possible to extrapolate the findings from specific laboratory populations to other populations of the same species in the field.

Once observation of resistance to pollution has been made, we suggest that compelling evidence for adaptive changes in the field requires several additional pieces of information: (a) demonstration that the changes in the trait studied are genetically determined and are subject to natural selection; (b) assessment of potential confounding environmental variables; (c) the demonstration that the increase in adaptive trait value can sustain a positive population growth rate and thus the long-term persistence of the population (Hansen *et al.* 2012). Field samples should always be accompanied by a complete ecological analysis of the soil/sediment/water from which organisms are obtained. An extra effort should be made to determine the bioavailability of the pollutant in question (De Coninck *et al.* 2014). In the case of laboratory studies, repeatable and highly controlled ecotoxicological tests should be accompanied by multi-generation experiments in which population growth rate is estimated. Additionally, if suitable molecular markers are available, in-depth assessment of genetic structure and genetic variation for the most advantageous traits should be attempted (Fig. 1.1). Besides a scarcity of demographic assays, we also found several sources of biases in the literature. These include publication, taxonomic, and methodological biases. The latter includes the lack of standardized methodologies among studies of similar species, studies covering only one generation (Appendix A, Fig. A.4) and studies focused on only a single life stage of the studied organisms (Table 1.2).

1.6 Conclusions

Despite decades of active research, it is still difficult to make broad statements about how likely, or how common, population adaptation is in the presence of environmental

contamination. Given the challenges of predicting the adaptive response of wild, populations based on data from a handful of populations or laboratory cultures with model organisms, we stress the need for: (a) long-term monitoring programs of populations in polluted habitats that integrate demographic studies with phenotypic, genetic, and selection assays; (b) use of standardized protocols among studies of similar species to make evolutionary toxicology studies more comparable (Oziolor *et al*. 2016); (c) an effort to deepen our understanding of evolutionary processes and underlying genetic mechanisms of resistance. Such approaches provide a great potential to advance our understanding of evolution in response to pollution in wild populations.

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Table 1.1. Synopsis of phenotypic, genetic, and selection assays for inferring phenotypic responses, presence of suitable genetic variation and a response to selection for resistance to pollution. Methods to find a link between the selection detected and the type of pollution studied are also shown. The numbers in parentheses are used in Table A.2 to classify the reviewed articles.

Phenotypic assays	**Genetic assays**		**Selection assays**	
Phenotype	*Quantitative traits*	*Molecular markers*	*Quantitative traits*	*Molecular markers*
Survival (1)	Quantitative trait locus (QTL) analyses (7)		Q_{ST}-F_{ST} comparisons (19)	F_{ST}-based outlier tests (23)
Growth traits (2)	Admixture mapping (8)		Trait direction of changes in the wild (20)	Detection of selective sweeps (24)
Physiological traits (3)	Association analysis (9)		Tests on neutrality of rates of evo (21)	Genetic association tests (25)
Developmental traits (4)	Additive genetic variance, heritability (10)	QTL mapping of mRNA expression (14)	Pedigreeing, animal model analysis (22)	Genome scan approaches (26)
Morphological traits (5)	Broad sense heritability (11)	QTL mapping of protein expression (15)	**Link of selection to pollution**	
Reproductive traits (6)	Reciprocal transplants (12)	Gene-specific mRNA expression (16)	Experimental selection (27)	
	Protein level estimates (13)	mRNA expression (17)	Phenotype-environment correlations (28)	Genotype-environment correlations (29)
		Tests on known candidate loci (18)	Phenotype-genotype correlations (30)	
			Other (31)	
Characteristics				
- Plasticity is not ruled out	- Identification of traits and loci to be likely under selection		- Investigation of adaptive changes/shifts	
- Synchronic and/or allochronic	- Genetic versus environmental bases for trait variation		- Synchronic and/or allochronic	
- Lab and/or field	- Laboratory and/or field		- Laboratory and/or field	
- Mainly phenotypic surveys	- Can be used to provide info prior to a population becoming subjected to selection		- Random genetic drift is ruled out	

Table 1.2. The reviewed results might be subject to biases such as publication bias, non-independence of studies; dominance of laboratory studies; poorly standardized methodologies; few generations covered during experiments; limited and noncomparable life stages investigated.

Factor	Bias	Description
Type of results	Publication bias	Positive results tend to be published more than negative ones. Publication bias is a common issue in the scientific literature and it may lead to distorted findings in systematic reviews and meta-analyses
Type of study	Only laboratory study	Almost all experiments on adaptive responses to pollution were conducted under laboratory conditions. In some cases, rearing certain species under laboratory conditions was not possible and few studies used microcosms in the original natural habitats (Bahrndorff *et al.* 2006; Piola & Johnston, 2006)
Approach	Lack of standardization of methodologies and parameters within studies of similar species	Studies are characterized by a range of methodologies and different combinations of measurements and observations. Methods are taxon-specific and even within the same general methodology there are major differences in duration of the experiment and concentrations tested among studies
Choice of populations	Comparison of populations already established in the field	Comparing populations from historically known polluted habitats and populations sampled from reference habitats give rise to problems concerning the unknown genetic history of the populations studied, the processes behind it and the fact that sensitive species may just disappear before investigations
Number of generations covered in an experiment	Only one or few generations	Most of the experiments looked at metal effects over few generations (Fig. A.5). Fifty studies among 108 remained vague regarding the number of generations covered. A handful of studies covered 8 to 10 generations (Postma & Davids, 1995; Vidal & Horne, 2003; Ward & Robinson, 2005; Leon Paumen et al., 2008; Fisker et al., 2011) and only two covered more than ten generations (Shirley & Sibly, 1999; Kafel et al., 2012)
Age class	Using only one life-stage	The susceptibility to toxic substances depends on the life stage of an organism. Initial structure of a population in an experiment influences its susceptibility to pollutants. The exploration of only early life stages excludes the investigation of reproductive traits

Figure 1.1. A diagram illustrating two populations that undergo different selection pressures and are used to study their phenotypic, genetic, and selective responses in laboratory and field assays. Pollution acts as a selective force for resistant phenotypes in population 2, which shows higher resistance to pollution than population 1. If the advantageous alleles reach fixation and the population growth rate is positive, then population 2 can recover and persist in the polluted environment by adaptation. However, if the number of selective deaths is too high, or if maladapted phenotypes lower the local absolute fitness below the replacement rate, then population 2 might go extinct. The degree of pollution, phenotypic variation, strength of selection, and population size and the interspecific interactions are all key factors in determining whether a population can persist through genetic adaptation in contaminated locations. Adaptation to pollution has been studied in the laboratory and field. When studied in the field, phenotypic trait variability and population sizes can be jointly monitored over time to reveal covariation that is consistent with increasing fitness. Reciprocal transplant and common garden experiment are possible in the field, which provides greater control over confounding environmental factors. Under laboratory conditions, a large number of repeated tests can be performed (phenotypic, genetic, selection, and population assays) in the short term and long term, either phenotypic and genetic assays with single individuals, or with entire populations, where demographic processes for invertebrates and annual plants are studied over multiple generations.

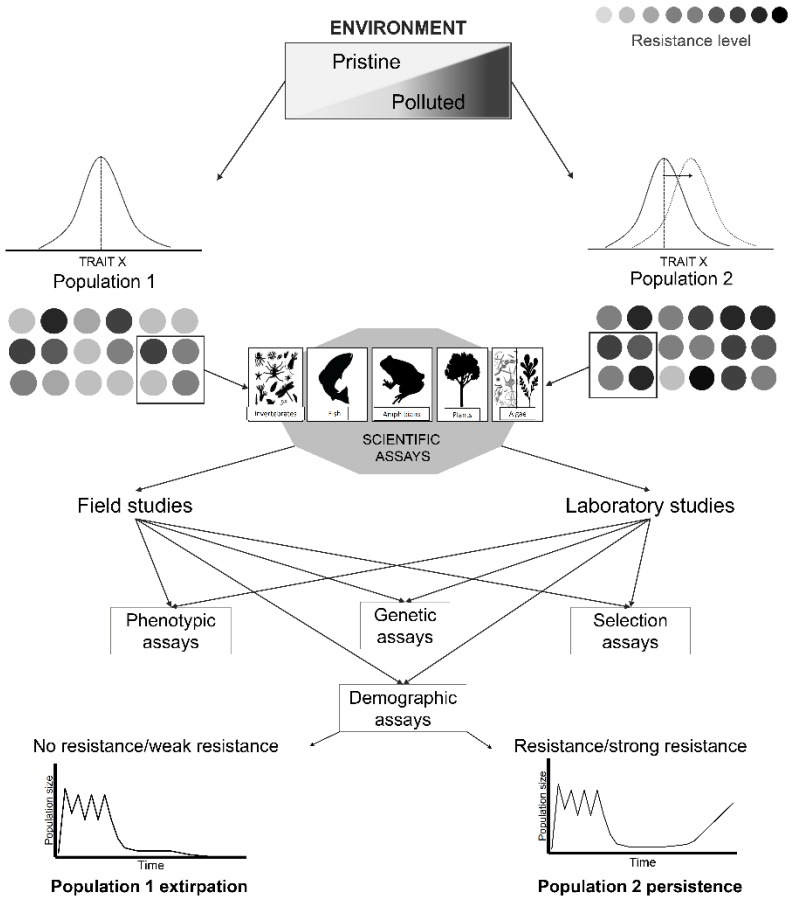


Figure 1.2. (a) Number of studies sorted by type of pollution and by taxa. (b) World map showing the localization of the contaminated sites from which populations were sampled. Different colors identify different types of pollution. Articles that made use of laboratory cultures were not considered.

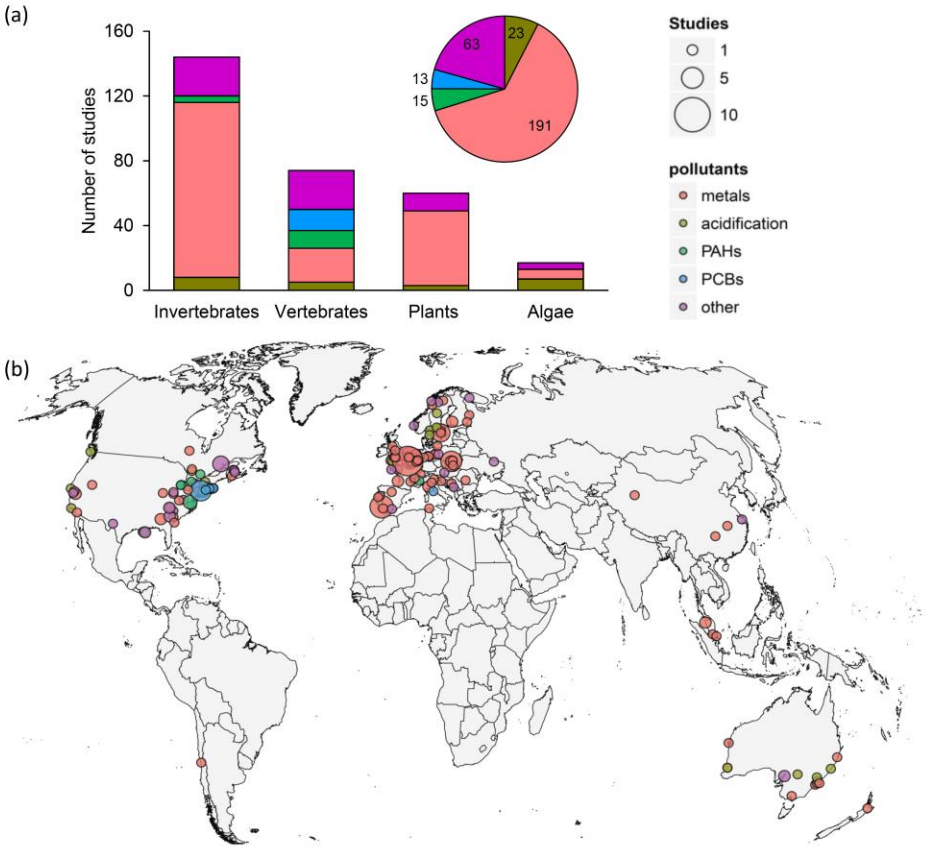


Figure 1.3. Number of studies on the different taxa that, through different approaches (phenotypic, genetic, selection, and demographic assays), found evidence for an adaptive response. The width of the lines represents the number of studies that belong to each approach. The numbers inside the boxes represent the number of species and, in brackets, the number of papers.

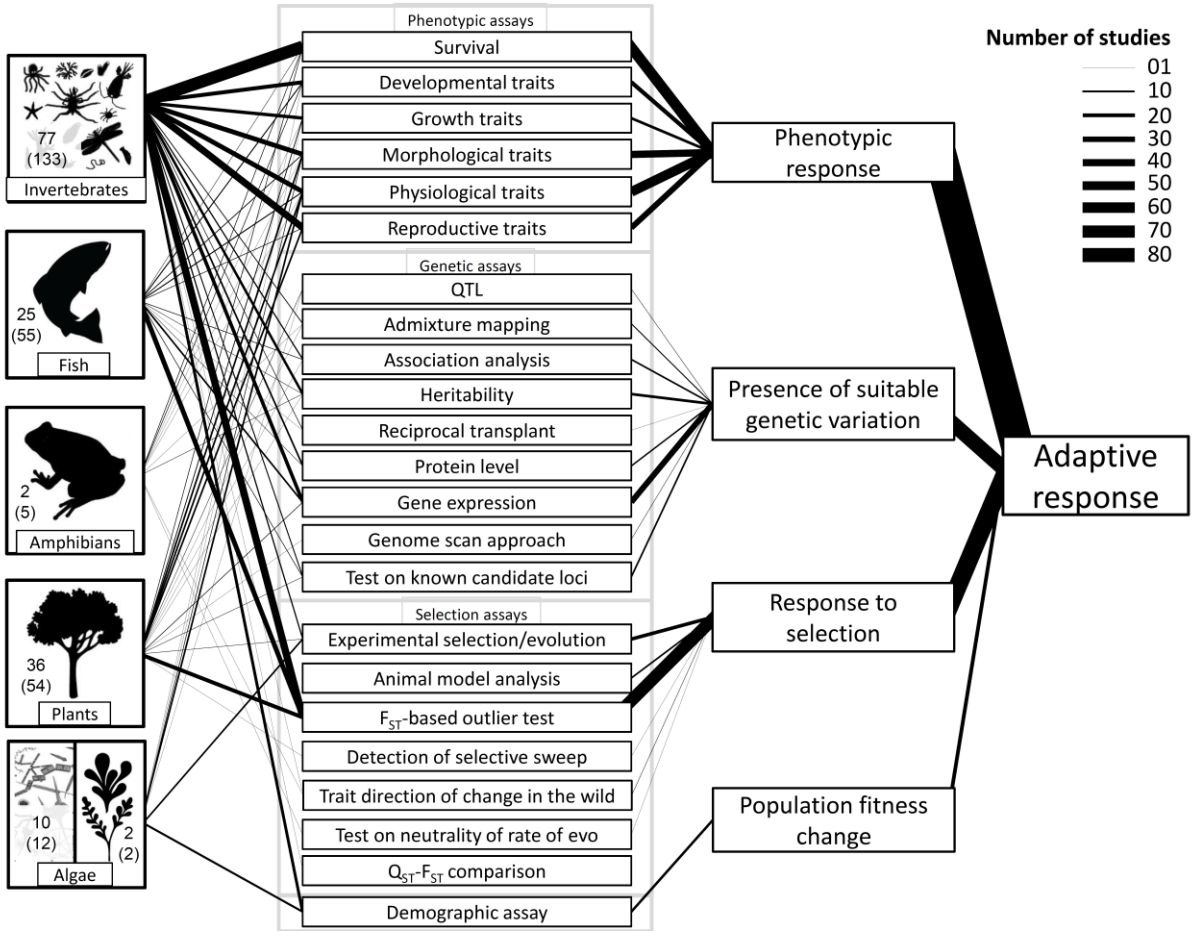


Figure 1.4. (a) Number of studies using different assays (phenotypic, genetic, selection, and demographic) that found evidence of a phenotypic response, presence of suitable genetic variation, a response to selection and population fitness change. (b) Number of studies on invertebrates, vertebrates, plants, and algae that found statistically significant evidence (or lack of) for a phenotypic response due to pollution (phenotypic assays), presence of genetic variation for resistance (genetic assays), responses to selection (selection assays), and population fitness changes (demographic assays). Number of studies in which these components were not considered are also shown (down right).

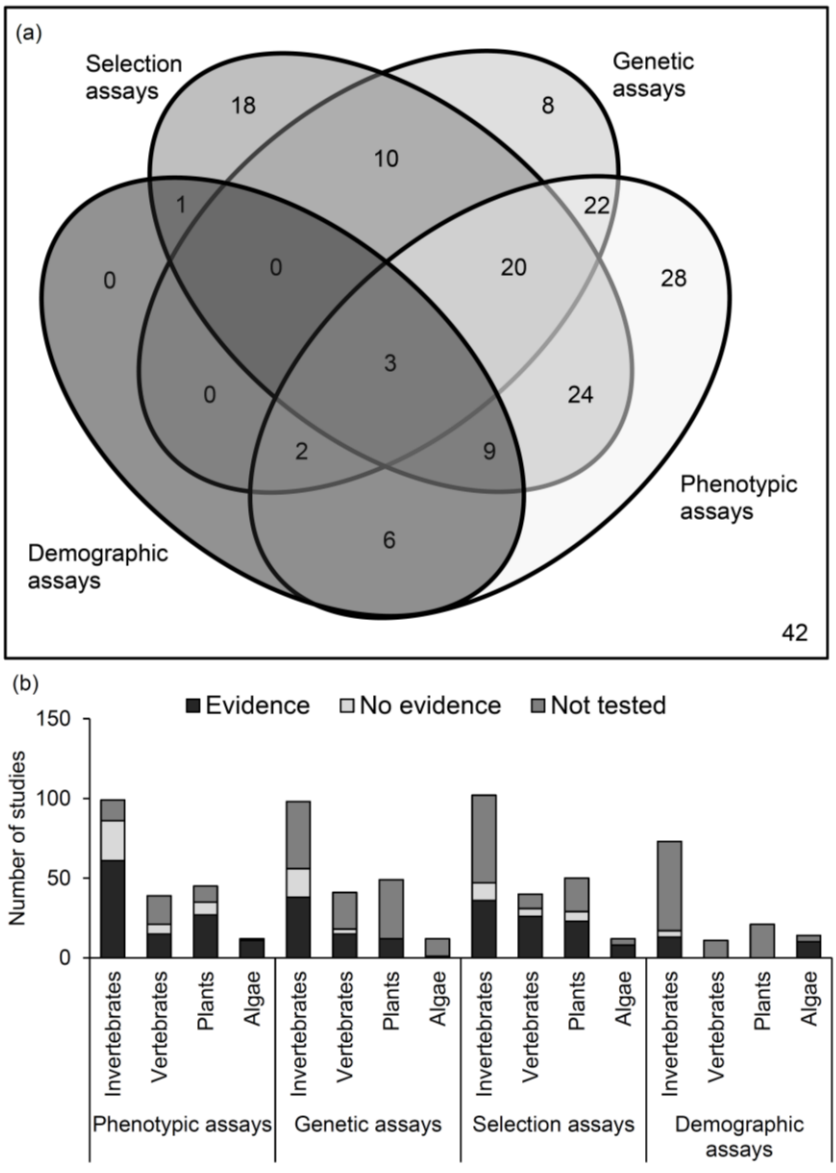
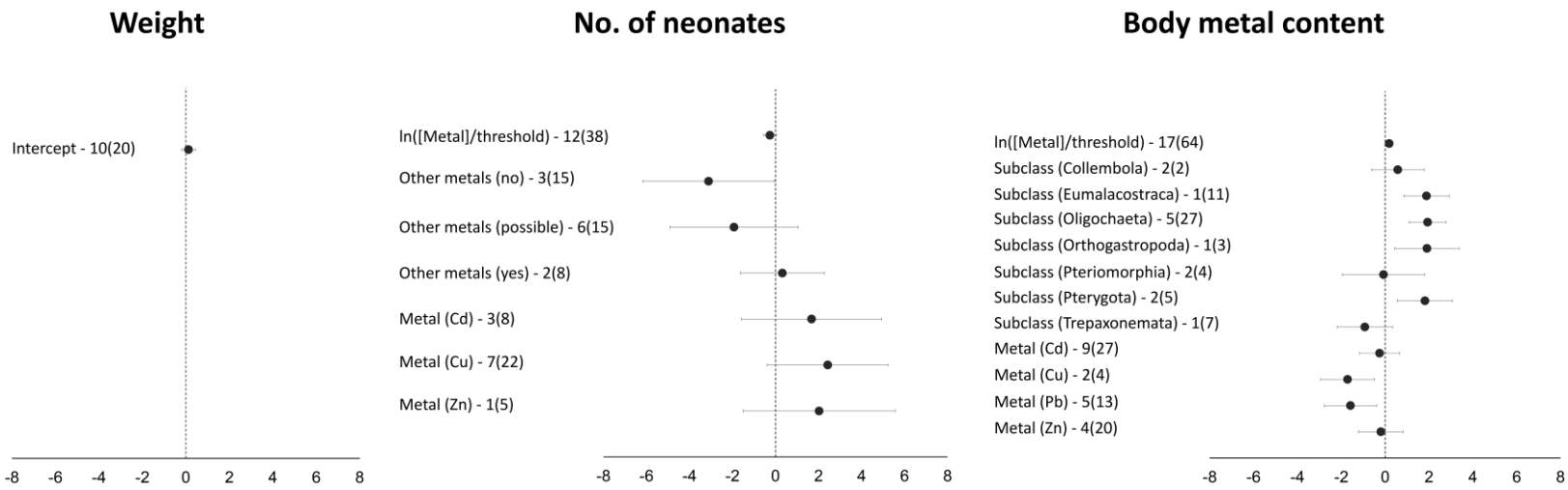


Figure 1.5. Fixed-effects estimates and confidence intervals of AICc-best models for weight, number of neonates and body metal content. The number of articles is shown beside each term and in brackets there is the number of datapoints.



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Connecting statement

In Chapter 1, I provided a comprehensive synthesis of strategies and trends in evolutionary toxicology research across multiple levels (genetic, individual and population level), taxonomic groups, methods, and pollutants. Studies were systematically assessed based on the approach used to study the evolution of resistance to pollution: analysis of phenotypic responses; characterization of the genetic basis/underlying genetic variation of the advantageous phenotypic traits; tests for evidence of selection against random genetic drift and gene flow; assessment of population growth rate. Most of the reviewed studies were focused on phenotypic responses to heavy metal pollution, at the individual level. Only about 12% of the studies included population growth rate estimates and most of them found that negative effects induced by pollution were exacerbated over multiple generations, even in the presence of tolerant phenotypes (Postma & Davids 1995; Haimi *et al.* 2006; Medina *et al.* 2009; Anderson *et al.* 2013; Dutilleul *et al.* 2014). Collectively, the studies are demonstrating that the presence of an advantageous trait in a polluted environment can sustain a positive population growth rate and thus the long-term persistence of the population is a key element in the study of the likelihood of evolutionary rescue. Thus, Chapter 1 highlighted the need of high-controlled and repeatable laboratory studies covering multiple generations and integrating demographic estimates.

In Chapter 2, I focused on heavy metal contamination, the most widespread form of pollution, as highlighted in Chapter 1. I used *Daphnia*, a key stone species in freshwater habitats, as a model organism. Its life history attributes allow the performance of bioassays with satisfactory level of replication, repeatability, and relatively fast pace. I looked at the effects of copper on *Daphnia* demographic trends and whether intraspecific genetic variation provided higher persistence, and higher probability of population rescue. By conducting a multi-generation bioassay that included close monitoring of population dynamics affected by metal contamination and by providing insights on the occurrence of adaptation at the population level, I contributed by filling one of main research gap identified in Chapter 1.

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Chapter 2

Genotype diversity promotes persistence in *Daphnia* populations during copper stress

Alessandra Loria, Melania E. Cristescu, Andrew Gonzalez

2.1 Abstract

Genetic adaptation is often the only option a population has to avoid extirpation when environmental stressors of high intensity are sustained for long periods of time. The occurrence of adaptation relies on the presence of genetic variability in a population's gene pool which provides variable traits upon which selection can act. However, despite the presence of genetic variability, under strong selection pressure, demographic stochasticity can drive populations to extirpation before the occurrence of adaptation. We conducted an experiment to examine whether genetic diversity leads to evolutionary rescue in a controlled experiment with natural populations of *Daphnia pulex* exposed to Cu contamination. We created monoclonal and multiclonal populations and monitored their population sizes during a 227-day microcosm experiment. We initially applied Cu at a sub-lethal concentration and then increased the concentration every week until the population sizes reached about 10% of the carrying capacity (Cu at 180 μg/L). The concentration was then increased up to 186 μg/L and held stable until the end of the experiment. We tested whether genetic variation within natural populations of *Daphnia pulex* increased the likelihood of evolutionary rescue and population persistence in a Cu-contaminated environment. We also quantified the change in genetic diversity over time. Treatments started to show population decline at a Cu concentration of 180 μg/L and eventually all treatment populations went extinct. A survival analysis showed that multiclonal populations had a higher probability of survival than monoclonal populations. As expected, loss of allelic richness was faster in copper treatments than in controls. In this experiment, genetic diversity was not sufficient to ensure long-term persistence and genetic adaptation to the copper contamination. We stress the need for more empirical studies manipulating genetic diversity and investigating its role in evolutionary and demographic responses to severe anthropogenic stress.

2.2 Introduction

Anthropogenic global changes are considered the main cause for the loss of biodiversity (Vinebrooke *et al.* 2004; Brook *et al.* 2008; Barnosky *et al.* 2011; Ceballos *et al.* 2015). Multiple drivers are known to drive the loss of diversity, including habitat loss, harvesting, climate change, exotic species and pollution (Diversity 2010; Tilman *et al.* 2017). Heavy metal contamination is one facet of environmental pollution that is thought to affect taxonomic and diversity, particularly in freshwater ecosystems (Bickham *et al.* 2000; Heugens *et al.* 2001; Loria *et al.* 2019).

The rate at which habitats are being degraded means that in many cases rapid adaptation will be required if extirpation is to be avoided (Bell & Collins 2008). Adaptation to heavy metal contamination can occur at different rates, depending on the presence of genetic variability (standing genetic variation and de novo mutations), in a population's gene pool, the strength of selection, and initial population size. When it occurs quickly enough to prevent extinction due to maladaptation a population is said to undergo evolutionary rescue (ER; Lynch *et al.* 1991; Burger & Lynch 1995; Gomulkiewicz & Holt 1995; Bell & Collins 2008; Orr & Unckless 2008; Gonzalez *et al.* 2013). ER is characterized by a U-shaped population time series showing phases of sharp decline, stabilization, and recovery corresponding to an increase in the frequency of an adaptive phenotype (Gonzalez *et al.* 2013; Carlson *et al.* 2014). A growing number of laboratory studies have provided strong support for evolutionary rescue theory (Bell & Gonzalez 2009); important factors include the history of exposure (Gonzalez & Bell 2013), the initial population size (Samani & Bell 2010), the strength of selection (Perron *et al.* 2008), dispersal (Bell & Gonzalez 2011), and the presence of advantageous genetic variation (Lachapelle & Bell 2012; Ramsayer *et al.* 2013) and beneficial mutations (Lindsey *et al.* 2013).

A handful of studies have suggested that standing genetic variation has an important role in facilitating rapid adaptation to novel environments (Frankham *et al.* 1999; Feder *et al.* 2003; Colosimo *et al.* 2005; Pelz *et al.* 2005; Steiner *et al.* 2007; Tishkoff *et al.* 2007) and in ER (Agashe *et al.* 2011; Lachapelle & Bell 2012; Ramsayer *et al.* 2013). However, manipulating different levels of genetic variation and collecting empirical evidence for factors influencing ER is challenging because time series data are required, along with high

levels of population replication and rigorously controlled selective environments. These requirements have resulted in experiments on ER with unicellular organisms such as bacteria, algae, yeast, although examples with insects (Agashe 2009; Agashe *et al.* 2011), fish (Oziolor *et al.* 2019), and wild populations of vertebrates are now available (Vander Wal *et al.* 2013). To date, few experiments have challenged populations with pollutants (Low-Décarie *et al.* 2015) such as heavy metals (Loria *et al.* 2019).

The freshwater microcrustacean cladoceran *Daphnia* (Anomopoda) is an excellent candidate model organism for ER experiments. It is easy to raise, has a wide distribution, and its parthenogenetic reproduction and short-generation time allow long-term demographic experiments. *Daphnia* has been largely used as model organisms to study aquatic environmental toxicity (Sarma & Nandini 2006; Morgan *et al.* 2007) for its relatively high sensitivity to environmental contaminants (Tomasiks & Warren 1996; Lampert 2006; Altshuler *et al.* 2011; Kim *et al.* 2015) but also their inter- and intra-population variability in phenotypic responses to metals (Hairston Jr *et al.* 2005; Suresh 2019). Whether this variability can lead to micro-evolutionary responses following long-term exposure to heavy metals (Stoddard & Harper 2007; Hochmuth *et al.* 2015) remains largely unclear. Copper has been widely used in industrial processes and agriculture (Nriagu 1996; Gledhill *et al.* 1997). Although Cu is an essential micronutrient for both prokaryotes and eukaryotes, high concentrations of copper in aquatic systems can have toxic effects that can alter individual physiology, increase mutation rates, and potentially reduce population fitness. Lastly, Cu tends to bio-accumulate in food webs (Tomasiks & Warren 1996; Long *et al.* 2004) in many natural water bodies where *Daphnia* is a key mediator of productivity.

In this study, we conducted a long-term experiment to test whether: 1) high levels of genetic variation would allow populations of *Daphnia* to undergo ER and persist under high Cu exposure; and 2) Cu stress affects genetic variation by driving different clonal responses through selection and, in general, genetic erosion. We expected that populations with high clonal diversity would outperform mono-clonal populations because greater standing variation is expected to support greater allelic richness for tolerance to Cu (Hochmuth *et al.* 2015). We also expected that the toxic effect of Cu and its potential selection of resistant

genotypes (together with genetic drift) would more rapidly erode clonal diversity in the treatments relative to control populations. Genetic erosion due to chemical contaminants has been observed in natural populations (Bickham *et al.* 2000; De Meester *et al.* 2006) including *Daphnia* in relation to heavy metals (Lopes *et al.* 2004; Agra *et al.* 2010, 2011; Ribeiro *et al.* 2012).

2.3 Methods

2.3.1 Experimental populations

We used *Daphnia* isolates collected from six habitats in Illinois (USA) during Spring 2013: three populations of *D. pulex* sampled from ponds (Dump, Bridge North and Center), and three populations of *D. pulicaria* sampled in lakes (Long, Clear and Sportsman's; Fig. B.1). To create the multiclonal experimental assemblage (MTC), an equal number of clonal lines were randomly chosen from each collection location for a total of 36 lines (six from each of the six habitat).

2.3.2 Experimental design

A total of 24 populations were grown in 12 L circular polyethylene tanks with 9 L of FLAMES medium (Celis-Salgado *et al.* 2008). The experiment consisted of a two-treatment factorial design with two diversity treatments: monoclonal (MNC) multiclonal (MTC) populations, and a Cu control and Cu treatment. The *Daphnia* were seeded at a density of 72 individuals per tank (36 clonal lines x 2 individuals), at the juvenile life-stage. All MTC populations contained 36 clonal lines each (six per habitat) and were replicated six times for a total of twelve MTC populations (six Cu control replicates and six Cu treatment replicates, t1-t12). The MNC populations consisted of six monocultures, one per habitat, six controls and six Cu treatments (t13-t24; Table 2.1) and each seeded by a randomly selected clonal line from each collection location that was also part of the MTC assemblage. MNC replicate populations consisted of different clonal lines because our goal was to test for an effect of the level of genetic diversity regardless of clone identity. All MTC tanks were seeded with two individuals per each clonal line for a total of 72 individuals; the MNC tanks were started with 72 replicate lines (same clonal line).

All 24 populations were kept in a walk-in growth chamber at a temperature of 18°C and humidity of 70% with a 12hr:12hr light-dark cycle. Populations were fed with a total of 1-mL mixture of microalgae (*Ankistrodesmus* sp., *Scenedesmus* sp., and *Pseudokirchneriella* sp., at an approximate target concentration of 55,000,000 cells/mL, 220,000,000 cells/mL, 250,000,000 cells/mL, respectively). The feeding regime was initially twice a week but starting from day 60 we switched to three times a week. This adjustment was conducted to sustain large population sizes. The positions of the tanks in the shelves was randomized on a weekly basis. The randomization ensured that at least two tanks of the same category (i.e. two MTC Cu treatments and two MNC Cu controls, etc.) were placed on the same shelf in order to allow a potential test for an effect of the position of the tanks on the shelves on population sizes.

The population size in each tank, and the abundance of neonates, juveniles and adults were estimated once a week until the thirteenth week (day 86) and then twice a week starting from the fourteenth week (day 93). For a detailed description of the counting method we refer to Appendix B and Figure B.2 and B.3. Resting eggs were regularly collected (day 26, 41, 67, 83, 109, and 226).

The populations were allowed to reach carrying capacity, and then on day 31 the treatment tanks were spiked with ionic Cu solubilized in 5% nitric acid at the non-lethal concentration of 150 μg/L. This initial spike was achieved by replacing 2L of Cu free medium with 2L at a Cu concentration that, brought the total concentration to 150 μg/L. The rate of copper contamination was then cautiously adjusted based on the response of populations. The concentration of Cu in the tanks was increased by 10 μg/L every 7 days until it reached 170 μg/L, then by 5 μg/L reaching 175 μg/L at day 49. At this point (week 7 and 8), the concentration was maintained stably for two weeks in order to monitor the population sizes after the change in the feeding regime (described above). After week 8, the concentration was increased by 1 μg/L a week and then at intervals of 2 μg/L until it reached a concentration of 184 μg/L (day 98). This concentration was kept stable for two weeks (weeks 14 and 15) and then was increased up to 186 μg/L. At this concentration, population sizes dropped to 10% of the carrying capacity and the increase of Cu was stopped. The Cu level was kept stable at 186 μg/L for the rest of the

experiment by replacing 2 L with fresh medium once a week. The controls were also refreshed with 2L of Cu free medium once a week. pH was measured every two weeks during the first 100 days of the experiment, and then every week until the end of the experiment. Cu levels were determined by inductively-coupled plasma optical emission spectroscopy (ICP-OES) situated at the Department of Earth and Planetary Science (McGill University, Montreal, Canada) on day 38, 101, and 164. The last surviving population was sampled for Cu concentration analysis on day 227.

To ensure the maintenance of diversity in the MTC populations during key stages of the experiment (day 27, before the copper was applied and day 74, when the Cu concentration of 178µg/L was reached), one individual of each of the 36 clonal lines (36 individuals) was added to each of the MTC tanks. For consistency in density replenishment, thirty-six individuals (clones) were also added to their respective MNC population.

2.3.3 Microsatellite analysis

Before the start of the experiment, tissue from each clonal line included in the experiment was collected. During the experiment, all *Daphnia* populations were sampled on day 24 (100 individuals per tank) and, then, on day 47 and 94 (50 individuals per tank). A final sample of 100 individuals was collected from control populations at the end of the experiment (day 227). *Daphnia* individuals were always collected in aliquots of 10 individuals ("replicated samples") and stored immediately at -80 °C in a 1.5mL Eppendorf tube. Genotyping was conducted at 10 previously mapped microsatellite loci (Table B.1) in order to determine allelic richness in the populations and its change over time using the protocol described by Cristescu *et al.* (2006). Multiplexed samples were genotyped using an ABI 3730XL Analyzer and chromatographs were evaluated using GeneMapper Software v3.0 (Applied Biosystems). The microsatellite analysis was carried out using a pooling approach assuming that the detected pattern of fluorescence peaks reflected the composite pattern of the individual alleles (Eschbach & Schöning 2013). Alleles were binned for manual allele identification based on the fragment sizes of the individually amplified clonal lines and were considered present if a peak was detected within their fragment bin (regardless of their fluorescent peak intensity relative to the LIZ

size standard). The average number of alleles for the 10 microsatellite markers, across the 36 clonal lines was 15.5 (SD = 1.87), with an average of 14.2 (SD = 1.0) in lake clonal lines and an average of 16.7 in pond populations (SD = 1.7; Table B.2). The total number of unique alleles was 24, two of which were part of lake populations while the rest belonged to pond populations. However, lake populations could be distinguished from pond populations by diagnostic alleles, unique to either lake or pond habitats as whole. The three pond habitats could also be distinguished from one another thanks to presence of unique alleles while the three lake habitats shared most of the alleles and could not be distinguished from one another (Table B.3).

The probability of presence for each clonal line at each time point was calculated by counting the number of alleles detected for each of the ten microsatellite markers. For a marker to be considered positive for the presence of a particular clonal line its diagnostic allele(s) had to be detected. The probability of presence of a clonal line was calculated by counting the number of markers that retrieved the genotype (both alleles) for each replicate sample and then averaged across the replicate samples. When data on a particular marker was missing, the probability of presence was calculated based on the number of markers that were successfully amplified. The probability of presence of each clonal line was represented by a value included between 0 (absent) and 100 (present). However, some clonal lines did not contain diagnostic alleles and could not be reliably distinguished in this process. Consequently, clonal lines that shared, for example, the same exact alleles (mainly lake clones) share also the same probability of presence. For a detailed description of the methods I refer to section "Microsatellite genotyping" of Appendix B.

2.3.4 Statistical analyses

Population growth rates were calculated as $\ln(N_{t+1}-N_t)$ where ln is the natural logarithm, N the estimated population size and t is time. To reduce the effects of the transient phase (initial population growth followed by a decline and a subsequent smaller peak), we only considered data collected after day 59. Mean growth rate and standard deviation (SD) were calculated for each treatment population and for each control group. Changes in

growth rate exceeding the SD of the overall control group were used to verify the presence (or attempts) of putative ER events by calculating the ratio between the peak value and the SD of the control group. The abundance of neonates estimated for each tank throughout the experiment was compared with growth rate estimates.

Time to extinction for each population was estimated and used as the response variable in a survival analysis using the KM (product-limit) method (Kaplan & Meier 1958) with the R package survival (Therneau 2015). Survival curves between monoclonal and high diversity populations were compared with a log-rank test. We also tested whether there was any difference in time to extinction in MNC treatments containing clonal isolates from lakes and ponds (*D. pulicaria* and *D. pulex* respectively) and thus replaced tank ID with habitat type. The same dataset was analyzed with a generalized linear mixed model (GLMM), family Poisson, with the package lme4 (Bates *et al.* 2014). The candidate models included time to extinction as a response variable, categorical fixed factor of genetic diversity (MTC and MNC) or population (mixed vs. *D. pulicaria* and *D. pulex*) or habitat ID (mixed vs. individual habitats). The best-fit model was selected through the Akaike information criterion (AIC; Burnham KP 2002). We then tested whether the treatment group replicates (high diversity and monoclonal) showed any within-group difference with a generalized linear model with tank number as a factor followed by a Tukey's range test with R package Multcomp (Hothorn *et al.* 2008). MTC populations and MNC populations were also analyzed separately, with a generalized linear model including only the factor tank ID and then we tested the results with a Least Square Means analysis.

Trends in allelic richness were analysed with a liner mixed-effect model in which population ID and population size (z-transformed) were the random effects and treatment (categorical) and time were the fixed effects. Allelic richness was z-transformed before the analysis. We tested few combinations of models by removing factors or including interaction factors between the fixed and random terms. We, then, ranked the models based on the AIC selection criterion and then focused on the best-fit model. For the first time point (day 24) only, when Cu was not yet introduced, we tested whether populations differed in allelic richness with a linear model and a Least-Square Means analysis with

the R package difflsmeans (Tukey adjustment; Kuznetsova *et al.* 2017). All analyses were done in in Rstudio version 3.6.1. (R Team 2019).

2.4 Results

2.4.1 Population growth dynamic

We did not observe any successful ER event. All populations experienced an initial rapid growth which culminated with a total abundance of 1300-2000 individuals, between day 17 and day 23 (Transient phase; Fig. 2.1) followed by cyclical dynamics. Control and treatment tanks followed different population dynamics starting from day 85. Both MTC and MNC controls continued to show cyclical dynamics while all treatment populations, both MTC and MNC populations, started to decline at a Cu concentration of 182 µg/L. Eventually, all populations went extinct. On day 103 the first extinction was observed in a monoclonal (Center Pond) population. This first extinction was soon followed by a series of other extinctions of monoclonal populations (Bridge Pond on day 139, Clear Lake on day 142, Long Lake on day 166). Multiclonal populations started to go extinct from day 173. The experiment finished on day 227 (~ 15 generations of *Daphnia*) when the last treatment population (multiclonal) went extinct.

However, from day 59, during the period of stable fluctuations in the controls, some treatment populations showed U-shaped patterns of growth that could be considered as putative ER attempts (Fig. 2.2, Fig. B.4, Table B.4 a,b). The highest population peaks (in relation to the control group SD) were all observed when the expected Cu concentration was at its highest value (186 µg/L). The highest peak was observed in the MTC population t2 at day 124 (6.19 times the control SD), the second peak in size was observed at day 149 in the Dump Pond population (5.28 times the control SD) followed by MTC t3 (4.75 times the control SD) at day 132 and Long Lake population (4.75 times the control SD) at day 132. MTC populations showed a higher number of total positive population peaks that exceeded SD of the control group (28 in MTC population vs. 15 in MNC populations). Moreover, MTC showed 11 positive peaks that were double the control SD against the 7 observed in the MNC population (Fig. 2.2). No putative ER attempts were observed in Clear Lake, Bridge Pond and Center Pond populations. By

inspecting the abundance of neonates throughout the experiment, we observed a fairly uniform distribution of neonates in the control populations (with an exception between day 160 and day 200 where they displayed a prolonged peak in abundance; Fig. B.5). In the treatment populations, neonates were detected until day 145 and then their abundance dramatically diminished.

2.4.2 Survival analysis

Survival analysis revealed a statistically significant difference between the time to extinction of MNC and MTC treatments (Fig. 2.3; $p = 0.025$) confirming that MTC treatments had a higher probability of survival. Multiclonal populations showed a median persistence time of 186 days compared to 154 days for monoclonal populations. The best-fit generalized linear mixed model (AIC = 117.5; Table 2.2) contained the fixed factor of diversity (multiclonal 5.24, SE = 0.06; monoclonal: 5.02, SE = 0.06) and tank number as a random effect (variance 0.01; standard deviation 0.12). Tukey's range test showed a statistically significant difference between MTC populations and MNC populations (estimate = -0.23, SE = 0.08, $p = 0.007$, Holm adjustment). We found no statistically significant difference among time to extinction in replicates of the MTC treatment group. Monoclonal populations showed some statistical differences and in particular the Center Pond population (the first population that went extinct) differed from Long Lake ($p = 0.002$), Sportsman Lake ($p = 0.0002$) and Dump Pond ($p = 0.00002$).

2.4.3 Microsatellite analysis

A total of 10 microsatellite loci were analysed to track genetic variation (allelic richness) throughout the experiment and to examine specific clonal composition. Populations originally collected from ponds (*D. pulex*) showed higher allelic richness (higher number of unique alleles across the 10 microsatellite markers) than populations sampled from lakes (*D. pulicaria*; Table B.3). The initial total allelic richness, across all clonal lines, was 83 alleles. By the first sampling date (day 24, before Cu application) the MTC control populations lost an average 29% of alleles across the 10 loci while the MTC treatment populations lost 33% (Fig. 2.4). Allelic richness continued to drop (despite the re-introduction of all clonal lines) so that by day 47 it had declined by 50% in MTC

control populations and 58% in the MTC treatment populations. By day 94 the controls had lost 58% of alleles while the treatments 65%. By the end of the experiment the MTC controls had lost 77% of alleles. A mixed model showed that the effect size of MTC control was slightly larger than MTC treatments (Table 2.2).

From an assessment of the microsatellite allele dynamic of MTC populations, it appeared that clonal lines collected from ponds (*D. pulex*) declined faster in comparison to those collected from lakes (*D. pulicaria*) and by the end of the experiment, only *D. pulicaria* lines were detected. This observation was confirmed by the best-fit mixed model which consisted of an interaction effect between the treatment effect (control vs. treatment) and time and, as random terms, tank ID and an interaction term between time and population size (Table 2.2). Before Cu application, there was no statistically significant difference between the allelic richness of control and treatment populations (Least-Square Means analysis, $p = 0.14$) while after Cu application and during the whole experiment the loss of alleles was not equal in controls and treatments ($p = 0.000002$).

D. pulicaria lines (originating from lakes) appeared to be more persistent and dominant than *D. pulex* lines (originating from ponds) in MTC populations (Fig. 2.5). *D. pulex* lines were absent or rare, regardless of their extinction time.

2.4.4 pH and Cu concentrations

During the entire course of the experiment, pH values ranged from 5.8 to 6.7 (Table B.5). Cu concentrations ranged from 164.1 to 176.5 µg/L on day 38 (expected 160 µg/L); from 157.4 to 162.4 µg/L on day 101 (expected 186 µg/L); from 193.5 to 197.8 µg/L on day 164 (expected 186 µg/L). The last surviving population was exposed to a Cu concentration of 168 µg/L, rather than the expected 186 µg/L for this stage of the experiment. However, we also estimated copper concentrations using a unique ICP-OES calibration curve obtained by averaging absorbance values of the calibration curves created at each sampling time point. In this last case, copper concentration estimates were closer to those expected (Table B.6).

2.5 Discussion

The main goal of this study was to test whether populations of *Daphnia* consisting of different clonal lineages would show higher probability of ER and longer population persistence than monoclonal populations during sustained exposure to copper stress. Although genetic diversity conferred an advantage for population persistence, no population recovered completely during the experiment. Genetic diversity decreased over time with *Daphnia pulicaria* replacing *Daphnia pulex* lineages and becaming dominant. The overall genetic decay was faster in the treatment populations.

2.5.1 The limits of evolutionary rescue

The population decline phase was characterized by abrupt increases in population growth rates that could be interpreted as failed ER attempts. The most evident ER attempts occurred within some of the most persistent MTC and MNC populations: MTC t3 (4.75 times the control SD; extinction at day 226), Dump pond (5.28x, day 187) and Long lake (4.75x, day 166) populations (Fig. 2.2). The most persistent MTC population (t3) showed the strongest putative ER attempt just 18 days prior to extinction and Dump population showed three attempts in a row between 38 and 21 days before extinction. Moreover, these two populations contained neonates during the putative ER attempts and toward the end of the experiment, indicative of successful reproduction (Fig. B.5). The failure of these bouts of population growth to sustain a full ER may be due to ecological and/or genetic constraint. Previous studies have found that, despite the presence of high variability in intrinsic rates of increase, competitive abilities, and lifespans during acute exposure to contaminants, *Daphnia* clones appear to have a consistent negative response to chronic intense environmental stress. This has been observed both in *D. pulex* in response to temperature stress (Loaring & Hebert 1981) and heavy metals (Yan *et al.* 1996, Yan *et al.* 2004), and *D. magna* in response to cadmium, 3,4-dichloroaniline (Baird *et al.* 1990), and food deficiency (Robinson *et al.* 2013). In this experiment, we were able to reach a copper concentration of 186 μg/L without causing immediate population extirpation. However, it is likely that the rate of population increase, which is considered sensitive to toxic substances within cladocerans (Sarma & Nandini 2006), was seriously impacted and that demographic stochasticity played a great role in the extirpation of experimental populations.

Demographic stochasticity can trigger population extirpation even under pristine conditions (MacArthur &Wilson 2001). According to the evolutionary rescue theory, demographic stochasticity is one of the main limits to successful ER events (Haldane 1957; Bell 2013). In this experiment, *Daphnia* populations were kept in 9-L medium. This restricted environment likely affected carrying capacity and demographic dynamics. Habitat size has been found to significantly impact the genetic structure of *Daphnia* populations by reducing clonal diversity and consequently leading to an increased genetic drift (Vanoverbeke *et al.* 2007). Another common limit to ER is genostasis or the lack of appropriate genetic variation. Despite the presence of neutral genetic variation in the MTC populations, there may have not be enough genetic variance underlying the absolute fitness to ensure a sustainable positive growth rate of the most fit genotypes (Bell 2013).

Another factor that may have played a role in the failed ER events is the lack of a pre-exposure to Cu stress prior to the experiment. *Daphnia* species are known to acclimate to their environment and it has been observed that certain populations can persist in metal contaminated habitats (Muyssen *et al.* 2002; Lopes *et al.* 2006; Agra *et al.* 2010; Saro *et al.* 2012). Pre-exposure provided a survival advantage even in laboratory populations (Lopes *et al.* 2006; Agra *et al.* 2010). In this study, *Daphnia* populations were not exposed to metal stress before the experiment and, despite copper concentration was increased gradually during the experiment, this lack of pre-exposure could have represented a disadvantage for the long-term persistence in a highly contaminated environment.

Moreover, differences in the toxicity of Cu between adults and early life-stages might have contributed to the failure of ER. During the assessment of population sizes in treatment populations, the presence of neonates was observed even during the last stages of population decline preceding extinction (Fig. B.5). However, early life-stages could not survive for more than a few days and were not detected during the following population count. These observations suggested that Cu was more detrimental for neonates and juveniles than for adults. In their review of ecotoxicological studies on Cladocerans, Sarma & Nandini (2006) highlighted similar findings where neonates

appeared to be, in some cases, twice as sensitive as adults. The same pattern has been seen in other crustaceans by Medina *et al.* (2009) that studied the responses (survival, development, and fecundity) of populations of the copepod *Tigriopus angulatus* to Cu and found juveniles to be the most sensitive life stage.

2.5.2 Genetic diversity promoted longer persistence

Despite the lack of successful ER events, MTC populations showed longer persistence than MNC populations. A positive effect of genetic diversity on persistence could be due to selection effects resulting from a higher probability for MTC populations to contain lines with some advantageous traits for surviving in the contaminated environment (Fisher 1930). A positive effect of genetic diversity on the ability of aquatic populations to resist disturbances has been previously observed in bacteria (Boles *et al.* 2004), plants (Schmitt & Antonovics 1986; Peacock *et al.* 2001; Hughes & Stachowicz 2004; Reusch *et al.* 2005), invertebrates (Jones *et al.* 2004) and vertebrates (Pearman & Garner 2005). There is also evidence that multiclonal populations are expected to reach higher densities compared to monoclonal populations (Vrijenhoek 1979; Vrijenhoek 1984). In our experiment, clonal competition and the subsequent clonal selection (resulting from differences in growth rates and time of brood production) might have led to the dominance of genotypes that conferred an increased overall population fitness compared to monoclonal populations.

2.5.3 Clonal responses to long-term exposure to Cu

We observed a consistent decrease in allelic richness and consequently in clonal diversity both in treatment and control MTC populations. Clonal diversity in *Daphnia* populations is known to be usually maintained by temporal and/or spatial heterogeneity in natural habitats (Loaring & Hebert 1981). The concomitant presence of *D. pulex* and *D. pulicaria* in the same environment (contaminated or not) may have led to the disappearance of certain genotypes due to competition. Moreover, it is possible that our experimental conditions may have favoured certain genotypes over others. However, a linear mixed model revealed that the treatment populations lost alleles at a faster rate compared to control populations. This faster loss can be explained by both the contemporary reduction

of population size due to Cu toxic effect and the consequent demographic stochasticity and a selection effect exerted by Cu contamination. Clonal erosion (loss of genetic diversity) has been previously observed in parthenogenetic zooplankton species both in pristine environments (Loaring & Hebert 1981; De Meester *et al.* 2006; Vanoverbeke *et al.* 2007; Vanoverbeke & De Meester 2010), and in heavy metal contaminated environments (Lopes *et al.* 2004; Ward & Robinson 2005; Coors *et al.* 2009; Agra *et al.* 2010; Agra *et al.* 2011; Ribeiro *et al.* 2012). In our experiment, because treatment populations were lastly assessed on day 94 while control populations were also assessed at the end of the experiment (day 226), we are missing information about which clonal lines persisted longer in the MTC treatment populations and this makes it harder to determine whether there was a shift in clonal selection due to Cu stress. Assuming that there was a common pattern of clonal selection both in control and treatment populations (in which *Daphnia pulicaria* became dominant since day 94, Fig. 2.5), then, the observed genetic erosion may be due predominantly to demographic stochasticity.

The overall predominance of *D. pulicaria* clonal lines could be linked to life-history traits differences between this species and *D. pulex. D. pulex* is a common inhabitant of temporary spring ponds that owes its seasonal colonization to the resting egg bank. *D. pulicaria*, on the other hand, inhabits permanent lakes and is characterized by low clonal selection (Thielsch *et al.* 2009). These different population dynamics could have been the cause of the common pattern of *D. pulicaria*'s predominance.

The two re-introductions of all clonal lines may have played a stabilizing effect on both MTC and MNC populations due to the subsequent increase in population size. Moreover, in the MTC populations, it ensured that all clonal lines were present and able to respond during copper stress. However, environmental conditions and repeated competition patterns might have led to a similar clonal distribution compared to the distribution prior to re-introductions of the clonal lines (Fig. 2.5). Loaring & Hebert (1981) found that even a small fitness advantage would soon lead to the exclusion of all but one/few clonal lines in populations of *D. pulex.*

2.5.4 Future directions

This experiment revealed how *Daphnia* populations reproducing asexually and containing naturally occurring genetic variation respond to exposure to copper contamination. The consistent removal of resting eggs minimized the occurrence of sexual reproduction and allowed us to track the fate of each genotype. However, sexual reproduction, through recombination, could have increased the likelihood of adaptation and ER (Felsenstein 1974; Waxman & Peck 1999). Moreover, including a pre-exposure factor, which is a determinant factor for the success of ER events (Carlson *et al.* 2014), could have resulted in a different outcome. It is likely that a slightly lower copper concentration than 186 µg/L would lower the cost of selection which, in the long run, would delay or, perhaps, avoid extirpation in certain populations. It is also possible that, by increasing the scale of this experiment (microcosm size and the heterogeneity of experimental conditions), genetic erosion and demographic stochasticity could have been reduced perhaps leading to a different final outcome. Lastly, the design of a similar experiment in which MTC populations contain the exact clonal lines consisting the MNC clonal populations (e.g. MTC populations consisting of six clonal lines and the six MNC clonal populations) would allow us to test for the ecological effect of genetic diversity on persistence and its correlation with other ecological variables (Hughes *et al.* 2008).

2.6 Conclusion

This experiment demonstrated that clonal diversity can extend the persistence of *Daphnia* populations in metal polluted environments but is not sufficient to facilitate successful ER. The genetic diversity of *Daphnia* populations decreased faster under Cu contamination and this greatly compromised overall population fitness (Medina *et al.* 2007; Ribeiro *et al.* 2012). Finally, differences in sensitivity to Cu contamination among life stages likely played an important role in the dynamics of *Daphnia* populations in this experiment. More studies of population fitness in the context of contamination are needed to deepen our understanding of the erosion of population fitness despite the presence of tolerant phenotypes (Postma & Davids 1995; Haimi *et al.* 2006; Medina *et al.* 2009; Anderson *et al.* 2013; Dutilleul *et al.* 2014; Loria *et al.* 2019).

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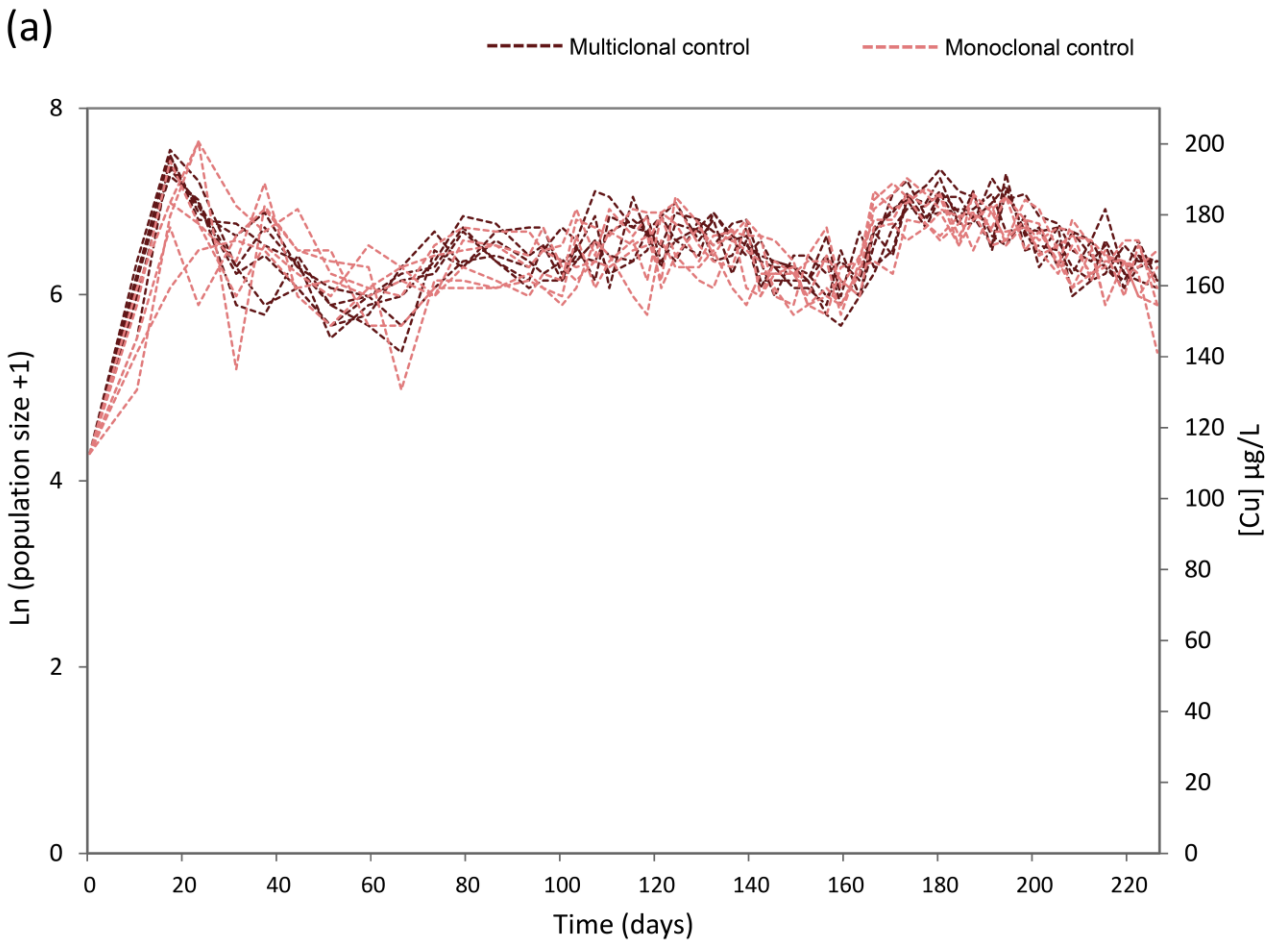
Table 2.1. Summary of the experimental design including the diversity treatment groups, their composition, the level of replication and the total number of samples (tanks). Each diversity group (high diversity and monoclonal populations) consisted of 6 control replicates and 6 treatment replicates for a total of 24 tanks. Clone ID in bold represent the clones used to create the monoclonal group populations.

		# Haplotypes	Haplotypes	# Replicates	# tanks
Multiclonal	Lake Long	6	LL1, LL3, LL10, LL11, LL13, **LL16**	6	12
	Lake Clear	6	CL3, CL4, CL11, CL12, **CL13**, CL28		
	Lake Sportsman	6	**SP8**, SP12, SP14, SP18, SP19, SP21		
	Pond Center	6	CT10, CT11, CT16, CT18, **CT24**, CT26		
	Pond Dump	6	DP1, DP6, DP8, **DP9**, DP21, DP25		
	Pond Bridge	6	**BN2**, BN9, BN11, BN13, BN20, BN27		
Monoclonal	Lake Long	1	LL16	1	12
	Lake Clear	1	CL13	1	
	Lake Sportsman	1	SP8	1	
	Pond Center	1	CT24	1	
	Pond Dump	1	DP9	1	
	Pond Bridge	1	BN2	1	

Table 2.2. Model-averaged coefficients from AICc-best models for time to extinction (generalized mixed linear model) and allelic richness (linear mixed model). The symbol * represents p value 0.01-0.05; ** represents p value 0.001-0.01; *** p value represents 0-0.001.

Time to extinction (glmm, poisson function)			**Allelic richness** (lmm)		
Fixed terms	**Estimate**	**SE**	**Fixed terms**	**Estimate**	**SE**
diversity group			*treatment group*		
multiclonal	5.24	0.06	control	0.39***	0.09
monoclonal	5.02	0.06	treatment	0.36***	0.09
			time	-0.004***	0.0005
			treatment x time	-0.004**	0.001
Random terms	**Variance**	**St.dev**	**Random terms**	**Variance**	**St.dev**
tank ID	0.02	0.12	tank ID	0	0
			population size	0.14	0.37
			residual	0.01	0.12
Least Square Means	**Estimate**	**SE**	**Least Square Means**	**Estimate**	**SE**
multiclonal vs. monoclonal	0.2*	0.08	control vs. treatment	0.31***	0.063

Figure 2.1. Population dynamics during the experiment in **(a)** controls and **(b)** treatments. In (b) the expected copper concentrations and the ICP-OES measures (averages and standard errors) are also shown. The latter were calculated according to the pooled calibration curve.



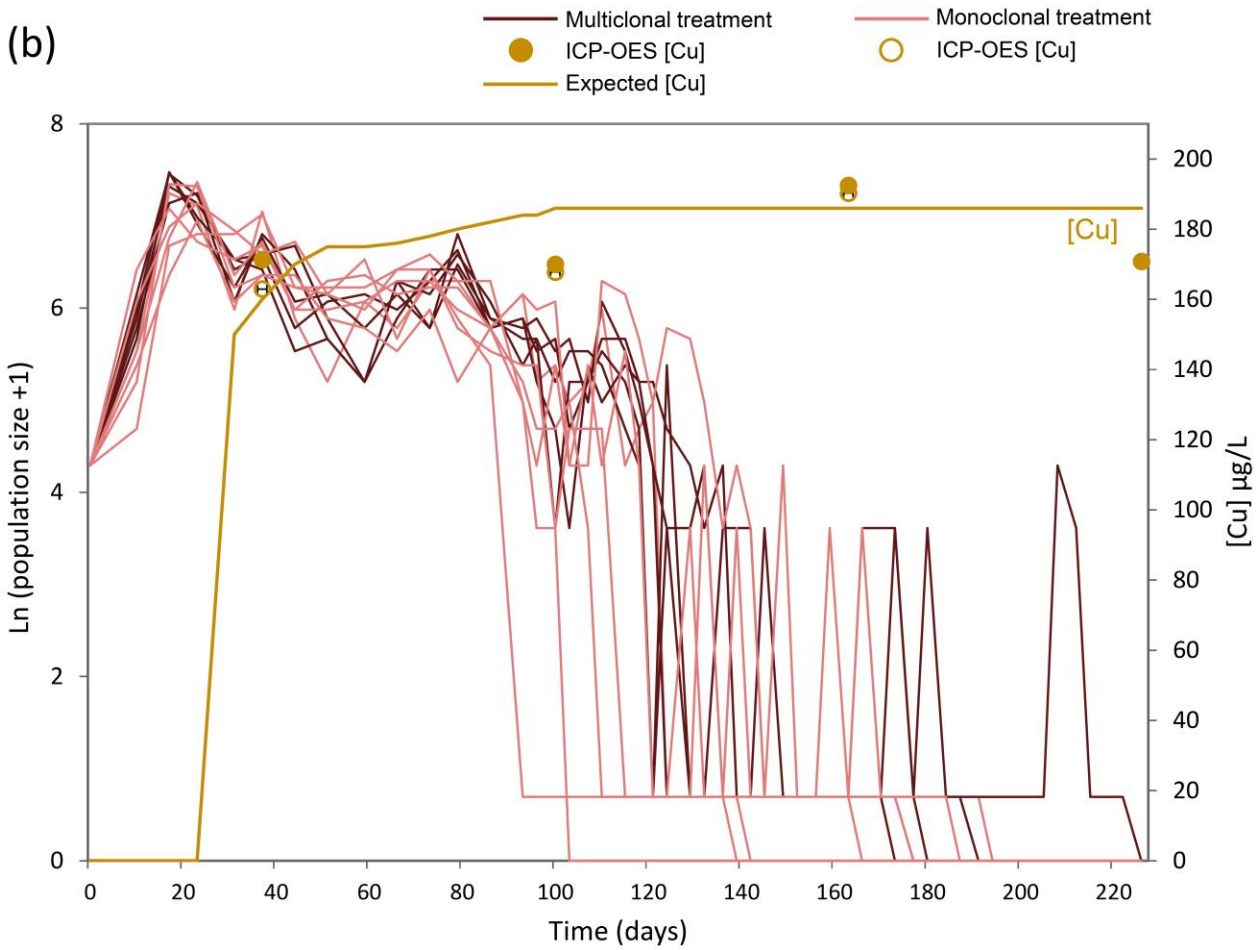
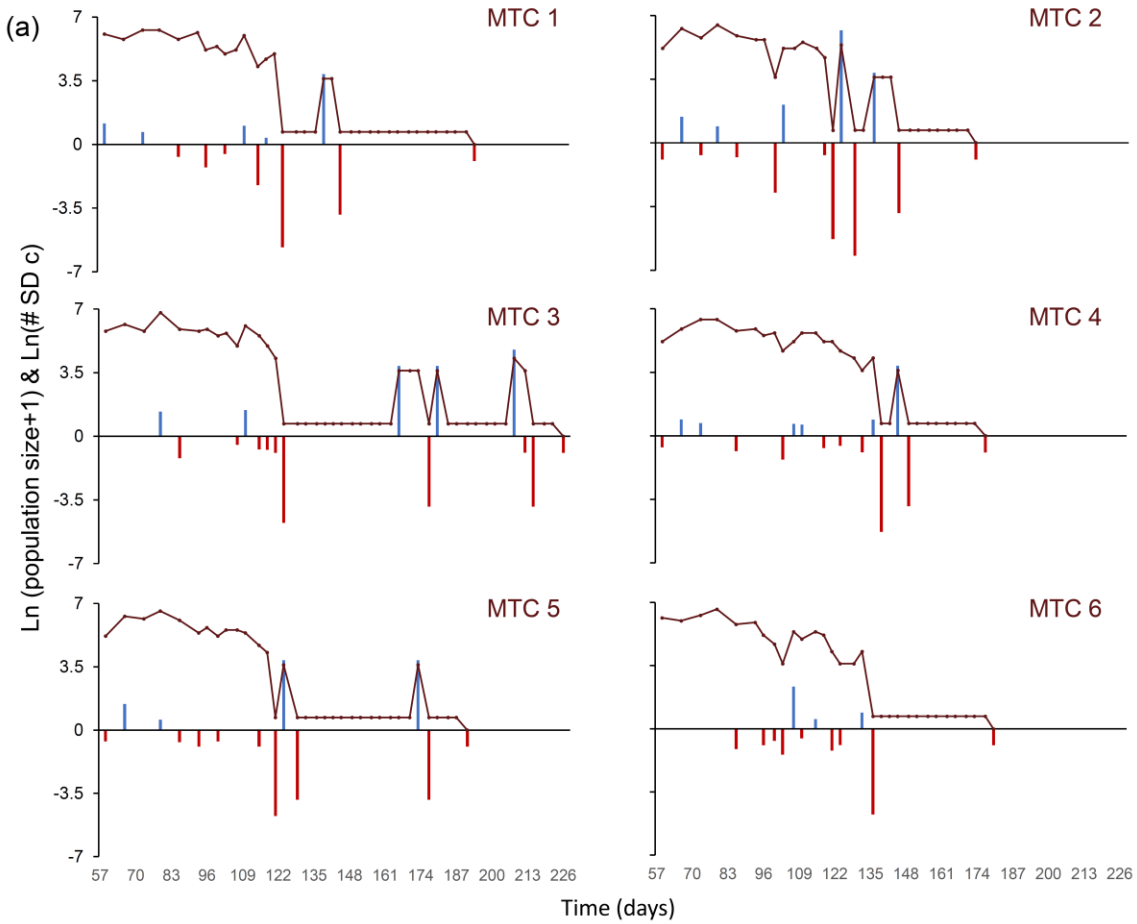

(b)
Multiclonal treatment
ICP-OES [Cu]
Expected [Cu]
Monoclonal treatment
ICP-OES [Cu]
[Cu]
Ln (population size +1)
[Cu] µg/l
Time (days)
0
2
4
6
8
0
20
40
60
80
100
120
140
160
180
200
0
20
40
60
80
100
120
140
160
180
200
220

Figure 2.2. Failed attempts of evolutionary rescue in **(a)** MTC populations and **(b)** MNC populations. Bars represent population sizes expressed as the ratio between the natural logarithm (Ln) of population growth rate (N_{t+1}-N_t) over the standard deviation of the overall control group. Lines represent population growth trends (as Ln).



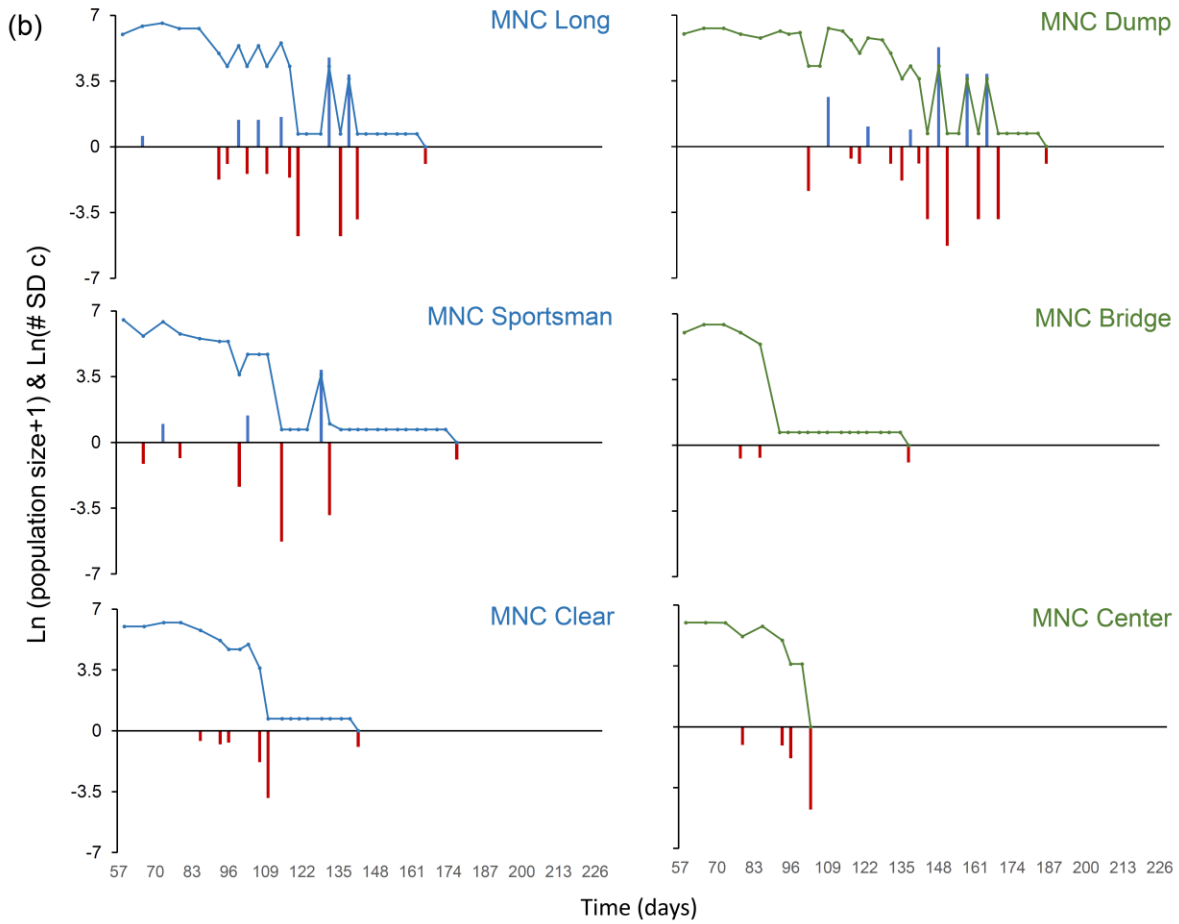

(b)
MNC Long
MNC Dump
MNC Sportsman
MNC Bridge
MNC Clear
MNC Center
7
3.5
0
-3.5
-7
Ln (population size+1) & Ln(# SD c)
57 70 83 96 109 122 135 148 161 174 187 200 213 226
Time (days)

Figure 2.3. Survival curves of the high diversity and monoclonal populations. The test of significance was calculated with a log-rank test. The risk table represents the number of populations that were still alive and whose assessment extended to that day in the experiment.

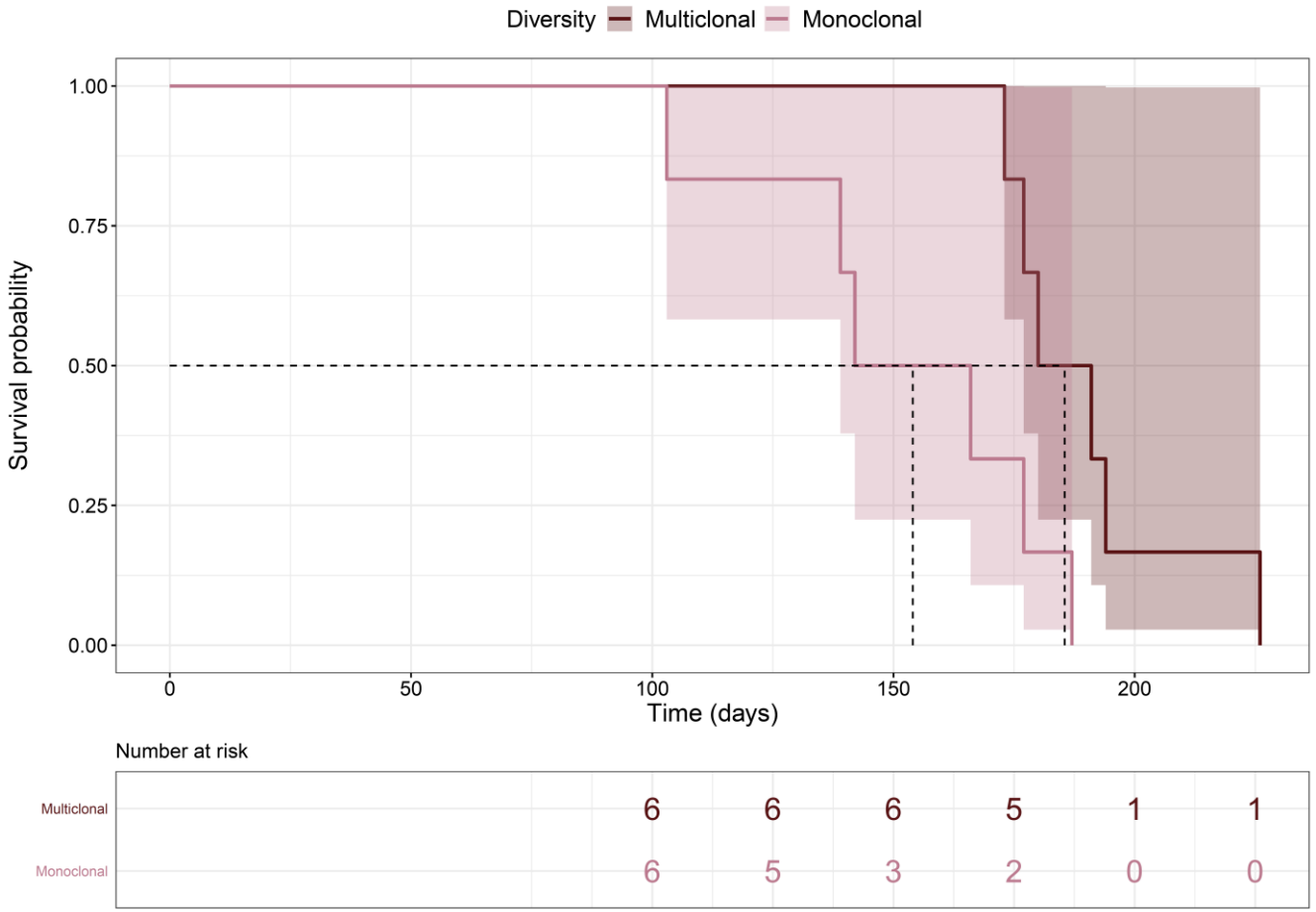


Figure 2.4. Allelic richness declines for the multiclonal treatment and control groups. The natural logarithm (+1) of the mean population size for both groups and expected copper concentration are also shown.

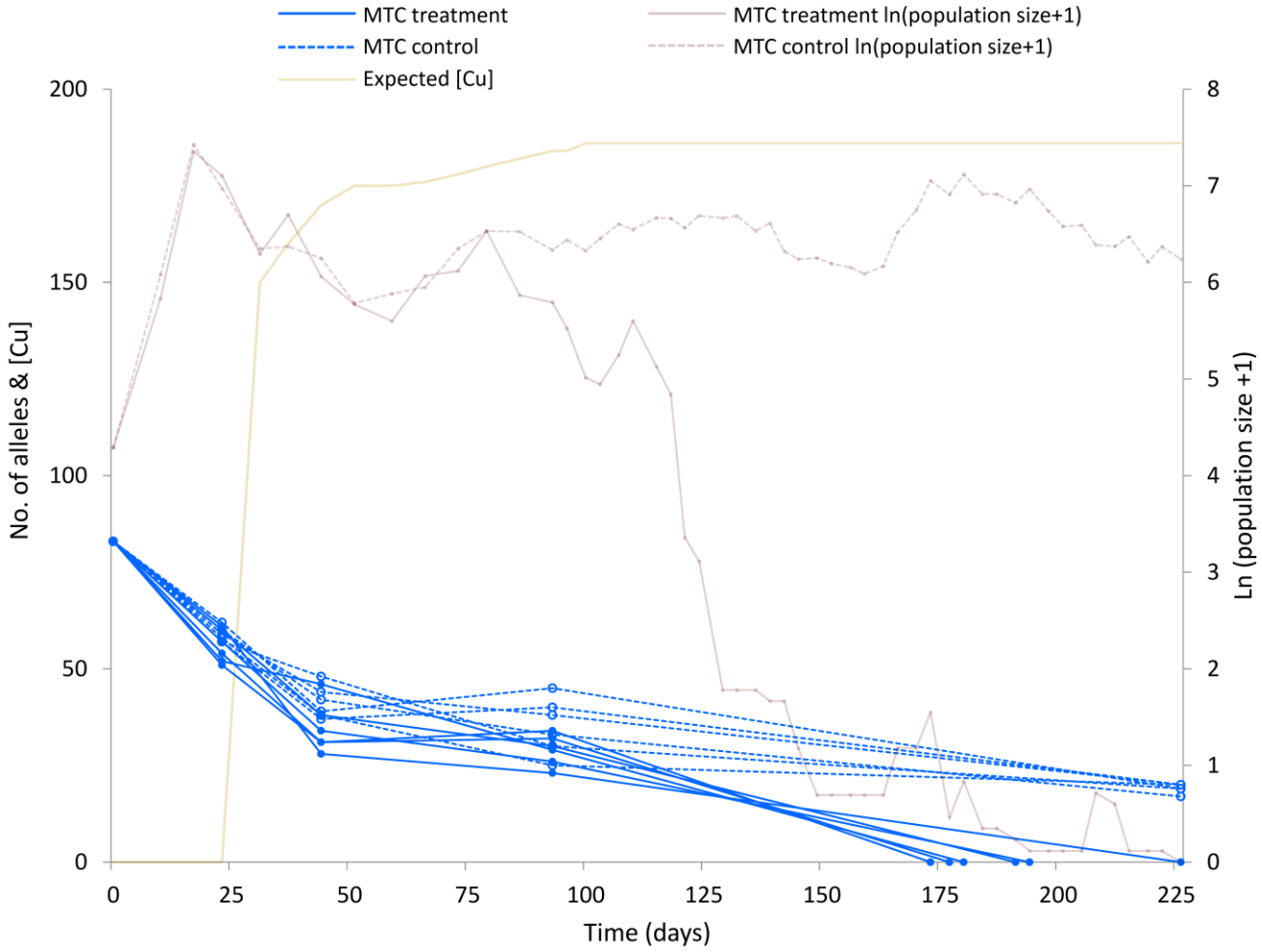
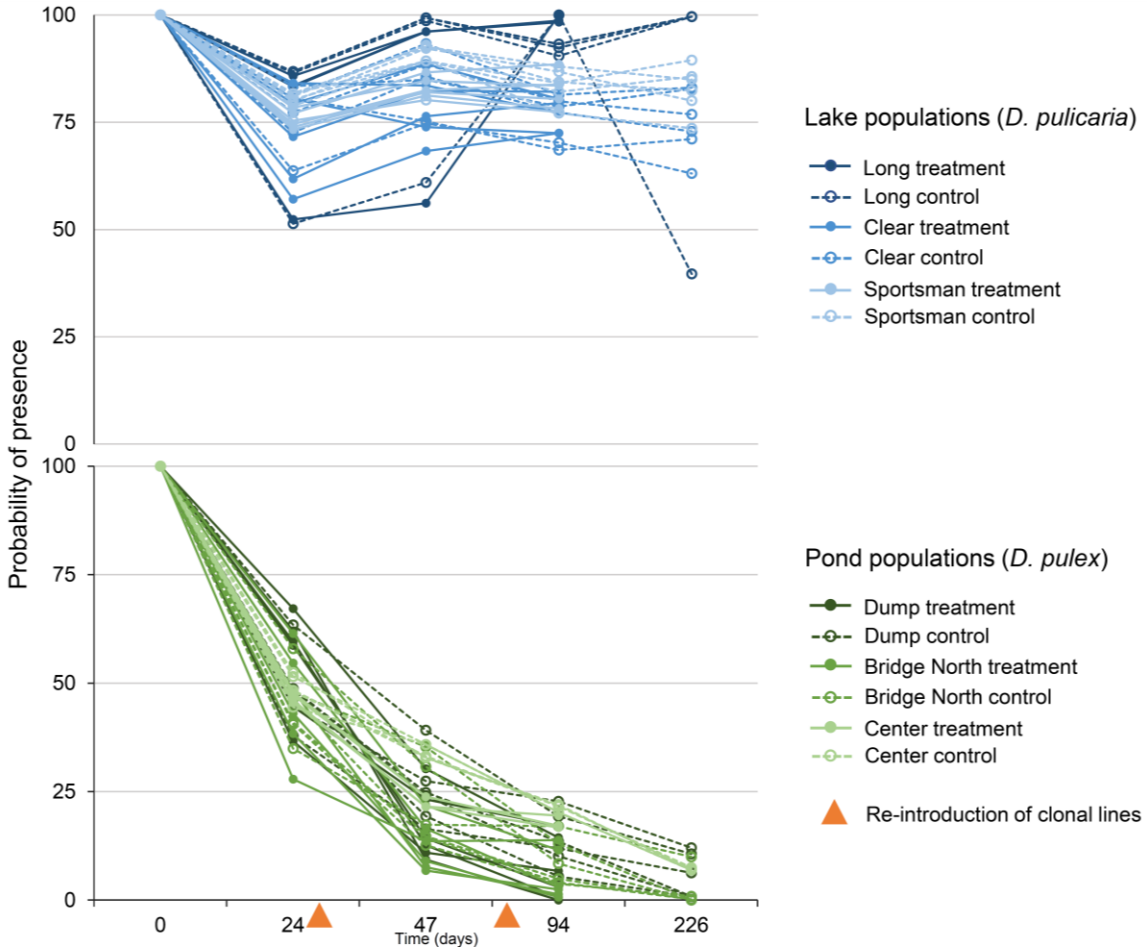


Figure 2.5. Averaged probabilities for the presence of lake (*D. pulicaria*) and pond (*D. pulex*) populations. A value of 100 or 0 represent presence and absence, respectively. From 1 to 25 likely absent, from 26 to 50 unlikely present, from 51 to 74 possibly present, and from 75 to 99 likely present.



Connecting statement

In Chapter 2, I conducted a bioassay using *Daphnia* populations to test whether high levels of intraspecific genetic variation enhance population stability, persistence and the probability of ER events in a copper-contaminated environment. As predicted, intraspecific genetic variation provided higher probability of survival in *Daphnia* populations, some of which persisted for ~15 generations. However, the detrimental effect of copper manifested as a higher mortality at the juvenile stages, and as an overall genetic erosion. No population experienced a complete rescue.

In natural environments, intra-specific genetic erosion and population extirpation generally occur within complex communities and are not the only effects that can be observed in an ecosystem undergoing a pollution event. Extirpated populations may be replaced by less sensitive ones, species interaction may be altered, and other species may persist through acclimation and/or adaption (Medina *et al.* 2007). Scientists have to deal with intricate eco-evolutionary feedbacks at the community and ecosystem level, which are still largely unknown (Loeuille 2019).

A key aspect in evolutionary and ecological studies is the ability to easily identify species, and track changes in the dynamic of community tracking its richness and abundance, as well as intraspecific genetic variation. In Chapter 3, I test the feasibility of eDNA-based metabarcoding to efficiently assess rapid biodiversity changes following pulses of glyphosate, a commonly used herbicide found to exert a double effect, toxic and fertilizing, on non-target aquatic organisms. Besides comparing findings with traditional monitoring methods, I also provide additional information on the effects of contamination to the intra-specific genetic variation of species with a key ecological role in aquatic environments.

By testing a new and innovative approach for estimating biodiversity and tracking its changes, Chapter 3 contributes to the implementation of an integrative approach to study the eco-evolutionary consequences of pollution at multiple levels of biological organization, in the context of natural environments.

Literature cited

Loeuille, N. (2019). Eco-evolutionary dynamics in a disturbed world: implications for the maintenance of ecological networks. *F1000Research*, 8.

Medina, M.H., Correa, J.A. & Barata, C. (2007). Micro-evolution due to pollution: possible consequences for ecosystem responses to toxic stress. *Chemosphere*, 67, 2105-2114.

Chapter 3

The performance of eDNA-based metabarcoding in estimating rapid zooplankton diversity changes during herbicide contamination

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3.1 Abstract

Evaluating the effects of human-induced environmental stressors on population and community persistence requires fast and accurate biodiversity estimates. The current genomic revolution offers a variety of candidate methods that have the potential to replace time-consuming morphological assessments. Metabarcoding based on environmental DNA (eDNA) is rapidly emerging as a candidate tool for quick biodiversity assessments but there is a scarcity of data on its performance when species turnover is expected to be fast. Here, we test the feasibility of using eDNA-based metabarcoding to assess rapid biodiversity changes following strong pulses of environmental stressors. We carried out a mesocosm experiment consisting of two types of nutrient environments (mesotrophic and eutrophic) and pulses of the herbicide glyphosate (Roundup). We first compare estimates of zooplankton diversity and taxonomic turnover at the family level obtained with metabarcoding and morphological assessments. Then, we test whether differences in species detections obtained with the two methods affect their correlation with glyphosate concentration, time, and phytoplankton density. Finally, for a target number of species, we assess the change in intraspecific diversity in response to glyphosate pulses. Compared to morphological assessment data, metabarcoding showed an average detection coverage of 38% of zooplankton families throughout the experiment. Metabarcoding detected several families of aquatic insects (likely aquatic larvae) that were not sampled for morphological assessments. Both metabarcoding and morphological approaches show that glyphosate has a strong effect on overall zooplankton diversity. Intraspecific genetic variation is positively correlated with estimates of abundance and is negatively affected by glyphosate, particularly after the severe pulse. Metabarcoding techniques can efficiently detect biodiversity and track its change. Presently, their potential pairing with morphological assessments could provide useful and complementary data on difficult-to-detect organisms and the dynamics of intraspecific genetic variation in the context of anthropogenic environmental stressors.

3.2 Introduction

The implementation of large-scale conservation programs aiming to reduce current extinctions relies on long-term ecological assessments and biodiversity surveys that allow the identification of species and provide reliable estimates of spatio-temporal biodiversity change (Larigauderie *et al.* 2012; Thomsen & Willerslev 2015). Traditionally, biodiversity data has been acquired by morphological characterization of organisms. However, surveys can be time-consuming, labor-intensive, expensive, and inaccurate, particularly when identifying larvae or juvenile life stages of closely related species, cryptic species (Thomsen & Willerslev 2015) or when detecting species with very low abundance (Darling & Mahon 2011) or elusive behavior.

Morphological assessments of organisms do not provide any information on the levels of population genetic diversity. This is problematic given that, often, a reduction in the levels of genetic diversity or an alteration of genetic structure may indicate a recent bottleneck or impending population extirpation (Wiens 2016). Moreover, recent research has shown that intraspecific genetic diversity has also an ecological significance at community, and ecosystem levels – sometimes with effects as large as those of species and community diversity (Hughes *et al.* 2008). In this regard, extraordinary progress has been made due to the use of molecular methods of species identification, determination of genetic diversity and to the rapid advancements of sequencing technologies (Hoffmann & Willi 2008; Vacher *et al.* 2016; Bohan *et al.* 2017; Valdez-Moreno *et al.* 2019).

One of the most promising approaches for the identification of entire communities of organisms is metabarcoding which involves the combination of DNA-based identification techniques (through the use of mass-amplification of DNA barcodes) with high-throughput DNA sequencing. Metabarcoding is particularly promising when applied to environmental DNA (eDNA), the DNA molecules that are released by organisms into the environment. Monitoring biodiversity through eDNA-based metabarcoding is becoming increasingly common (Gibson *et al.* 2014; Barnes & Turner 2016; Carraro *et al.* 2018; Holman *et al.* 2018; Pont *et al.* 2018; Valdez-Moreno *et al.* 2019). Metabarcoding datasets were usually limited to the study of inter-specific trends in α and β diversities at the family and order levels due to the difficulty in distinguishing erroneous DNA

sequences produced during DNA amplification and sequencing from true diversity within groups of similar sequences (Turon *et al.* 2019). The development of new bioinformatics tools and algorithms (e.g., DADA2, UNOISE3; Callahan *et al.* 2016; Edgar 2016) is facilitating this distinction and the first empirical validations of the assessment of intraspecific genetic variation through eDNA-based-techniques are promising (Sigsgaard *et al.* 2017; Tsuji *et al.* 2018; Adams *et al.* 2019; Turon *et al.* 2019). However, metabarcoding cannot be applied to natural systems without strict measures for calibration due to high rates of false positives (taxa are detected but were not present) and false negatives (taxa are not detected but were present; Cowart *et al.* 2015).

To understand the prevalence and the origin of false negatives and false positives, there is need for empirical testing of the accuracy, reliability, and repeatability of such methods through careful comparison with data obtained through traditional methods (Cristescu 2014). Several studies have closely looked at differences in detection estimates of aquatic invertebrates obtained with metabarcoding and morphological assessments (Lobo *et al.* 2017; Yang *et al.* 2017; Cahill *et al.* 2018; Leasi *et al.* 2018; Serrana *et al.* 2019; Sun *et al.* 2019). Leasi *et al.* (2018), for example, compared how differences in detection of meiofaunal taxa obtained with metabarcoding and morphological assessments influenced the correlation of richness, species composition, and phylogenetic diversity with environmental parameters such as salinity, depth, grain size, etc. They found that diversity estimates were highly dependent on the method used and that different phyla were affected differently by this bias. Metabarcoding reported lower species richness compared to morphological taxonomy. Serrana *et al.* (2019) compared diversity metrics and their correlation with environmental variables (stream width and depth, dissolved oxygen, total nitrogen and total phosphorus) of stream macroinvertebrates obtained with metabarcoding and morphological assessments. They obtained higher diversity estimates using metabarcoding but β diversity estimates obtained with the two methods based on both incidence and abundance matrices were correlated.

eDNA-based metabarcoding techniques have also been applied and compared with morphological assessments in the context of anthropogenic stressors (Frontalini *et al.* 2018; Sun *et al.* 2019). Frontalini *et al.* (2018) looked at the effect of mercury (Hg)

pollution on benthic foraminifera community diversity estimates obtained with eDNA-based metabarcoding and morphological assessments during a mesocosm experiment. Both sets of diversity estimates were negatively influenced by Hg pollution. Moreover, metabarcoding detected foraminiferal taxonomic groups that are usually overlooked with traditional methods and among these groups they found potential bioindicators of Hg pollution. Sun *et al.* (2019) tested the effect of pond size and pollution level (mainly metals) on the structure of macroinvertebrate communities. In this case, metabarcoding reported higher species richness compared to morphological taxonomy. However, data obtained with morphological assessments showed a higher explained variation for differences among ponds, probably due to the provision of abundance data. In both of these cases, pollutants were either already in the environments and organisms were sampled from them (Sun *et al*. 2019) or they were applied in a certain concentration since the beginning of the experiment (Frontalini *et al*. 2018). There is a lack of studies testing the efficiency of e-DNA-based metabarcoding and its coupling with bioinformatics tools and algorithms in capturing rapid biodiversity changes (including intraspecific genetic diversity) in response to sudden environmental change within a short-time scales. Being able to assess both community composition and within-species genetic variation in contexts where aquatic populations and communities are threatened by sudden environmental change would represent a big step forward and an undeniably auspicious for biodiversity conservation efforts.

Chemical pollution is a constant threat for freshwater habitats and biodiversity. A prevalent contaminant of aquatic ecosystems is the organophosphorus herbicide glyphosate. In the last 30 years, its use has increased 100-fold due to the use of Roundup ready crops and the emergence of resistant weeds and the subsequent need of larger quantities (Myers *et al.* 2016). This led to the detection of glyphosate in natural environments (Anderson 2002, 2005; Byer *et al.* 2008; Struger *et al.* 2008) and to rising concerns about its toxicity to aquatic organisms (Relyea 2005; Annett *et al.* 2014). The toxic effect of glyphosate in non-target species is highly species-specific and differs depending on the surfactant portion of the formulation (Folmar *et al.* 1979; Tsui & Chu 2003; Relyea 2005; Annett *et al.* 2014). Phytoplankton species are more sensitive than fish and invertebrates (Ma *et al.* 2001; Ma *et al.* 2002; Tsui & Chu 2003; Saxton *et al.*

2011; CCME 2012). These differences in sensitivity along the food chain play an important role in the effects of the herbicide on different freshwater organisms because the direct toxicity of glyphosate could be worsened or reduced by predator-prey interactions (Fussmann & Gonzalez 2013; Osmond & de Mazancourt 2013). For example, phytoplankton-feeders could be impacted by both direct toxicity and lack of food. Furthermore, if their predators are resistant to the stressor, phytoplankton-feeders would also be negatively impacted by a stronger predation pressure. This scenario is further complicated by the fact that glyphosate has been found to be a relevant source of phosphorus when it degrades (Hébert *et al.* 2019). The double-sided effect of glyphosate: toxic in the short-term and fertilizing in the long-term increases the need for elucidating studies on the overall effects of this herbicide at the community and ecosystem level (Fugère *et al.* 2020). eDNA-based metabarcoding could be a valid tool to unravel these interlinked multi-level responses within freshwater communities and reveal underlying changes at the genetic structure. Its future application depends on our efforts to calibrate and validate its application.

In this study, we test the feasibility of using eDNA metabarcoding to assess rapid biodiversity changes following strong pulses of environmental stressors. The mesocosm experiment consisted of two phases. Phase I involved two pulses of the herbicide glyphosate and phase II consisted of a severe dose of glyphosate expected to be lethal to phytoplankton species (CCME 2012). In phase I, mesocosms were distinguished based on the concentration of ghyphosate that they reached through the pulses application (moderate and high doses; see below). We compare estimates of zooplankton diversity and taxa turnover at the family level obtained with metabarcoding and morphological assessments with a particular focus on diversity within rotifers, crustaceans, and insects. We also test whether zooplankton diversity estimates obtained with metabarcoding and zooplankton density estimates obtained with morphological assessments are correlated with each other and whether they show the same ecological signal in response glyphosate concentration and phytoplankton density estimates throughout the experiment as predictors (phase I and phase II). Testing for a correlation between estimates obtained with microscopy and estimates obtained with metabarcoding not only may show support for similarities between the two databases but may also contribute in informing us about

the possibility of using eDNA-based metabarcoding to quantitatively assess zooplankton in freshwater ecosystems. Finally, for a target number of species, we assess the response to glyphosate at the intraspecific level obtained with the metabarcoding approach during the experiment.

We test the hypothesis that metabarcoding and morphological approaches show a similar pattern of taxonomic turnover. Moreover, this similarity will also be reflected in the ecological signal in response to glyphosate stress during the experiment. Specifically, we expect glyphosate doses in phase I to exert an indirect effect on zooplankton diversity and abundance through changes in phytoplankton abundance and to exert a more toxic effect on the zooplankton community in its severe dose. Finally, we expect intraspecific genetic variation to be positively correlated to population size but negatively correlated to glyphosate in the phase II of the experiment.

3.3 Methods

3.3.1 Experimental design

The experiment was conducted in 2016 at the Large experimental array of ponds (LEAP), an aquatic mesocosm facility built at McGill University's Gault Nature Reserve (Mont-Saint-Hilaire, Québec, Canada) and designed to allow highly replicated experiments. Each tank (1136 L Rubbermaid plastic tanks) was filled with ~1000 liters of water and organisms from Lake Hertel (a glacially formed lake situated about one km upstream from LEAP). The experiment involved pulse contamination with the herbicide Roundup® Super Concentrate (a commercial glyphosate formulation, Monsanto, St-Louis, MO, USA), in three different concentrations: a moderate, a high and a severe dose. The experiment consisted of two phases in which a 2-level nutrient treatment (mesotrophic and eutrophic) was crossed with a 3-level glyphosate treatment (no glyphosate, moderate, high) in phase I, and with a two level glyphosate treatment (no glyphosate, severe) in phase II. The moderate (0.3 mg/L) and high (15 mg/L) doses of glyphosate were considered sublethal for phytoplankton while the severe dose (40 mg/L) was considered lethal (CCME 2012; Fig 3.1). The severe dose was applied to all mesocosms including one of the control replicates for each nutrient treatment which represented a "no-pre-

exposure treatment". The purpose of the 2-level nutrient treatments was to disentangle the fertilizing effect of glyphosate from its toxic effect and to test the indirect effect of phytoplankton on zooplankton composition. The trophic state was maintained with bi-weekly applications of nitrogen and phosphorus (N:P molar ratio of 33, similar to Lake Hertel) to reach two different concentrations (15 µg/L TP in the mesotrophic ones, and 60 µg/L TP in the eutrophic ones; Fugère *et al.* 2020). The original experiment comprised 48 ponds where roundup doses varied in their target concentration (from 0 to 15 mg/L). However, eDNA was sampled in a subset (eight mesocosms) of these, which are the ponds on which we focus our analyses in this thesis. These eight ponds were represented by four controls: two mesotrophic replicates (MC and Mc) and two eutrophic replicates (EC and Ec) and four treatments: mesotrophic moderate-glyphosate (Mg), mesotrophic high-glyphosate (MG), eutrophic moderate-glyphosate (Eg) and eutrophic high-glyphosate (EG; Fig. 3.1a). The experiment lasted eight weeks (57 days) starting from August 17th, 2016 (day 0) to October 12th, 2016 (day 56; Fig. 3.1b). Glyphosate was applied on day 6 and 34, while the severe pulse was applied on day 45. In total, the ponds were sampled 11 times for eDNA (days 0,6,14,29,34,37,40,42,44,48,56) by filtering 250 mL with 0.22 µm filters for a total of 88 samples. Together with eDNA samples, phytoplankton and zooplankton samples were collected, except for day 44. Water samples were collected for phytoplankton biomass in the upper 35 cm of the water column in five random locations within each mesocosm using integrated samplers made from 2.5 cm diameter PVC tubing. Samples were then combined in a 1-L Nalgene bottle. Zooplankton samples were collected in ten random locations and filtered with a 64 µm sieve for a total of 2L of water. Zooplankton were anesthetized using carbonated water and then preserved in 75 % ethanol. To measure in-pond glyphosate concentration, 1 L water samples were collected in clear plastic bottles immediately after applying glyphosate. Samples were then acidified with sulfuric acid to a pH < 3 and frozen until analysis with liquid chromatography heated electrospray ionization tandem mass spectrometry using an Accela 600-Orbitrap LTQ XL (Thermo Scientific, Waltham, MA, USA). In-pond glyphosate concentration was measured for day 6 (after the first pulse of glyphosate), 14, 29, 34 (after the second glyphosate pulse), and 44 (the day after the

severe pulse). For more details on the original experimental design, see Fugère *et al.* (2020).

3.3.2 Library preparation

DNA was extracted using PowerWater (MoBio) extraction kit, with a few modifications. In the first step the filters were incubated with the extraction buffer for 10 minutes at 65 °C. To monitor for potential contamination, two blank extractions were also conducted. DNA extracts were quantified using NanoDrop microvolume Spectrophotometer (ThermoFisher, Waltham, MA, USA) and diluted using ultrapure water to a concentration of 2.5 ng/μL. The samples were amplified using the primers developed by (Leray *et al.* 2013) to target a 313 bp fragment within the classical Folmer region (Folmer *et al*. 1994) of the Cytochrome c oxidase subunit I (COI) gene. COI provides discrimination among closely related species as well as intraspecific information (Hebert *et al.* 2003). It has been for a long time the marker of choice in population genetics and phylogeographic studies and its use is now gaining momentum in metabarcoding studies targeting eukaryotes (Elbrecht *et al.* 2019; Hajibabaei *et al.* 2019). Libraries were prepared following the protocol "16s Metagenomic Sequencing Library Preparation" created by Illumina Inc. (Illumina *et al.* 2013) with few modifications. Library preparation involved a first PCR followed by a first cleaning, the indexing reaction (Nextera Index kit), a second cleaning, quantification of samples (Agilent technologies bioanalyzer, DNA 1000 kit) and equo-molarization prior to next-generation-sequencing (NGS). PCR reactions were conducted in five technical replicates. Two blank extractions, three PCR negative controls, and two mock communities were also included, for a total of 96 libraries. For detailed information about the mock communities see Appendix C ("Mock communities", Table C.1)

Each DNA extract was PCR amplified five times. The PCR consisted of a total volume of 12.5 mL: 0.2 μM of forward and reverse primers, 7.5 μL of 2xKAPA HiFi HotStart ReadyMix (KAPA Biosystems Inc., USA), and 1 μL of diluted DNA extract. The PCR thermocycler regime used followed Leray *et al*. (2013) and the amplification success was assessed on a 1% gel electrophoresis. The five replicate PCR products were pooled. The library was purified using ultrapure beads (AMPure XP beads) at ratio of 0.875 according

to the manufacturer's protocol (in 20 µL DNA solution). Indexing reactions were performed using the Nextera XT Index kit (24-index, V3). This second reduced-cycle PCR was performed in 25 µL reaction volume containing 2.5 µL of unique pairs of Illumina Nextera tags per sample, 2.5 µL of cleaned DNA amplicons, 12.5 µL of 2xKAPA HiFi HotStart ReadyMix (KAPA Biosystems Inc., USA), and 5 µL of ddH20. PCR cycling conditions included 3 minutes at 95 °C, followed by eight cycles of 95°C for 30 seconds (s), 55°C for 30 s, 72°C for 30 s, and a final extension of 5 minutes at 72°C. The final clean-up was done with the same type of ultrapure beads but this time the volume of the solution was 30 µL. After quantification following manufacturer's instructions (Agilent technologies bioanalyzer, DNA 1000 kit), the samples were all diluted to the target concentration of 12.5 ng/µL (the lowest concentration observed). The sequencing-ready library was submitted to Genome Quebec Innovation Center facility (Montreal, Quebec, Canada) for sequencing in one run using pair-end Illumina MiSeq sequencer of 300 bp.

3.3.3 Bioinformatics analyses

The bioinformatics pipeline consisted of demultiplexing, quality filtering, trimming raw reads based on quality score, and assigning taxonomy (Fig. 3.2). The quality plot function in the DADA2 (Divisive Amplicon Denoising Algorithm 2) package version 1.12.1 (Callahan *et al.* 2016) was used to identify the low quality end trimming position. The latter was then used to trim the reads along with adapters and primers with Cutadapt version 1.18 (Martin 2011). Sequences were denoised with the amplicon sequence variant (ASV) method in DADA2, which was originally developed for microbiology studies to correct erroneous sequencing due to amplification and sequencing errors. This method produces ASVs in each library as an alternative of OTUs for which a dissimilarity threshold is needed. This new procedure assumes that biological differences occur more often than erroneous sequences and it represents an efficient current approach to unravel intraspecific genetic variation. DADA2 generates an error model tailored to an individual sequencing run and employs algorithms that use the model to distinguish between true biological sequences and those generated by error. The detailed algorithm of DADA2 is thoroughly described in Callahan *et al*. (2016).

For quality assessment, mock community libraries were tested with different combinations of quality filtering parameters: 'minimum quality average' allowed before trimming (18, 20, 23, 25, and 28), 'maximum number of expected errors' allowed in a forward read (4 and 5), 'maximum number of expected errors' allowed in a reverse read (5 and 6), and 'minimum size of an ASV' (4 and 8). The rest of the parameters were kept constant: the 'window size' to compute average quality (1), 'minimum amplicon length' expected (200 bp), 'minimum overlap for merging' (20 bp), 'maximum amplicon length expected' (600 bp). More specifically, after trimming and quality filtering, BLASTN (Altschul *et al.* 1990) was used to perform a BLAST search of each ASV versus local reference databases. The local reference databases consisted of COI sequences of the mock communities species downloaded from NCBI GenBank (Appendix C, Table C.2). For the taxonomic assignment, we used a sequence similarity of 98% and a sequence coverage of 95%. We selected the combination of parameters that maximized the detected species from the mock community libraries and minimized the amount of contamination from blank and control libraries (Table 3.1). The selected pipeline consisted of a quality filtering score of 23 (when mock community *a* detection showed a slight increase), a maximum number of "expected errors" allowed in a forward and reverse read respectively of 4 and 5, and a minimum size of an ASV of 4 sequences. The experimental libraries were analysed using these parameters (Table 3.1).

3.3.4 Taxonomic assignment

Taxonomic assignment at the family level used ASV sequences based on nucleotide BLAST searches against a local database of COI sequences of Eukaryota species from NCBI GenBank. The best BLAST hit was identified with > 90% identity, e-value 0.01, and a minimum query coverage > 95%. After obtaining a list of families for each library, we identified and removed the ones associated with microalgae, protists, fungi, fish, mammals, sessile organisms (e.g., polyps), and terrestrial invertebrates that do not have an aquatic stage in their life cycle (Appendix C, Table C.3). Other contamination such as sequences belonging to the mock communities or species recovered in the negative controls were removed from the analyses (Appendix C, Table C.4).

For the investigation of genetic diversity within species, the taxonomic assignment at the family level was followed by assignments at the species level using BASTA (Basic Sequence Taxonomy Annotation; (Kahlke & Ralph 2019) which assigns taxonomies to sequences based on the Last Common Ancestor (LCA; Wood & Salzberg 2014). The BASTA parameters (e-value threshold, minimum number of hits to use, percentage of hits that are used for LCA estimation) were selected based on the output of an optimization algorithm run on the mock communities' sequences. The optimization algorithm suggested an e-value threshold of 1E-80, a minimum number of hits of 1, and a minimum percentage of hits of 60%. We used these parameters to assign taxonomies to sequences based on the LCA of the best BLAST hit identified with > 90% identity (Fig. 3.2).

3.3.5 Diversity estimates

The number of taxonomic families for rotifers, crustaceans and insects, and the *effective numbers of species* (Hill numbers; Jost 2006) were estimated for each mesocosm pond at each time point. The *effective numbers of species* was calculated at the family level using the R package 'vegan' (Oksanen *et al.* 2013) and we used abundance data (number of individuals) within each taxonomic family for microscopy data, while we used ASVs abundance (number of ASV reads) data within each taxonomic family, for metabarcoding data. For the latter, we used the package 'vegan' as well upon removal of sequences reported in Tables C.3 and C.4. Diversity within families (metabarcoding data) was represented by the number of different ASV sequences assigned to a particular family. Diversity within rotifers, crustaceans and insects was estimated by the sum of the diversity (number of ASVs) within families belongings to each taxonomic group (Fig. 3.2).

Intraspecific genetic diversity consisted of the number of different ASV sequences assigned to a particular species and was estimated only for species that were consistently detected using BASTA, across libraries. We graphically assessed the dynamics of genetic diversity of these selected species throughout the experiment and we statistically compared their trends with the abundance estimates obtained through morphological assessments (section *3.3.7*).

3.3.6 Comparison between metabarcoding and morphological assessment data

Detailed abundance estimates were available for the first five time points of phase I (day 0, 6, 14, 29, 34) and for day 42 (before the application of the severe pulse of glyphosate). Zooplankton abundance was not available for phase II except for the total number of cladocerans and copepods at day 48. Rotifers abundances were available for the controls that underwent the severe pulse (Mc and Ec) and for the high-glyphosate ponds (MG and EG). This data was only used as information of presence/absence of zooplankton after glyphosate severe pulse. For phase I, we first assessed whether there was any taxonomic group (among rotifers, crustaceans, and insects) that were systematically detected with one method but not the other one, and vice-versa. Further analyses were dedicated to taxonomic families that were either: i) not detected with the metabarcoding approach or ii) detected but not consistently with detection with morphological assessments. We used different approaches to test whether a failure to detect some families was attributable to low quality of reads. We first tested taxonomic assignment with a lower percentage identity (80% instead of 90%). We then used the DADA2 pipeline that retrieved the highest number of final reads (quality 28, ef4er5, min4; Appendix C, Table C.2) and we assigned taxonomy with a percentage identity of both 80% and 90%. We also tested whether the missing families were retrieved by assigning taxonomies to quality filtered unmerged reads (forward and reverse), separately.

Sequences of the taxonomic family Cyclopidae, a common taxon found in the zooplankton bulk samples collected during the experiment, were likely assigned to Maxillopoda sp. as a best hit. Since no other families belonging to this class are generally found in Lake Hertel, we assumed that they represented taxa of the Cyclopidae family. For this reason, when sequences assigned to Maxillopoda sp. were retrieved, we considered them as sequences belonging to the family Cyclopidae.

For the families of zooplankton identified by both approaches, we visually compared their presence-absence dynamics for each mesocosm pond and we calculated the percentage of families detected with morphological assessments that were also detected by metabarcoding and then, for each family of rotifers, crustaceans, and insects, we calculated the number of libraries in which they were detected with metabarcoding and

morphological assessments, the percentage of detection with metabarcoding of taxonomic families found by morphological assessments and the number of false positives and false negatives.

3.3.7 Statistical analyses

Comparative assessments between the response of zooplankton diversity unveiled by morphological assessments and metabarcoding were only conducted for phase I while data relative to phase II were only qualitatively compared. First, we tested whether detections obtained with the metabarcoding approach of particular zooplankton taxa were related to their abundance (number of individuals/L). We selected the most dominant taxonomic families detected through morphological assessments and we used a generalized mixed model (GLM) fitted with the binomial family "logit" with the response variable as yes/no for detection with metabarcoding and the independent variable as the number of individuals of that particular families estimated with morphological assessments. A combination of other factors (time, nutrient level, chlorophyll *a* concentration, and glyphosate concentration) were also included. The random effect was represented by mesocosm ponds identities. A null model and two linear models were also tested (Table C.5).

With a linear model analysis (lm; package "lme4"; Bates *et al.* 2014), we tested the correlation between the number of families obtained with morphological assessments with the number of families estimated with metabarcoding and the same for the *effective numbers of species*. Rotifer and crustacean diversity and intraspecific genetic variation of selected species (number of ASV sequences) were tested for their respective correlation with abundance (number of individuals) estimated with morphological assessments.

To test whether diversity estimates obtained with metabarcoding and morphological assessments responded in a similar manner to glyphosate contamination, we analyzed, in parallel, the total number of taxonomic families, *effective numbers of species* (Hill numbers), diversity (number of sequences) and abundance (number of individuals) within rotifers and crustaceans obtained with both approaches using linear mixed models (lmer; package "lme4"; Bates *et al.* 2014). Glyphosate concentration was not measured at all

time points. However, since glyphosate degradation was very low (Fugère *et al.* 2020) we assumed that the concentration between measurements did not change. Using lmer, we modelled the response variables as a function of glyphosate concentration (z-transformed ppb), chlorophyll concentration (z-transformed $\mu g/L^{-1}$) or their respective interaction with time (days, categorical), and mesocosm identity (random effect; Table C. 7). Akaike information criterion (AIC; Burnham KP 2002) was used to select the best-fit models. Since the presence of insects was limited in the samples collected for morphological assessments, no comparison was done between the two methods but their diversity (number of ASV sequences obtained with metabarcoding) was tested with the same linear mixed models used for rotifers and crustaceans.

Finally, the same mixed models used for the number of families, *effective numbers of species* (Hill numbers), rotifer, crustacean, and insect diversity were used to test whether intraspecific genetic variation of selected species was impacted by glyphosate concentration (z-transformed ppb) or chlorophyll *a* concentration (z-transformed $\mu g/L^{-1}$) during phase I (day 0, 6, 14, 29, 34, 42) of the experiment. All analyses were conducted in R 3.6.1 (R Team 2019).

3.4 Results

3.4.1 Metabarcoding output description

The MiSeq pair-end sequencing yielded a total of 13,994,351 reads with an average quality score of 33 (out of 40). The number of raw reads in the experimental ponds ranged between 23,406 and 279,643 (Table 3.1). DADA2 tests with different combinations of quality filtering parameters gave similar results with a raise in species detection with increasing quality filtering scores (Table C.2). The detection of species in the mock community was 50% for the mock community *a* and was 63% for mock community *b*. Blanks' contamination increased from a quality filter of 23 and, overall, it ranged from 2 to 6 species. The lowest number of reads, after trimming, filtering, denoising, merging, and removing chimeras was observed in a sample collected at day 29 from the eutrophic pond moderate-glyphosate (16,729 reads) and the highest was observed in a sample collected at day 56 from the same pond (234,555; Table 3.1). Blank

samples ranged from 461 to 4,166 reads (Table C.2) while the extraction controls from 4,878 to 7,907 reads (Table 3.1). Both blank and control samples consisted of sequences belonging to Hominidae, Muridae, and other non-zooplankton organisms that were removed (Table C.4).

3.4.2 Comparison of detection rates between metabarcoding and morphological assessments

The taxonomic assignment generated a total of 127 families including eight families of microalgae, five families of protists, 19 families of fungi, 27 families of invertebrates, one family of fish, and two families of mammals that were not considered for this study (Table C.3). Four families of crustaceans (three families of Cladocera and one family of Copepoda), four families of Monogononta (Rotifera), and eight families of insects (six families of Diptera and two families of Odonata) were retained and considered for analysis. Family composition of each pond estimated through metabarcoding was compared with families detected with morphological assessments (Table 3.2). There were differences in detection rates between the two approaches (Table 3.2-3.3, Fig. 3.3). These differences included absolute false negatives and relative false negatives and false positives. Absolute false negatives were represented by taxa that were detected with morphological assessments but that were never detected with the metabarcoding approach while relative false negatives and false positives were represented by taxa detected by both methods but that showed inconsistent detection rates across time and mesocosm ponds. There were also taxa that were not detected with morphological assessments but were detected with the metabarcoding approach (one family of cladocerans, and seven families of insects; Table 3.2-3.3, Fig. 3.3). Taxonomic families that were not detected by the selected DADA2 pipeline (Table 3.2) were also not detected by using more relaxed parameters with both DADA2 and BLASTN (Altschul *et al.* 1990) or by analysing the forward and reverse reads separately. Metabarcoding detection rate across mesocosms comparing to detections with morphological assessments ranged from 8.3% (mesocosm mesotrophic high-glyphosate) to a maximum of 65.1 % (mesocosm Ec: eutrophic control, severe pulse only) with a total average percentage of detection of 38.3% (SD = 32.01; Table C.6). Chydoridae and Cyclopidae were the families tested for

a correlation between their detection with metabarcoding and abundance (no. of individuals) estimated with morphological assessments (Table C.5). A GLM analysis did not find any correlation between the detection of both families and their relative abundance estimated with morphological assessments. For Chydoridae the best-fit model was the null model (p = 0.005) while for Cyclopidae, the best-fit model included glyphosate concentration (p = 0.03) and time (p = 0.01; Table C.5).

3.4.3 Analysis of Last Common Ancestor (LCA) output

Analysis of Last Common Ancestor (LCA) led to the identification of three species of rotifers (*Asplanchna sieboldi*, *Euchalnis dilatata*, and *Keratella cochlearis*; 53 total haplotypes), two of crustaceans (*Chydorus brevilabris* and *Sida crystallina*; 5 total haplotypes), and four species of insect (*Callibaetis fluctuans*, *Cloeon dipterum*, *Smittia stercoraria*, and *Tanytarsus mendax*; 22 total haplotypes; Fig. 3.4 a). LCA detected also the genus of rotifera *Polyarthra* sp. Because morphological assessments found only one species of the genus *Polyarthra* and given the high haplotype diversity observed, we included it in the assessment of intraspecific genetic variation. *Polyarthra* sp. showed the highest haplotype diversity (24 haplotypes).

3.4.4 Assessing the relationship between taxonomic diversity and abundance

The number of families and the *effective numbers of species* (Hill numbers) estimated with the metabarcoding approach and morphological assessments were positively correlated (p = 0.0001 and p = 0.02 for the number of families and the *effective numbers of species*, respectively; Fig. 3.5 a,b). Rotifer diversity estimates obtained with metabarcoding were correlated with rotifer abundance estimates obtained through morphological assessments (p = 0.000112; Fig. 3.5 c) while crustacean diversity estimates obtained with metabarcoding were not correlated with crustacean abundance estimates (p = 0.86; Fig 3.5 d). Genetic variation in *K. cochlearis* and *Polyarthra sp.* was positively correlated with their abundance estimates obtained through morphological assessments (p = 9.32E-06 and p = 1.04E-05 for *K. cochlearis* and *Polyarthra* sp., respectively; Fig. 3.5 e, f, C.2).

3.4.5 Ecological signal during the experiment

3.4.5.1 Phase I

We observed different trends in the dynamic of the number of taxonomic families across experimental mesocosms (Fig. 3.6 a,b, C.1 a,b). The number of zooplankton families in controls was always above 2 in the mesotrophic mesocosm and above 3 in the eutrophic one. The highest number of zooplankton families (6 families) was always observed in glyphosate-free conditions. The first pulse of glyphosate caused a decrease in the number of families detected with both methods. The mesotrophic moderate-glyphosate mesocosm (Mg) seemed to be more impacted than the eutrophic moderate-glyphosate mesocosm. After the second pulse almost all treatment mesocosms showed a slight increase in the number of zooplankton families. According to the dynamic of the *effective numbers of species* (Hill numbers), high-glyphosate mesocosms were the most impacted by the first pulse, especially with estimates obtained with metabarcoding (Fig. 3.6 a,b). Conversely, the mesotrophic moderate-glyphosate (Mg) and the eutrophic moderate-glyphosate (Eg) mesocosms showed a slight increase followed by the first pulse according to morphological assessments and metabarcoding, respectively.

The best-fit lmer model for the response of the number of zooplankton families was the same for both morphological assessments and metabarcoding and included glyphosate concentration as explanatory variable (estimate -0.55, SE 0.12 and estimate -0.68, SE 0.12 for morphological assessments and metabarcoding, respectively; Fig. 3.7 a, Table C.7, C.8). Model selection by AICc on estimates of the *effective numbers of species* resulted in the same best-fit model including also glyphosate concentration (estimate -0.25, SE 0.21 and estimate -0.48, SE -0.29 for morphological assessments and metabarcoding, respectively).

Zooplankton diversity within families of rotifers, crustaceans, and insects estimated with metabarcoding showed substantial differences across treatments (Fig. 3.8 a,b). During the whole experiment, rotifers were better represented in the eutrophic controls which showed the highest diversity during phase I. Conversely, the moderate-glyphosate mesocosms (Mg and Eg) showed fluctuations in the presence of rotifers following glyphosate pulses. However, rotifers were mostly impacted in the high-glyphosate ponds (MG and EG) where also crustaceans showed the lowest diversity. The high-glyphosate

mesocosms were characterized by higher diversity within insects which seemed to dominate in the mesotrophic high-glyphosate pond (MG) with the highest peaks on day 6 and 56 (16 sequences assigned to insects; Fig. 3.8 a).

The abundance of rotifers estimated with morphological assessments and their diversity (number of sequences) were also best explained by a model including glyphosate concentration as independent variable (estimate -0.16, SE 0.14 for morphological assessments, and estimate -0.26, SE 0.14 for metabarcoding; Fig. 3.7, Table C.7, C.8). The abundance dynamic of crustacean species was best explained by the concentration of chlorophyll *a* (estimate 0.44, SE 0.13) while diversity estimated with metabarcoding was represented by a best-fit model including glyphosate concentration (estimate -0.50, SE 0.13). Diversity within insects (number of sequences) was explained by a model including an interaction factor between glyphosate concentration and time (categorical; Table C.8).

3.4.5.2 Phase II

The severe pulse caused a decrease in the number of zooplankton families and the *effective numbers of species* in all ponds according to the metabarcoding approach estimates (Fig. 3.6 a,b, C.1 a,b). No rotifers or crustaceans were detected by metabarcoding in the mesotrophic moderate-glyphosate mesocosm (Mg) after the severe pulse while in the high-glyphosate mesotrophic (MG) and eutrophic mesocosms zooplankton was still present at the end of the experiment according to both methods. However, both control mesocosms (mesotrophic and eutrophic) that were subjected to the severe pulse (Mc, Ec), but in particular the mesotrophic mesocosm (Mc), showed a sharp decrease in the *effective numbers of species* according to metabarcoding. Family diversity estimated with metabarcoding diminished in all treatment mesocosms but in the mesotrophic high-glyphosate pond (MG), diversity in insects increased. Rotifers disappeared from all the mesocosms that were subjected to the severe pulse except in the mesotrophic high-glyphosate mesocosm (MG; Fig. 3.8 a). According to morphological assessment data, crustaceans were present in all ponds after the severe pulse and rotifers were present in the cases their presence and abundance was assessed (Mc, Ec, MG, and EG; Fig. 3.8).

3.4.6 Dynamic of intraspecific genetic variation

The dynamics of intraspecific genetic variation throughout the experiment was consistent with what was observed with family diversity dynamics (Fig. 3.6 b). Mesotrophic controls showed the highest intraspecific genetic variation (Fig. 3.6 b) which was mostly represented by rotifer species. The lowest number of haplotypes was observed in the mesotrophic moderate-glyphosate mesocosm. The highest variation of insects was observed in the mesotrophic high-glyphosate mesocosm (8 haplotypes). Haplotypes were not uniformly distributed across controls and treatments but were better represented in control mesocosms (Table C.9). A percentage ranging from 38 to 47% of haplotypes were, in fact, observed in control mesocosms while the lowest percentage was recorded in the mesotrophic high-glyphosate mesocosm (MG). Insect species had the highest percentage of haplotype presence in the mesotrophic control (44%, MC). A percentage of 25% was the second highest level observed in the mesotrophic moderate- and high-glyphosate mesocosms (Mg and Mg). The insect species *Cloen dipterum* showed the highest percentage of haplotype diversity in the mesotrophic high-glyphosate mesocosms (MG; Table C.9).

We observed a high haplotype diversity in *K. cochlearis* in eutrophic control mesocosms (Fig. C.2 a, b). *Polyarthra* sp. showed a peak of intraspecific genetic variation (15 haplotypes) in the mesotrophic control (MC, day 14) while in the eutrophic control (EC) variation did not show large fluctuations (Fig. C.2 c, d). In all mesocosms, including controls *Polyarthra* sp., intraspecific genetic variation showed a decrease from the beginning of the experiment. Intraspecific genetic variation in both species was generally low in the moderate- and high-glyphosate mesocosm treatments. Genetic variation in *K. cochlearis* and *Polyarthra sp.* was further tested with mixed model analysis which resulted in a best-fit model including glyphosate concentration as the independent variable (estimate -0.17, SE 0.16 and estimate -0.27, SE 0.14, for *K. cochlearis* and *Polyarthra* sp., respectively, Table C. 10). In both species, genetic variation dropped after the severe pulse (Fig. 3.5 e, f). In the case of *K. cochlearis* this drop was very evident in the mesotrophic control (Mc, Fig. 3.5 e) that underwent the severe pulse only. Six haplotypes were present for two consecutive sampling times before the severe pulse (day

40 and 42) and declined to 0 at day 56. A similar pattern was observed in *Polyarthra* sp. whose haplotype diversity went from 5 to 0 in four days (Fig. 3.5 f).

3.5 Discussion

3.5.1 Detection rates and effectiveness of metabarcoding

We found that metabarcoding was efficient in detecting the majority of families of rotifers (57%) and crustaceans (75%; Table 3.2; Fig. 3.3) but there were also several false negatives and false positives. False negatives could be explained by a less than optimal primer specificity. Zhang *et al.* (2018) tested the effectiveness of different primers (COI and 18S) used separately or in combinations in the detection of zooplankton species. When they tested COI Leray primer alone, they obtained similar amplification success to what was found here. The detection rate increased with the number of primers and markers used. Similar results were also obtained by Alberdi *et al.* (2018) that found a link between amplification success and species group.

Compared to the overall detection of families observed with morphological assessments, the recovery rate of taxonomic families obtained with metabarcoding per pond and per time point was lower (Table 3.3, C.6). The presence of false negatives at certain time points could be due to an overall low sequencing depth that led to the underestimation of families with very low read numbers. Increases in sequencing depth was seen to increase diversity detection (Alberdi *et al.* 2018). In contrast, the presence of false positives throughout the experiment could be also explained by a longer persistence of eDNA in the mesocosm environments that may have biased detection of actual species' presences. eDNA samples were collected a few days apart during the experiment which may not be a sufficient time for complete degradation of e-DNA released in the past. An eDNA molecule ranging from 300 to 400 bp could in fact be detected up to seven days in aquatic controlled conditions (Matsui *et al.* 2001; Zhu 2006). This is the main reason why scientists are investigating the possibility of using eRNA (which appears to degrade faster than eDNA) as a more reliable tool to estimate current diversity in aquatic environments (Cristescu 2019).

COI metabarcoding recovered several taxonomic groups that were not found by morphological assessments (Table 3.2, Fig. 3.3). These groups could represent false positives (Cristescu & Hebert 2018) or taxa that were present but not sampled by traditional methods. Bottom fauna need to be sampled with specific sampling techniques. Insects larvae, for example, are usually sampled with emergence traps (Davies 1984) or benthic sampling techniques (Malison *et al.* 2010). A good performance of metabarcoding in identifying insect taxa was also observed by Ekrem *et al.* (2010), Silva & Wiedenbrug (2014), Lin *et al.* (2018), and Sun *et al.* (2019). Although there is a possibility that the detected insects are false positives, a pattern in their presence was observed across different treatments. Their family diversity was higher in the high-glyphosate treatments (MG, EG) and this was particularly evident in the mesotrophic treatment where they were consistently dominant through time (after the first pulse; Fig. 3.8 a, b)**.** The dominance of insects and the lower detection of zooplankton taxa by both morphological assessments and metabarcoding in the high-glyphosate treatments could be explained by an intensification of predation due to alterations in predation-prey interactions induced by glyphosate stress (top-down regulation; Chang *et al.* 2005; Fussmann & Gonzalez 2013; Kovach-Orr & Fussmann 2013; Yamamichi & Miner 2015; Bell *et al.* 2019). Insects are less susceptible to herbicide contamination (including glyphosate) compared to other zooplankton taxa (Folmar *et al.* 1979; Relyea 2005) and this could have exacerbated the detrimental effects of glyphosate on zooplankton preys.

3.5.2 Zooplankton dynamics and ecological response to glyphosate pulses

Despite the presence of false negatives, zooplankton diversity estimates and abundance estimates showed a similar ecological changes in response to the experimental conditions (Fig. 3.8 a,b, C.1). The dynamics of the overall number of zooplankton families and the *effective numbers of species* estimated with metabarcoding were correlated with the number of zooplankton families and the *effective numbers of species* estimated with morphological assessments. Both estimates (number of families and the *effective numbers of species*) were significantly and similarly affected by glyphosate concentration (Fig. 3.6 a, b, 3.8, Table C.8).

Rotifer diversity (number of sequences) and rotifer abundance (microscopy) were also both affected by glyphosate concentration in a similar way (Fig. 3.7). We found a different ecological response between crustacean diversity (number of sequences) and abundance (microscopy). The change in crustacean diversity was best predicted by glyphosate concentration while change in abundance was best predicted by chlorophyll *a* concentration. The possibility that the abundance of crustaceans increased with phytoplankton density while their diversity decreased due to declines caused by the detrimental effect of glyphosate cannot be excluded. This difference may be also due to differences in detection within specific taxonomic groups, which led to apparently different ecological responses. Leasi *et al.* (2018) and Cahill *et al.* (2018) found that differences in the recovery of particular taxonomic groups could bias ecological interpretations.

The two pulses during phase I and the severe pulse of glyphosate in phase II showed different effects on zooplankton diversity (Fig. 3.6 a, b, 3.8 a, b, C.1 a, b). The first pulse induced a decrease in the number of taxonomic families and the *effective numbers of species* but the second pulse led to an increase in zooplankton diversity and abundance. This can be explained by the putative long-term fertilizing effect of glyphosate (Hébert *et al.* 2019; Fugère *et al.* 2020). Fugère *et al.* (2020) found that while the first pulse of glyphosate negatively affected phytoplankton, the second pulse of glyphosate, did not affect chlorophyll *a*. The severe pulse of glyphosate had detrimental effects on zooplankton diversity, particularly in rotifers and crustaceans in the mesotrophic mesocosms (Fig. 3.8 a, b). A greater impact of glyphosate in the mesotrophic mesocosms suggests that the higher productivity of the eutrophic mesocosms likely provided a counteracting effect for the toxicity of glyphosate. Moreover, pre-exposure to phase-I doses of glyphosate may have represented an advantage for coping with the severe pulse as also observed by Fugère *et al.* (2020).

3.5.3 Glyphosate effect on intraspecific genetic variation

The dynamic of intraspecific genetic variation of the rotifers *K. cochlearis* and *Polyarthra* sp. showed a negative effect in relation to glyphosate concentration in both mesotrophic and eutrophic mesocosms (Fig. C.2 a, b, c, d). Genetic erosion due to

chemical stress represents a factor of concern in risk assessment and has been observed in several studies as reviewed by Van Straalen & Timmermans (2002). According to Van Straalen & Timmermans (2002), toxicants can affect genetic diversity by increasing mutational load, by natural selection on resistance genotypes, by inducing bottleneck events, and by altering migration. In this particular case, it is very likely that a bottleneck event and subsequent extirpation led to the observed genetic erosion. Rotifers represent biological indicators and are largely used in toxicity tests for their important ecological role in aquatic environments. They contribute substantially to secondary production and nutrient recycling and are dominant components of zooplankton communities (Larramendi & Soloneski 2016). In their review, Moreira *et al.* (2016) highlighted that there is a scarcity of studies on the chronic effects of pesticides on rotifers but that the existing studies reveal high sensitivity depending on genotype diversity and experimental conditions. On the other hand, other studies reviewed by Hanazato (2001) found that rotifers increase in abundance during pesticide contamination due to an alleviated competition with crustaceans taxa that are more sensitive. In this particular case, the advantage gained by the higher sensitivity of their competitors, may have been counteracted by an increased predation from insect larvae. The direct toxic effect of glyphosate could, then, have played a role in the genetic erosion and extirpation of these two rotifer species (Fig. C.2 a,b,c,d).

3.5.4 Future directions

Further studies are required to alleviate the shortcomings of eDNA-based metabarcoding and take full advantage of its strengths. To better understand the sources of false negatives and false positives and reduce the resulting biases, future studies should aim to maximize sequencing depth, primer specificity (Zhang *et al.* 2018) and experimental replication. Appropriate validations are needed to confirm whether metabarcoding could be used for quantitative assessments of changes in abundance of zooplankton taxa as it was suggested by the correlations we observed between diversity estimates (obtained with metabarcoding) and abundance estimates (obtained with morphological assessments; Serrana *et al.* 2019). Regardless of the method used, further studies are needed to better understand the impact of glyphosate in non-target organisms. In this study, glyphosate

likely interfered with predator-prey interactions and affected biodiversity at multiple levels (Bell *et al.* 2019). However, we are still far from understanding the long-term consequences for biodiversity and to what extent and in which abiotic and biotic conditions glyphosate exerts a toxic, fertilizing or synergistic effect when in combination with other pollutants.

3.6 Conclusion

Metabarcoding was found to be a promising tool for detecting biodiversity change. Despite discrepancies between detections with metabarcoding and morphological assessments, the two approaches converged in estimating the overall ecological impact of glyphosate on zooplankton communities. However, it is still too early to completely rely on eDNA-based metabarcoding to naively estimate biodiversity in aquatic environments. With further improvement and validation, eDNA-based metabarcoding can provide valuable complementary data when coupled to traditional methods and when reference libraries are well populated. Specifically, it can provide information on benthic taxa (e.g. insect larvae), and on intraspecific genetic variation and its changes through time. Moreover, when applied to biodiversity zooplankton communities facing rapid environmental stress, it can capture the correlation between declines of biodiversity and the intensity of the stressor. Thus, the use of metabarcoding techniques could greatly contribute to elucidating the effects of anthropogenic stressors on the ecological and evolutionary resilience of populations and communities.

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Table 3.1. Number of reads throughout the DADA2 pipeline with a minimum quality of 23 (before cutadapt), maximum numbers of "expected errors" allowed in forward and reverse read respectively 4 and 5, and a minimum size of an ASV of 4.

Mesocosm	day	raw data	cutadapt	filtered	denoised F	denoised R	merged	no chimeras
Mesotrophic control	0	154605	152349	142972	142423	142322	140058	129479
	6	118440	116628	109612	109118	109163	107567	99286
	14	129413	127355	121287	120827	120818	119250	111611
	29	107337	105764	99509	99213	99208	98138	89657
	34	127243	124947	117092	116870	116757	115939	109828
	37	100506	98345	90922	90671	90597	89923	86202
	40	127128	124653	116580	116289	116231	115381	111417
	42	138643	135465	126511	126107	126033	124400	117772
	48	190710	187686	175510	174126	174505	170935	153817
	56	182286	179177	168276	167194	167566	164726	147646
Mesotrophic control (severe pulse)	0	245924	240220	224218	223168	223077	219140	204772
	6	141793	138943	128109	127558	127521	125772	121511
	14	177750	173416	162203	161616	161487	159799	151317
	29	145165	140829	130600	130130	130136	128942	124385
	34	125523	120519	109867	109469	109502	108332	99875
	37	147463	143687	131294	130845	130777	129512	120912
	40	199887	193433	175965	175351	175178	172986	160796
	42	184348	177878	163739	163288	162919	161247	149549
	48	100368	84020	77142	76855	76853	76182	76094
	56	148594	139091	129243	129077	129002	128503	127880
Mesotrophic "moderate" glyphosate	0	103030	99241	92158	91845	91688	90823	87440
	6	105606	102535	95519	95227	95075	93804	90737
	14	124092	120166	111270	110798	110645	108982	108320
	29	23406	22745	21297	21123	21159	20669	19512
	34	126084	122710	115201	114373	114593	112501	103905
	37	129233	126157	117146	116727	116629	115132	108707
	40	114248	110346	101794	101452	101431	100726	96718
	42	120882	116413	108777	108314	108292	107271	100900
	48	110667	96558	89824	89621	89460	88890	88529
	56	156880	148388	139192	138732	138744	137150	132822
Mesotrophic, "high" glyphosate	0	184180	179658	168428	167704	167659	165385	155269
	6	157443	153438	144139	143506	143453	142136	140254
	14	146357	142166	132606	132174	132037	130364	127041
	29	113104	108597	99209	98737	98658	97402	93871
	34	194981	188803	174477	174146	174155	173403	166320
	37	154374	147426	133947	133586	133658	132472	130605
	40	170110	162758	148792	148422	148001	146281	145240
	42	220450	214388	200291	199738	199396	197622	191760
	48	149070	143211	131945	131699	131626	130561	122544
	56	220319	197353	184690	184202	184119	182528	177209

pond	day	raw data	cutadapt	filtered	denoised F	denoised R	merged	no chimeras
Eutrophic control	0	156414	153945	142614	142112	141972	140178	124244
	6	158681	155709	144883	144388	144239	142709	132973
	14	245232	240481	223632	223079	222893	220719	205635
	29	186104	181307	168081	167767	167597	166496	154183
	34	115691	112697	104254	103709	103757	102066	95036
	37	179472	174561	159709	159439	159221	158105	144675
	40	108107	103958	93401	93165	93012	92176	86346
	42	183452	177457	163606	163280	163160	161981	148904
	48	150889	146048	131408	131052	130912	129527	113897
	56	169131	163336	148363	148084	147958	146692	130256
Eutrophic control (severe pulse)	0	167930	162791	151503	151029	150801	149228	140416
	6	155644	151751	141520	140974	140927	139090	129314
	14	163041	159393	151181	150678	150558	148186	132435
	29	146177	143631	133028	132678	132530	130993	115714
	34	204840	199870	185900	185324	185327	183342	169641
	37	136187	130682	120223	120085	119919	119331	110389
	40	144907	143120	131433	131192	131114	130247	119346
	42	163393	160064	147744	147542	147203	146336	132051
	48	127572	116362	102237	102010	101783	101182	100542
	56	215727	205758	189829	189384	189385	187869	183373
Eutrophic “moderate” glyphosate	0	163727	160305	148330	147732	147562	145044	131251
	6	130306	125701	116300	115936	115782	114129	110602
	14	194022	189731	179356	178629	178548	176430	166433
	29	26449	22931	17916	17731	17511	17081	16729
	34	163373	158826	147118	146774	146460	144181	137479
	37	122617	119034	107747	107385	107312	103476	100325
	40	189273	183795	168646	168218	168003	157096	153948
	42	145145	140639	130019	129649	129351	125057	120043
	48	127846	114643	107314	107015	106799	105373	105169
	56	279643	270123	249404	248824	248328	246448	234555
Eutrophic “high” glyphosate	0	108693	105643	94186	93744	93749	92159	86784
	6	124049	121749	112397	112159	112093	111162	110983
	14	150538	146797	135191	134952	134694	132925	130359
	29	155565	152079	139222	138848	138811	131920	127747
	34	167308	160554	146695	146336	146242	145128	144754
	37	134273	130855	116157	115740	115777	114744	113038
	40	164549	161286	145609	145222	145262	144465	143316
	42	159794	155669	143138	142814	142723	141666	139491
	48	120274	113060	103773	103580	103559	102553	101762
	56	200970	196580	178254	177806	177751	176226	168865
Extraction control 1		29308	6108	4949	4902	4905	4884	4878
Extraction control 2		17571	9250	7970	7922	7927	7908	7907

Table 3.2. Comparison between presence/absence of taxonomic families of Crustacea, Rotifera, and Insecta obtained through morphological assessments (morph) and the metabarcoding approach (met) across mesocosms. Mesocosm ponds are coded as: MC (mesotrophic control), Mc (mesotrophic control with final severe pulse), Mg (mesotrophic moderate-glyphosate), MG (mesotrophic high-glyphosate), EC (eutrophic control), Ec (eutrophic control with final severe pulse), Eg (eutrophic moderate-glyphosate), EG (eutrophic high-glyphosate). Symbol * represents the families that were never detected by metabarcoding.

Taxonomic group		Family	MC		Mc		Mg		MG		EC		Ec		Eg		EG	
			morph	met	morph	met	morph	met	morph	met	morph	met	morph	met	morph	met	morph	met
Crustacea	Cladocera	Chydoridae	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
		Daphniidae*	x		x		x				x		x		x		x	
		Macrotrichidae		x		x		x		x		x		x		x		x
		Sididae	x	x		x	x	x		x		x	x	x	x	x		x
	Copepoda	Cyclopideae	x	x	x	x	x	x	x		x	x	x	x	x	x		x
		Nauplii	x		x		x		x		x		x		x		x	
Rotifera	Monogononta	Asplanchnidae		x				x			x	x	x	x		x		x
		Brachionidae	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x
		Conochilidae*	x		x		x				x				x		x	
		Lecanidae	x		x	x	x		x		x		x		x			
		Synchaetidae	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
		Testudinellidae*	x		x		x		x		x		x				x	
		Trochosphaeridae*									x							
Insecta	Diptera	Baetidae		x		x		x				x		x		x		x
		Chaoboridae				x						x						
		Chironomidae				x		x		x		x		x		x		x
		Culicidae														x		
		Limoniidae								x								
		Sciaridae		x						x						x		
		Other	x		x		x		x		x		x				x	
	Odonata	Lestideae		x														
		Libellulidae																x
Other											x							

Table 3.3. Comparison between morphological assessments and metabarcoding on the number of samples in which taxonomic families of Crustacea, Rotifera, and Insecta were detected during phase I. Also shown, the percentage of detection with metabarcoding of taxonomic families found by morphological assessments and the number of false positives and false negatives. Estimates of undetermined nauplii obtained with the morphological approach were not considered.

Taxonomic group		Family	No. of libraries		%	False +	False -
			Morphological assessment	e-DNA			
	Cladocera	Chydoridae	39	13	33.3	5	24
		Daphniidae	19	0	0	0	19
		Macrotrichidae	0	41	-	41	0
		Sididae	8	8	100	16	0
	Copepoda	Cyclopideae	28	17	60.7	7	11
Rotifera	Monogononta	Asplanchnidae	2	2	100	15	0
		Brachionidae	23	13	56.5	6	9
		Conochilidae	9	0	0	0	9
		Lecanidae	21	2	9.5	0	19
		Synchaetidae	26	17	65.3	7	9
		Testudinellidae	9	0	0	0	9
		Trochosphaeridae	1	0	0	0	1
Insecta	Diptera	Baetidae	0	15	-	-	-
		Chaoboridae	0	3	-	-	-
		Chironomidae	0	25	-	-	-
		Culicidae	0	1	-	-	-
		Limoniidae	0	1	-	-	-
		Sciaridae	0	5	-	-	-
	Odonata	Lestideae	0	3	-	-	-
		Libellulidae	0	2	-	-	-

Figure 3.1. Schematic representation of the (a) experimental design and (b) timeline. **(a)** The color of the cycles and the numbers within them indicates the glyphosate concentrations of the pulse additions. The first phase included two mild pulses (0.3 and 15 µg/L) and the second phase consisted of the application of a severe concentration of glyphosate (40 µg/L). White cycles represent control ponds. **(b)** Timeline of the experiment where black cycles represent sampling dates, light orange cycles represent the moderate and high pulses and the red cycle represents the severe pulse application. Modified from Fugère *et al.* 2020.

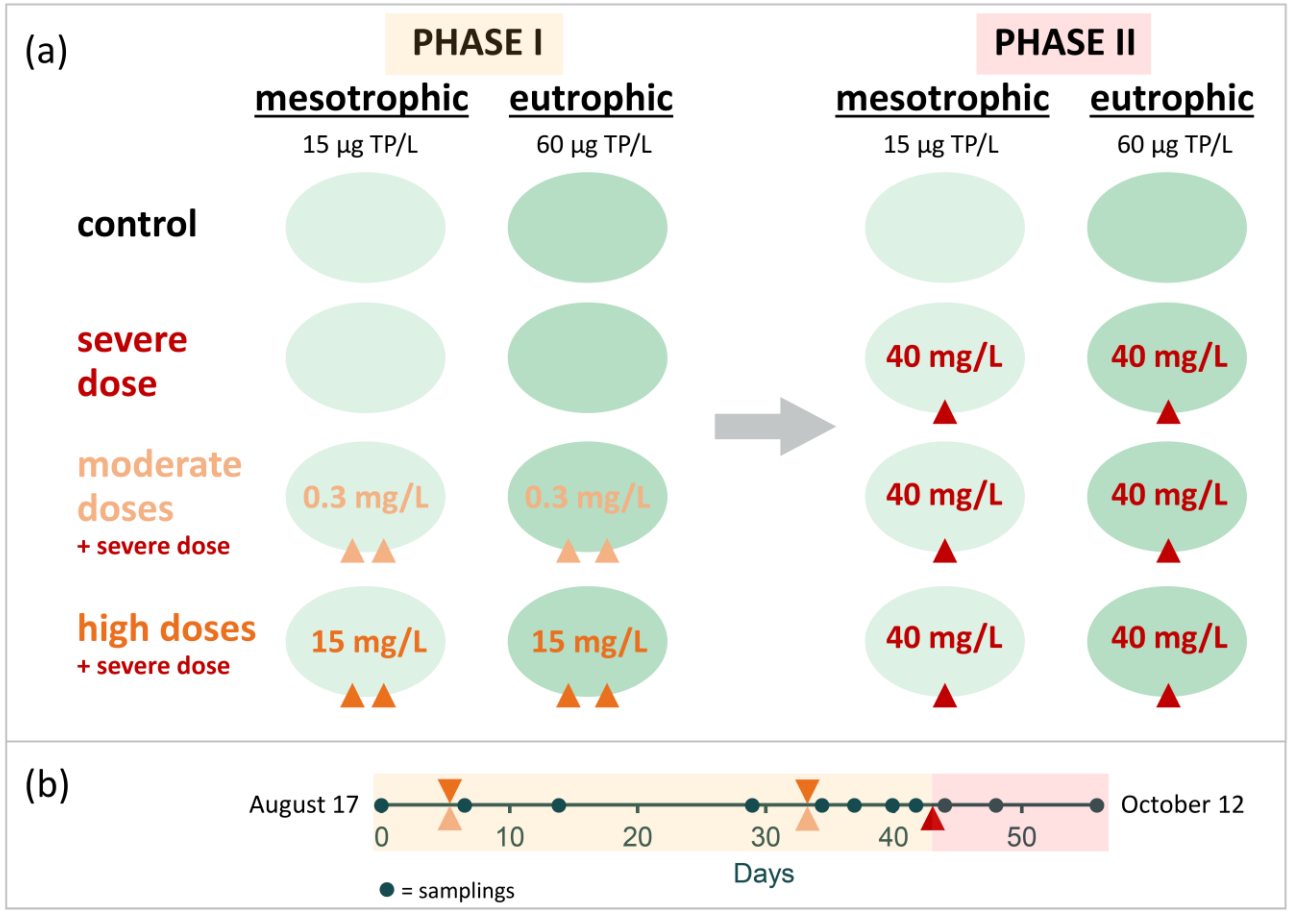


Figure 3.2. Flowchart of the bioinformatic pipeline. Raw reads were trimmed, filtered, denoised, merged, and chimeras were removed with the quality plot function in DADA2. Following a BLAST search against a local reference database, taxonomy was assigned using ASV sequences for analyses at the family level and it was based on LCA using BASTA for analyses at the species level. Best BLAST hits were identified with > 90% identity. For analyses at the family level, we looked at the number of taxonomic families, the *effective numbers of species* (Hill numbers), and ASVs abundance in rotifer, crustacean and insect families. At the species level, we focused on ASVs abundance within the species considered for analysis of intraspecific genetic variation.

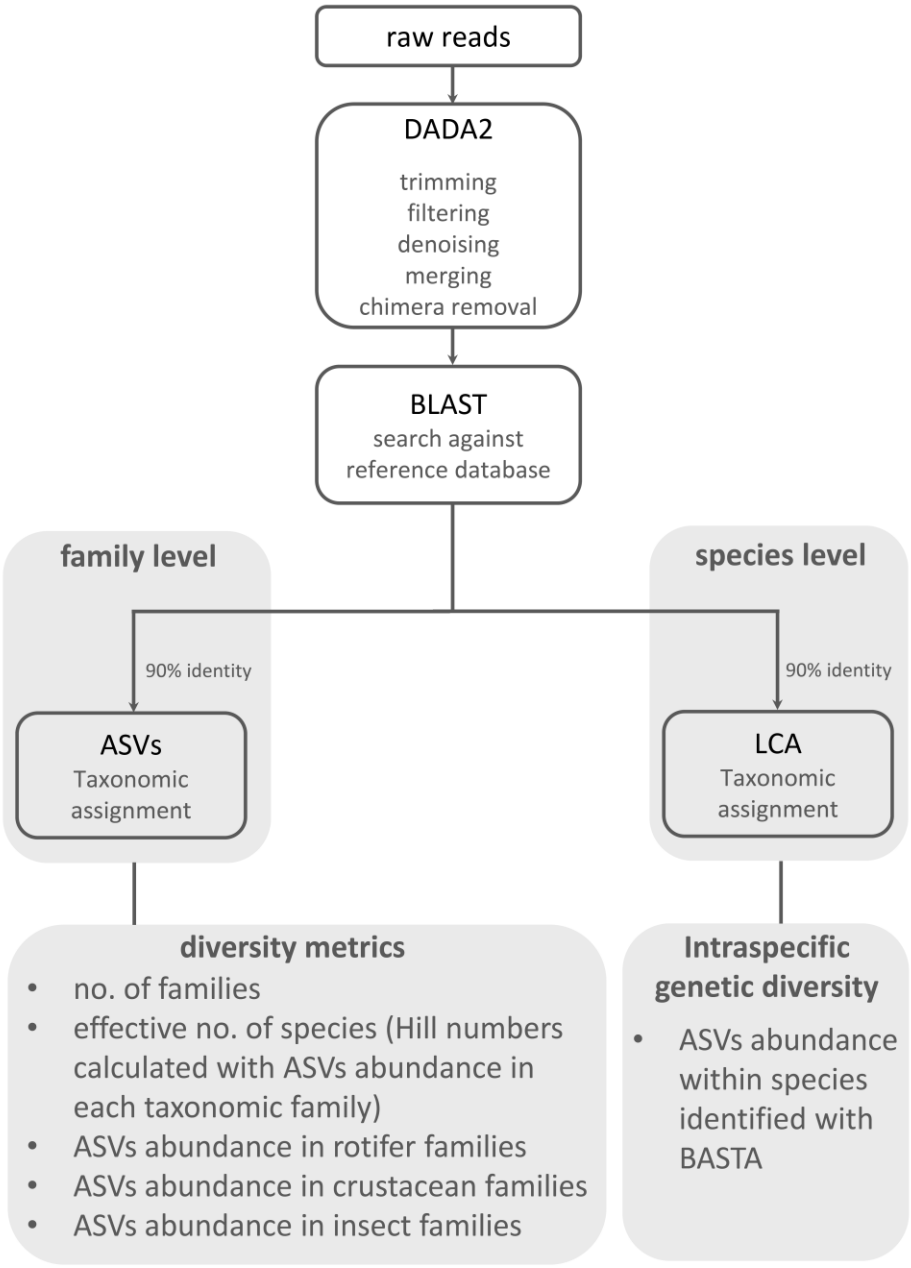


Figure 3.3. Comparison of morphological assessment estimates and metabarcoding data on the number of taxonomic families of rotifers, crustaceans, and insects.

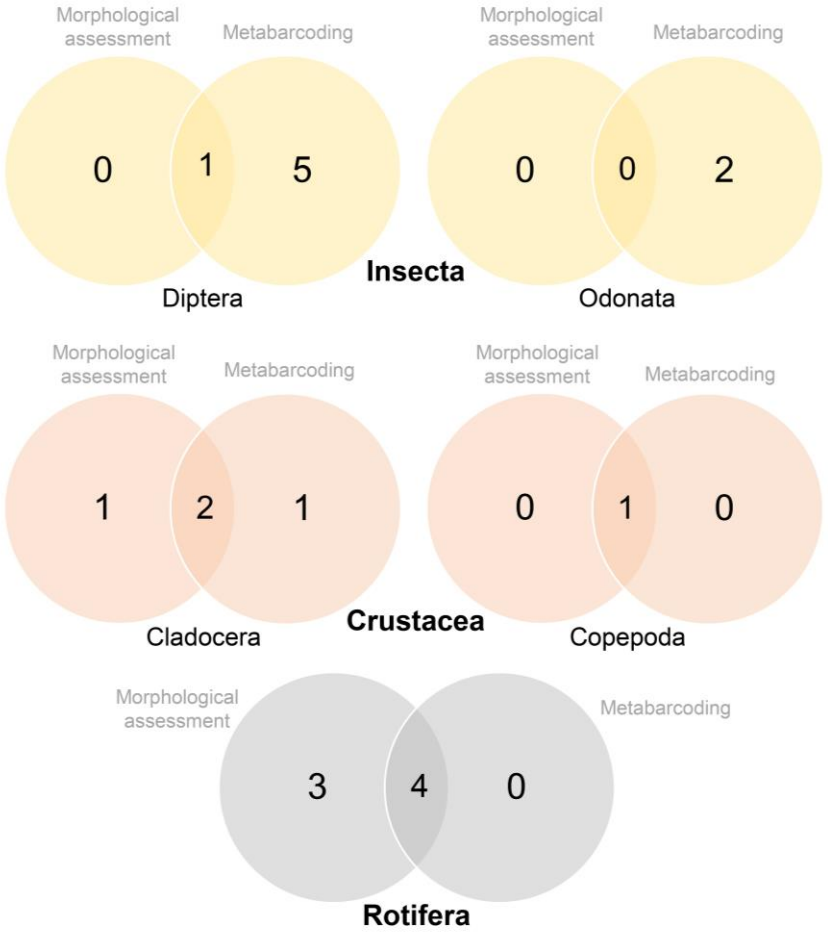


Figure 3.4. (a) Species and relative number of haplotypes estimated using a Last Common Ancestor Algorithm (BASTA). **(b)** Total number of haplotypes for rotifers, crustaceans and insects (species detected with the last common ancestor algorithm only) across ponds (relative estimates at the top right and percentages at the bottom right). Mesocosm ponds are coded as: MC (mesotrophic control), Mc (mesotrophic control with final severe pulse), Mg (mesotrophic moderate-glyphosate), MG (mesotrophic high-glyphosate), EC (eutrophic control), Ec (eutrophic control with final severe pulse), Eg (eutrophic moderate-glyphosate), EG (eutrophic high-glyphosate).

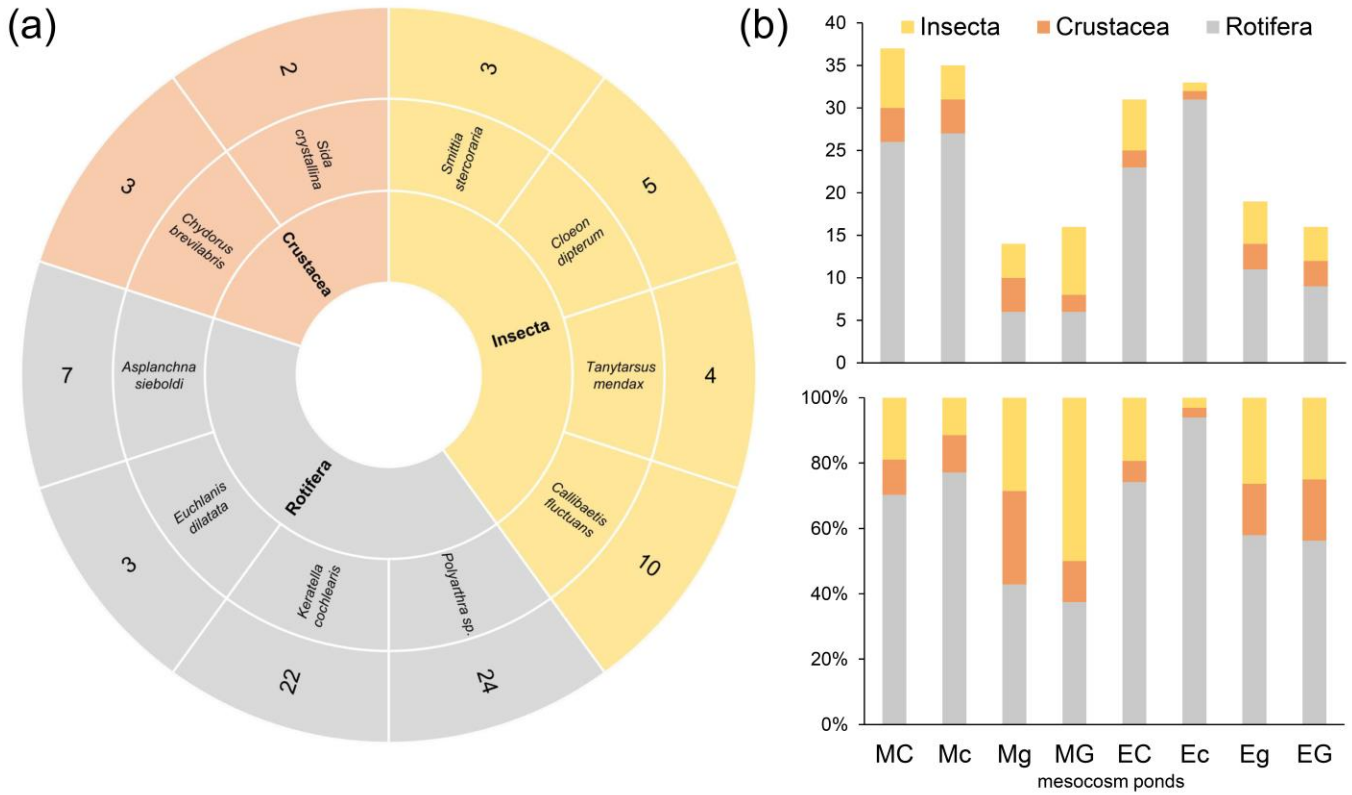
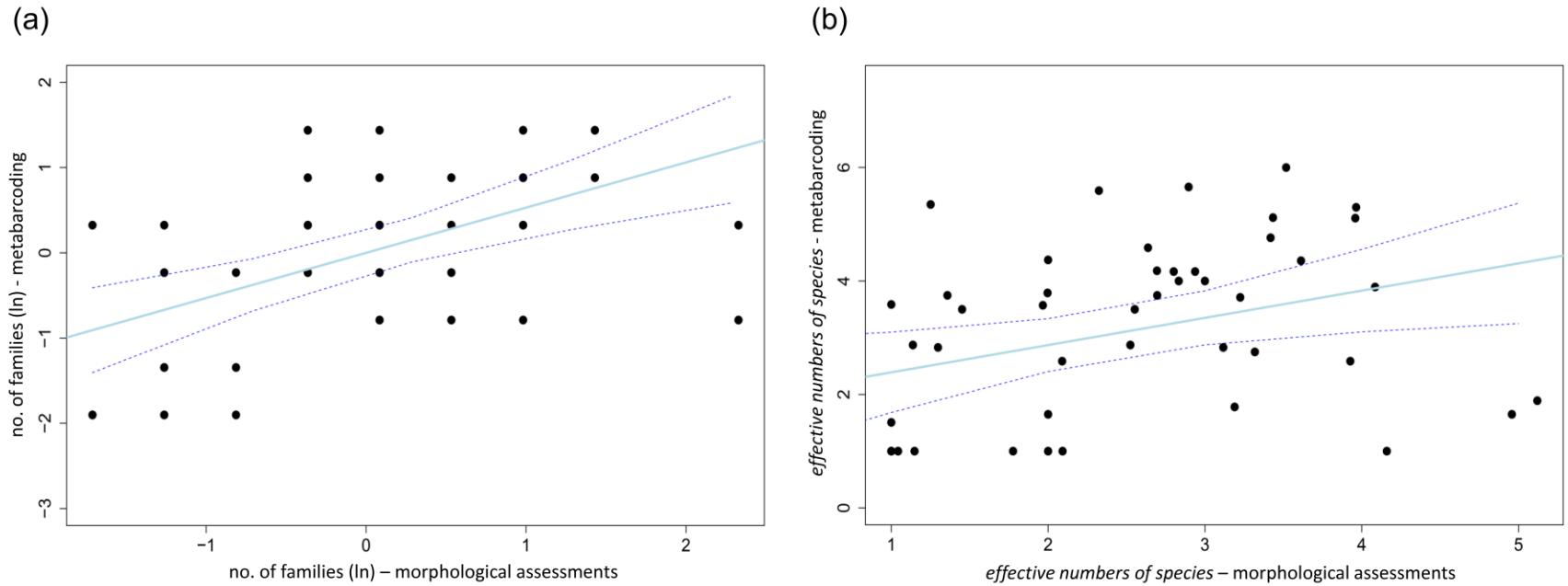
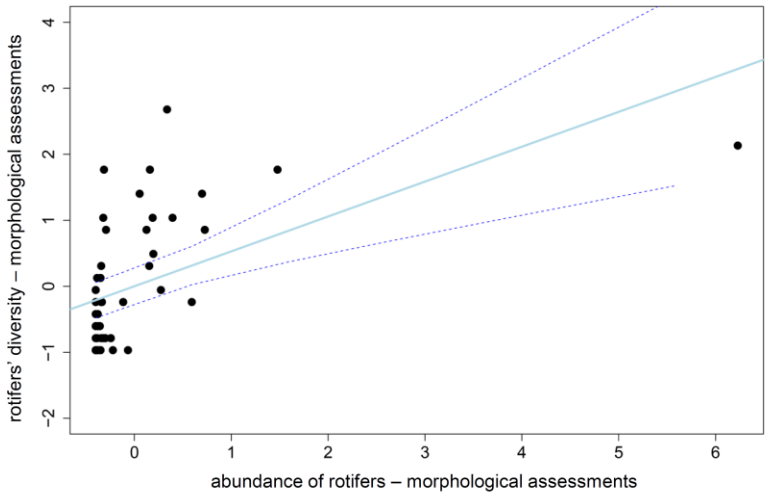


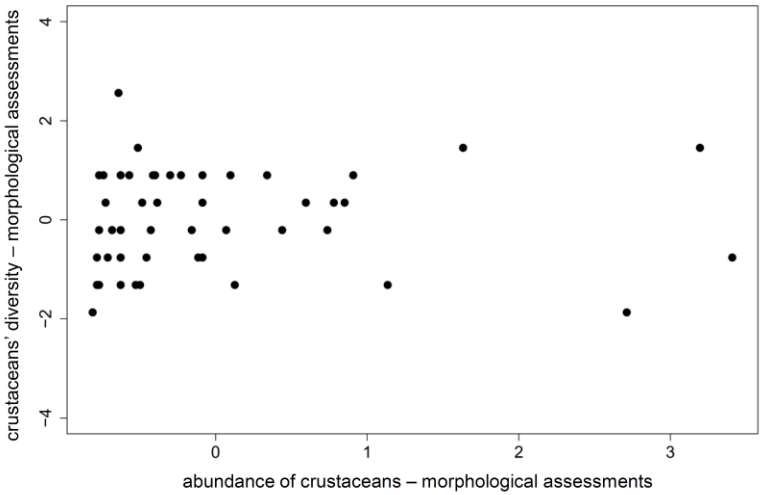
Figure 3.5. Correlation between **(a)** the number of families and **(b)** the *effective numbers of species* (Hill numbers) estimated with metabarcoding with the respective number of families and the *effective numbers of species* estimated morphological assessments. Correlation between the diversity of **(c)** rotifers and **(d)** crustaceans with the respective abundance estimated through morphological assessments. Correlation between the intra-specific genetic variation of the rotifer species **(e)** *Keratella cochlearis* and **(f)** *Polyarthra sp*. with the respective abundance (number of individuals) estimated through morphological assessments.



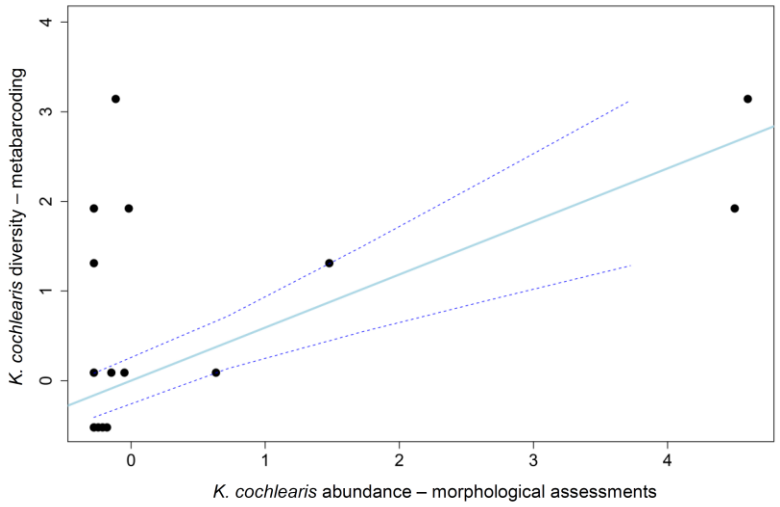
(c)


rotifers' diversity – morphological assessments
abundance of rotifers – morphological assessments
−2
−1
0
1
2
3
4
0
1
2
3
4
5
6

(d)


crustaceans' diversity – morphological assessments
abundance of crustaceans – morphological assessments
−4
−2
0
2
4
0
1
2
3

(e)


K. cochlearis diversity – metabarcoding
K. cochlearis abundance – morphological assessments
0
1
2
3
4

(f)

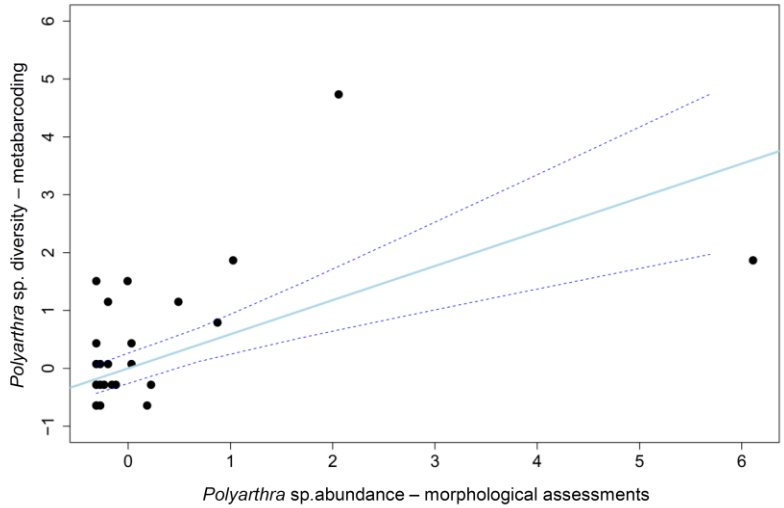
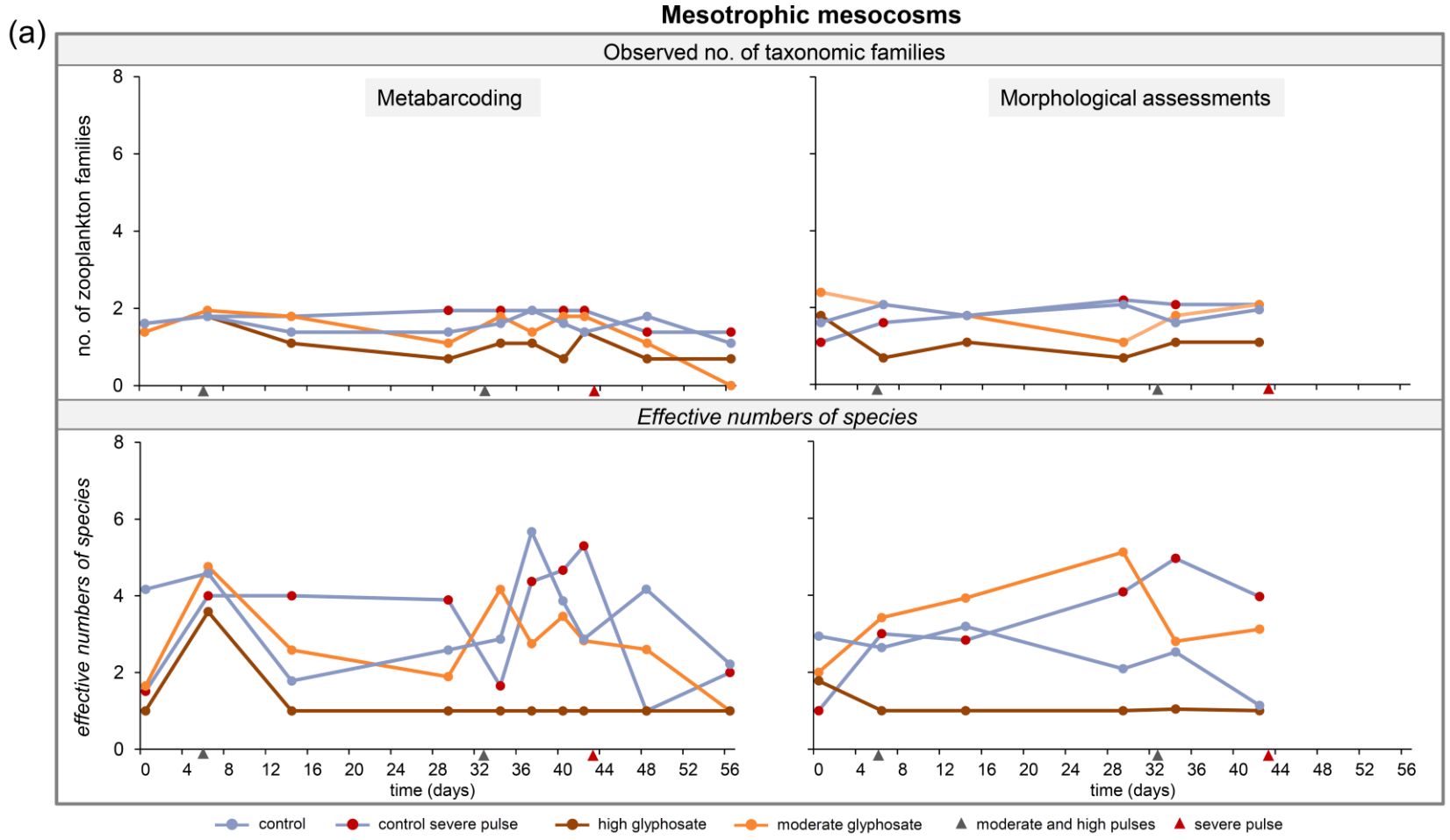

Polyarthra sp. diversity – metabarcoding
Polyarthra sp.abundance – morphological assessments
−1
0
1
2
3
4
5
6

Figure 3.6. Dynamics of the estimated number of taxonomic families and the *effective numbers of species* of rotifers and crustaceans (ln-transformed) obtained from metabarcoding ("zooplankton meta") and morphological identification ("zooplankton morpho") in **(a)** mesotrophic and **(b)** eutrophic mesocosms.



(b)

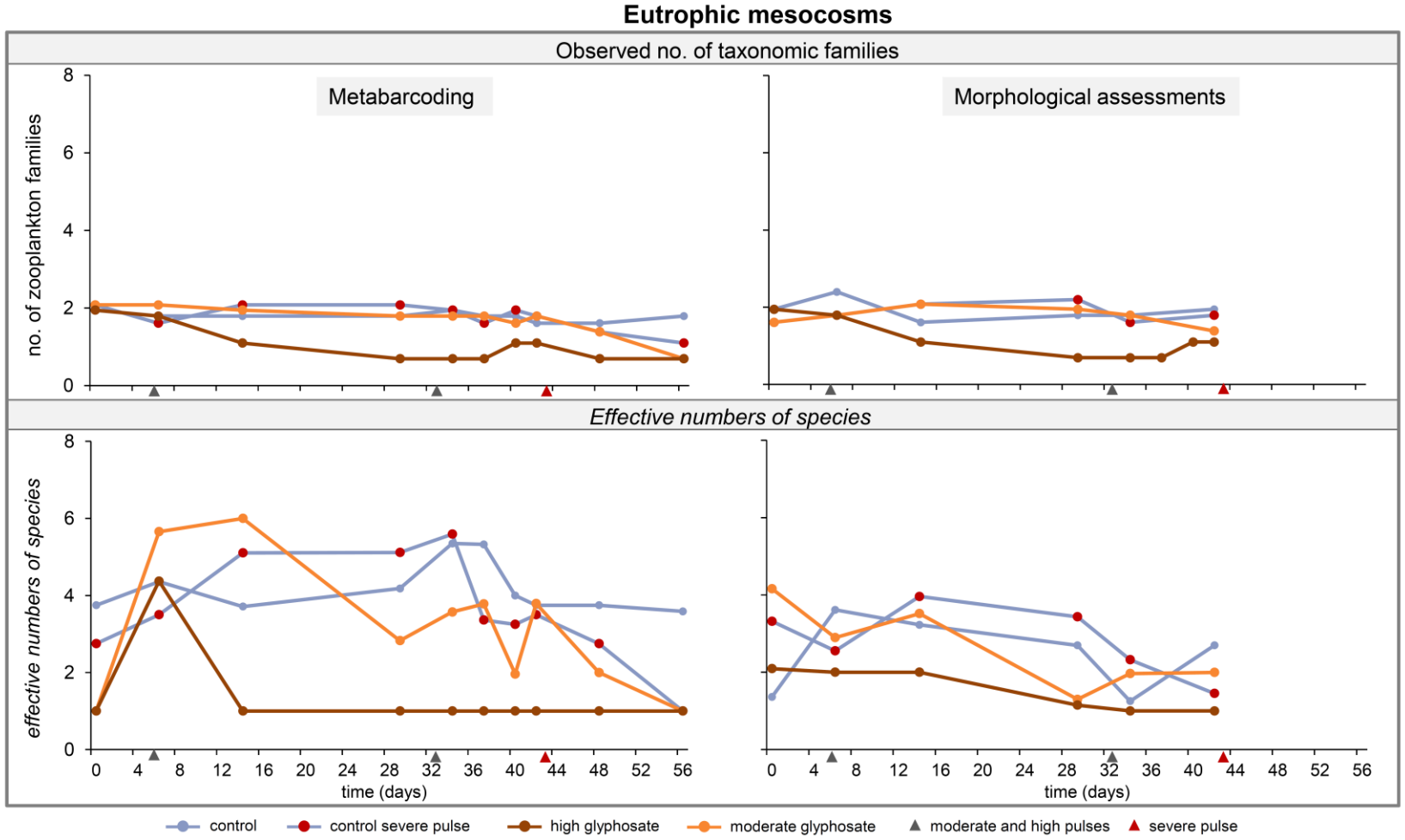

Eutrophic mesocosms
Observed no. of taxonomic families
Metabarcoding
Morphological assessments
no. of zooplankton families
Effective numbers of species
effective numbers of species
time (days)
time (days)
control
control severe pulse
high glyphosate
moderate glyphosate
moderate and high pulses
severe pulse

Figure 3.7. Fixed effects estimates and confidence intervals of best-fit models for **(a)** the total number of taxonomic families and, the *effective numbers of species* **(b)** number of sequences (metabarcoding) and abundance (morphological assessments) of rotifers, crustaceans and insects (metabarcoding only), and **(c)** haplotype diversity in the species of rotifers *Keratella cochlearis* and *Polyarthra* sp.

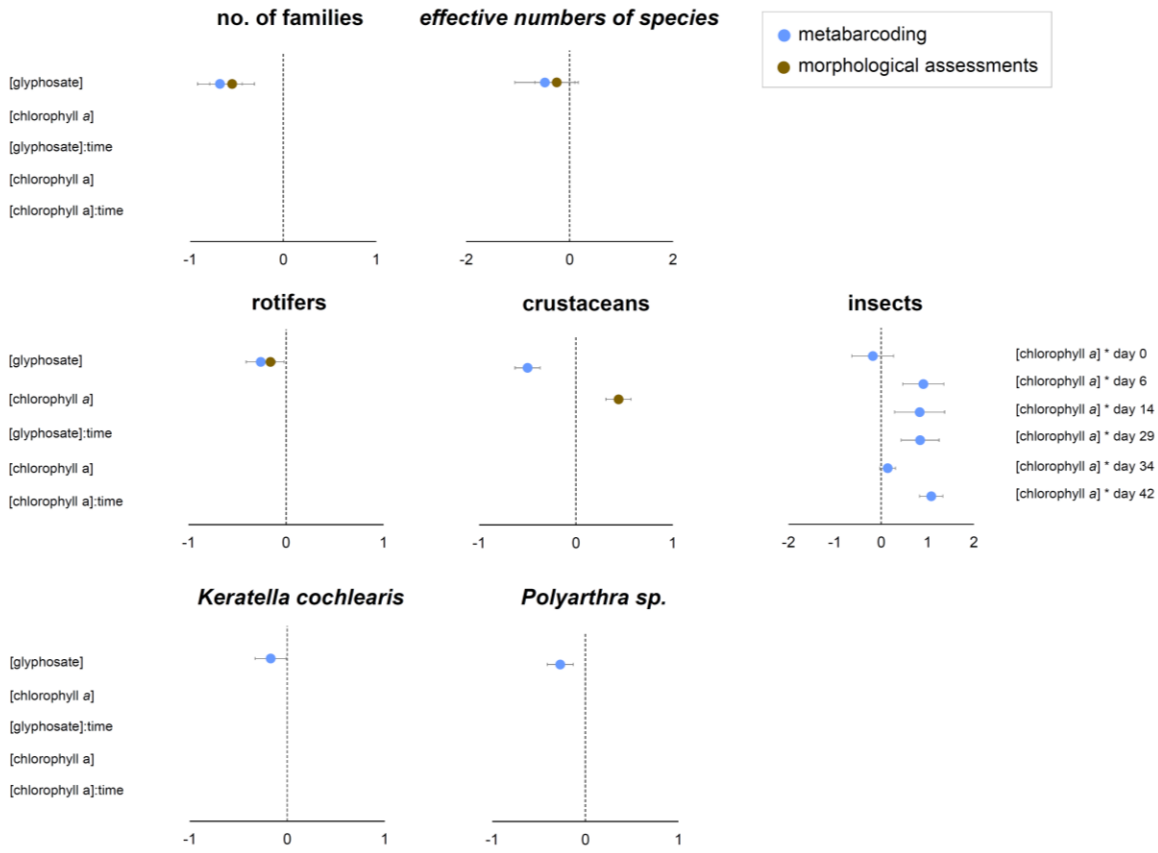
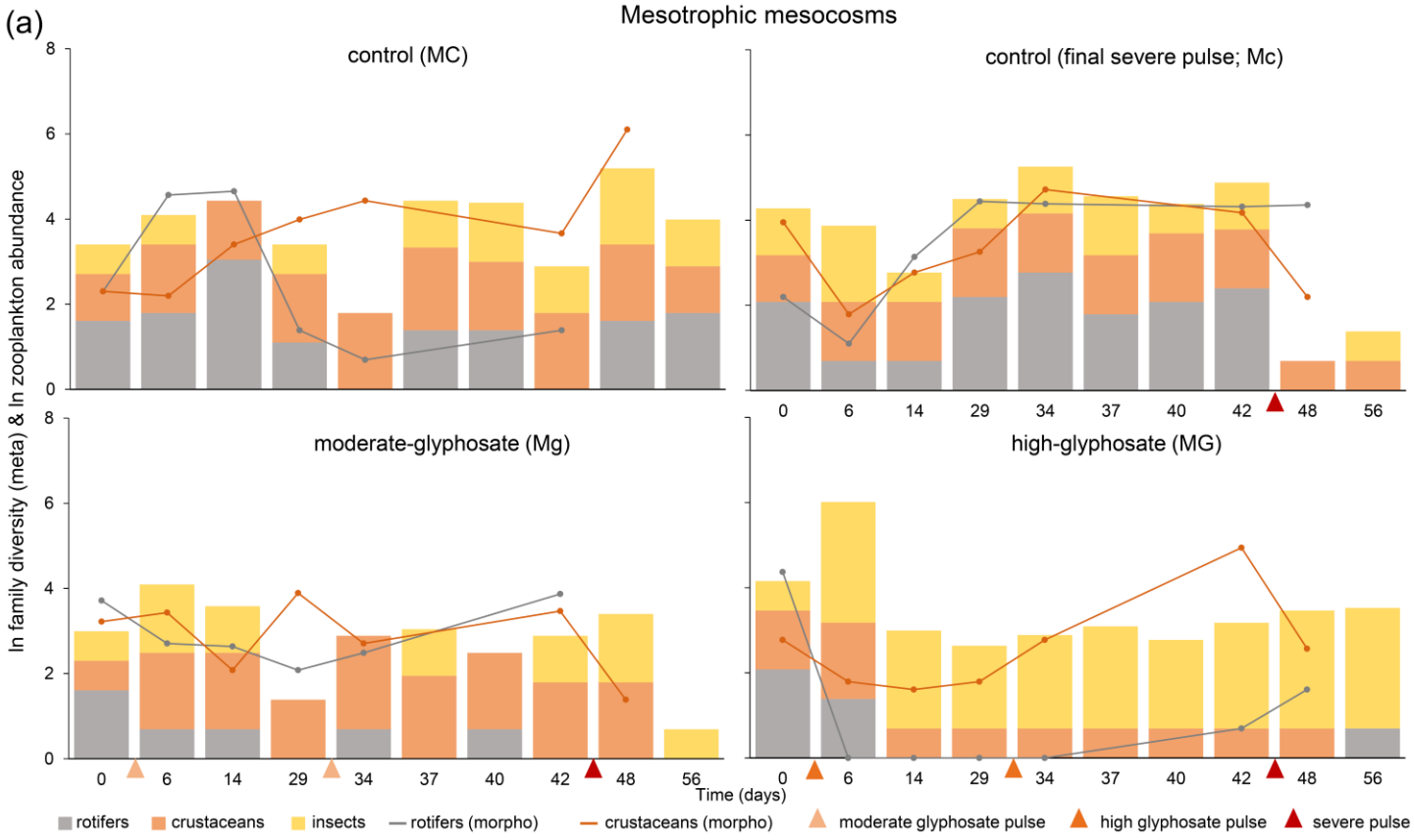
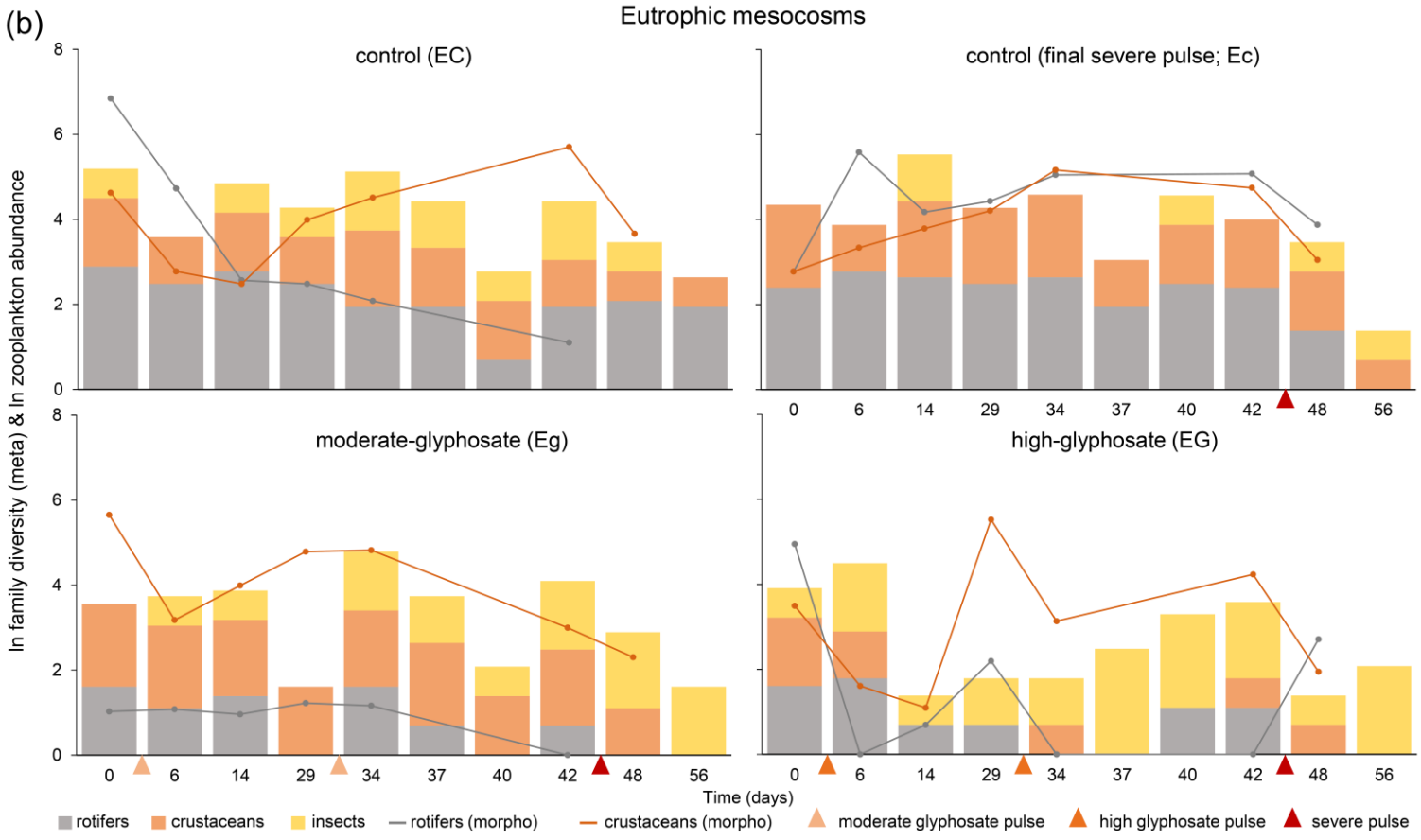


Figure 3.8. Family diversity in rotifers, crustaceans, and insects (ln-transformed number of sequences; "meta") and the relative abundance (ln-transformed number of individuals; "morpho") estimated through morphological assessment for **(a)** mesotrophic and **(b)** eutrophic mesocosms.




(b)
Eutrophic mesocosms
control (EC)
control (final severe pulse; Ec)
moderate-glyphosate (Eg)
high-glyphosate (EG)
ln family diversity (meta) & ln zooplankton abundance
Time (days)
rotifers
crustaceans
insects
rotifers (morpho)
crustaceans (morpho)
moderate glyphosate pulse
high glyphosate pulse
severe pulse

General discussion

Synthesis

In the past half-century global biodiversity has dramatically declined due to human impact on the environment through overexploitation, changes in land and sea use, climate change, pollution, and invasive species (Secretariat of the Convention on Biological Diversity, 2010; Tilman *et al.* 2017). Despite our good knowledge of the major drivers of biodiversity declines and their consequences for ecosystem properties, the role of micro-evolutionary processes remains largely unknown (Carroll *et al.* 2014; Loeuille 2019). Because most of the causes of biodiversity losses involve triggers that can be regulated by government policies, there is much room for action. As such, one of the most urgent steps toward biodiversity conservation is to understand the conditions that favor the persistence of genetic diversity and to define the thresholds in the response of populations and communities to altered genetic diversity (Chapin Iii *et al.* 2000; Brondizio *et al.* 2019).

This thesis explores the ecological, evolutionary and genetic consequences of pollution on biodiversity. Finding evidence of adaptation to pollution requires an integrative approach for testing whether observed increase in resistance to a contaminant is heritable, is under selection, promotes higher fitness and a positive growth rate at the population level. Chapter 1 showed that the integration of demographic assays with quantitative trait methods and molecular genetics provided clearer evidence for adaptive responses to pollution than studies that used only one approach. For example, studies that include measures of population growth rate often reveal negative effects on population trends despite the presence of tolerant phenotypes (Postma & Davids 1995; Haimi *et al.* 2006; Medina *et al.* 2009; Anderson 2013; Dutilleul *et al.* 2014). Unfortunately, studies that employ an integrative approach are in the minority. This underrepresentation, together with inconsistency in the set of approaches and methodologies used to study adaptation make it harder to infer how likely, or how common, population adaptation is in the presence of environmental contamination. Other literature reviews on micro-evolutionary responses to chemical pollution encountered similar challenges (De Coninck *et al.* 2014; Oziolor *et al.* 2016).

Chapter 2 contributes to filling the gap identified in Chapter 1 by combining molecular and demographic approaches in a multi-generation experiment and by testing the contribution of genetic diversity to population persistence of the freshwater microcrustacean *Daphnia* in the presence of copper contamination. *Daphnia* is an ideal model organism for an experimental test of evolutionary rescue (ER) because of its short generation time, ease of culture and control of genetic variability. However, few studies (Stoddard & Harper 2007; Hochmuth *et al.* 2015) have assessed the demographic and genetic effects of a strong selective pressure imposed by metal contamination on *Daphnia* populations differing in their levels of genetic diversity. A potential for adaptation to copper was observed in *Daphnia* and persistence was promoted by clonal diversity. However, no population of *Daphnia* underwent a full ER event. Juvenile stages were more susceptible to copper stress than adults. Selection effects led to substantial genetic erosion which was further exacerbated by genetic drift due to demographic stochasticity. The results of Chapter 2 were in accordance with findings of Chapter 1 (Loria *et al.* 2019) and to the genetic erosion hypothesis that explains the decline in genetic diversity following exposure to metals (Lopes *et al.* 2004; Coors *et al.* 2009).

Perturbations due to contamination by pollutants can cause compositional, structural and functional changes in natural communities that are often subtle and difficult to identify and track using morphological methods. Adopting molecular methods to detect these changes in a fast and sensitive fashion would greatly accelerate our understanding of the response of biodiversity to pollution and consequently the implementation of conservation actions. In Chapter 3, I tested the application of environmental DNA (eDNA) based metabarcoding on natural zooplankton assemblages collected during a mesocosms experiment involving herbicide contamination and eutrophication. Despite discrepancies deriving from false positives and false negatives, metabarcoding showed to complement well traditional methods. It provided diversity estimates of certain taxonomic groups that usually require specific sampling protocols (e.g., insect larvae) and provided estimates of intraspecific genetic variation that were correlated with estimates of abundance estimates obtained with morphological assessments. Ekrem *et al.* (2010), Silva & Wiedenbrug (2014), Lin *et al.* (2018), and Sun *et al.* (2019) observed similarly good performances of metabarcoding in detecting difficult-to-sample taxonomic groups and Serrana *et al.* (2019) too found a positive correlation between intraspecific genetic diversity and abundance estimates obtained with morphological assessments. Metabarcoding revealed negative effects on

zooplankton diversity attributable to herbicide contamination. With additional calibration and improved reference databases metabarcoding can greatly complement traditional approaches in the study of eco-evolutionary responses to pollution.

Contribution to knowledge

This thesis confirms the need for integrative approaches combining knowledge of genetics, ecology, evolution and demography. It highlights how the evidence for adaptation to pollution is fragmented and characterized by unbalanced experimental designs (laboratory-based studies focusing on one life stage over few generations) as well as methodological and publication biases (Chapter 1). Similar shortcomings were observed in relation to climate change, selective harvesting, and landscape alterations (Hansen *et al.* 2012). This thesis shows that genetic diversity had a positive effect on persistence but that adaptation to pollution was limited by detrimental effects on population size and consequently on genetic diversity. It confirmed that an increased negative effect of pollution on early life stages may largely contribute to population extirpation (Chapter 2). This has also been found by McKim (1977), Stark & Banken (1999), Staples *et al.* (2011) and Anderson *et al.* (2013). Finally, this thesis revealed that the combination of traditional freshwater biodiversity assessments with eDNA-based metabarcoding provide a more in-depth picture of how glyphosate pollution and nutrients directly and indirectly affect zooplankton community dynamics (Chapter 3). Specifically, this work provides additional information on the presence and diversity of difficult-to-sample taxa and provided estimates of intraspecific genetic variation. It shows that insects are more abundant in severely impacted ponds (intensification of top-down regulation) and that intraspecific genetic variation of two selected species of rotifers is negatively impacted in the context of glyphosate contamination. The thesis shows that metabarcoding supports empirical evidence of the putative long-term fertilizing effect of glyphosate as found by Fugère *et al*. (2020). Moreover, the positive correlation between intraspecific genetic variation estimated through metabarcoding with estimates of abundance obtained with morphological assessments suggests that, with further validation and calibration, metabarcoding represents a promising tool for the quantitative assessment of changes in abundance of zooplankton taxa.

Significance

This thesis provides a synthesis of the evidence for adaptation to pollution across multiple levels of biodiversity, taxonomic groups, methods, and pollutants. It fills several research gaps including multi-generation studies of rapid evolution at the population level that integrates demography, ecology and evolution. My thesis focused on life in freshwater ecosystems, which represent some of the most productive yet threatened ecosystems by chemical pollution (Costanza *et al.* 1997; Dudgeon *et al.* 2006; Dodds *et al.* 2013). Empirical tests of the ability of *Daphnia* populations to adapt to metal pollution provides not only useful insights on the applicability of ER theory to aquatic invertebrates but also contributes to our understanding of their capacity to adapt to pollution. This work also contributes to the advancement of the fields of evolutionary ecotoxicology and community ecology by testing innovative molecular and bioinformatic techniques. These approaches are likely to advance our understanding of the eco-evolutionary feedbacks between corresponding processes at two scales, the evolutionary recovery of populations and the ecological responses of community to chemical pollution (Chapter 3). Moreover, despite the challenge posed by the relative long persistence of eDNA in the environment, this thesis shows that eDNA can be used to reveal rapid community shifts during environmental stress. Being able to determine the composition of communities and their temporal and spatial shifts together with changes in intra-specific genetic variation allows the design of long-term monitoring programs for endangered populations.

Future directions

To understand the conditions that favor the persistence of genetic diversity, populations, and communities, research efforts should be characterized by integrative approaches combining knowledge of genetics, ecology, evolution and demography (Pelletier *et al.* 2009). The gaps identified in Chapter 1 can be filled by designing long-term experiments characterized by standardized protocols that integrate demographic studies with phenotypic and genetic assessments. Conducting comparable studies will expand our knowledge of the ability of organisms to persist in the presence of anthropogenic stressors. These experiments will also provide future researchers with a multitude of highly comparable and statistically powerful data that can deepen our understanding of complex eco-evolutionary dynamics. As stressed by De Coninck *et al.* (2014), an important factor that could facilitate the comparability of studies and improve their value is the determination of the bioavailability of the pollutants studied. Biotic

and abiotic conditions may largely differ among aquatic environments and this can considerably affect the bioavailability of various substances. Moreover, the bioavailability of transformation and degradation products should also be determined as they can be even more toxic/problematic than the original substance (Belden & Lydy 2000). Glyphosate, for example, represents a source of anthropogenic phosphorus when is degraded which can persist in the environment and contribute to eutrophication (Hébert *et al.* 2019; Fugère *et al.* 2020).

The stage of the life cycle in which organisms are assessed also deserves further attention and when investigating the ability of organisms to develop resistance and survive pollution. Early life stages have shown to be the most sensitive components of aquatic populations (McKim 1977; Anderson *et al.* 2013) and different population structures can lead to different outcomes (Stark & Banken 1999). Experiments should include in-parallel assessments of both adults and juveniles because of their differential ranges of tolerance. As stressed by Anderson *et al.* (2013), these age-related differences should be carefully determined for each toxic substance and taken into account during ecological risk assessments and policy.

Another fundamental aspect in the study of adaptation to pollution is the evaluation of the presence and fate of genetic diversity in populations. Genetic diversity is the basis for evolution by natural selection (Fisher 1930) but, as found by recent research, it has also an ecological significance at the population, community and ecosystem level, sometimes with effects as large as those of inter-specific diversity (Hughes *et al.* 2008). In Chapter 2, genetic diversity promoted population persistence, but it was also severely impacted by copper contamination. Genetic diversity of natural populations is inversely proportional to the rate and severity of environmental stress (Bell 2013) and is predicted to decrease through genetic drift, gene flow, and selection. In order to identify the specific environmental, demographic and genetic conditions that facilitate a population to recover (leaving "phase II" of ER; Carlson *et al.* (2014), the one in which populations are most vulnerable), ER experimental designs should include, within the same experiment, different combinations of intensities of the studied pollutant, initial population sizes, and initial levels of genetic diversity. Moreover, the study of population genetic diversity should not be limited to pollution events. Populations that have been previously impacted can experience long-term ecological consequences, such as an increased sensitivity to novel environmental stresses due to the loss of appropriate genetic variation to deal with new

stressors or to small population sizes (Posthuma & Van Straalen 1993; Van Straalen & Hoffmann 2000; van Straalen & Timmermans 2002; Medina *et al.* 2007) and fitness costs (altered life-history parameters; Belfiore & Anderson 2001; Morgan *et al.* 2007; Ribeiro *et al.* 2012). Monitoring the genetic diversity of natural populations and exploring to what extent pollution matters to ecological and evolutionary dynamics of population persistence during and after contamination is a topic in need of further study.

Another question deserving of further research is the extent to which neutral genetic diversity is correlated to functional trait diversity. For example, by looking at the allele frequencies change of metallothionein genes during the experiment described in Chapter 2 one can evaluate whether resistant genotypes were subjected to selection, and to what extent. The identification of candidate genes that influence the variation in particular traits and that undergo changes in allele frequency in response of pollution is indispensable for a deep understanding of the mechanisms of selection and adaptation (Hoffmann & Daborn 2007; Hoffmann & Willi 2008) to pollution.

In Chapter 3, eDNA-based metabarcoding was shown to be a promising tool in the field of eco-evolutionary biology for its potential to simultaneously capture biological diversity (including intra-specific genetic diversity) of different taxonomic groups including taxa that are usually difficult to sample and classify. However, metabarcoding approaches still require careful validation and interpretation and cannot be naively applied in natural systems without appropriate calibration. There is still great need for more densely populated reference libraries which would allow us to extend detections to more taxonomic groups and refine taxonomic assignments (Hajibabaei *et al.* 2007). The design of markers that target different taxonomic groups and the subsequent use of multiplexed markers would further improve detections and perhaps align diversity estimates obtained with metabarcoding with those obtained through morphological assessments (Zhang *et al.* 2018). Moreover, thoughtful attention needs to be given to the influence of spatial, temporal, and ecological factors on the presence and distribution of eDNA (Cristescu & Hebert 2018). For example, in very polluted environments eDNA could be degraded faster and this would bias diversity estimates; or, in other environments, it could be degraded too slowly creating a virtual overlap of contemporary and past presences (Barnes *et al.* 2014). Moreover, the detection of particular taxa could, in reality, be attributable to the transport of eDNA from other environments. Some of these issues could be alleviated increasing the level

of replication and the use of mock communities, or performing pre-trials testing DNA degradation in different environments. Other issues, such as the general long persistence of eDNA in the environment are more challenging to address. More effort should be given to finding solutions or good alternatives to approaches based on slow-degrading eDNA. For example, eRNA-based markers could detect biotic assemblages more efficiently than eDNA-based techniques. Despite being dismissed in the past for its putative *in vitro* instability, in the light of recent findings (Laroche *et al.* 2016; Pochon *et al.* 2017), eRNA-based techniques are being re-evaluated and found to be promising tools for biodiversity monitoring (Cristescu 2019) especially when paired with eDNA-based techniques (Laroche *et al.* 2016; Cristescu & Hebert 2018). Due to the multitude of research opportunities that eDNA metabarcoding offers, there has been a rise in publications involving metabarcoding techniques in the last few years (Thomsen & Willerslev 2015; Barnes & Turner 2016). This has also created a multitude of protocols and pipelines whose evaluation and comparison is needed.

The above recommendations could further advance the study of biodiversity and the factors that can promote evolutionary resilience in response to pollution. Moreover, given the links between biological diversity and the stability of communities and ecosystems (Gonzalez & Loreau 2009), it is presumed that by preserving biodiversity and its components we could limit the impact of human-induced environmental stressors on ecosystem functions (Isbell *et al.* 2015). In the long run, results from eco-evolutionary studies will contribute to the understanding of how biodiversity is changing and how we can best invest our efforts to conserve it in the future.

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Appendix A

Supplementary material for Chapter 1

The use of molecular markers

Molecular markers represent an essential tool in studies on evolutionary change (Klerks *et al.* 2011). They can be used for the assessment of the genetic diversity and genetic structure at the population level, to evaluate the potential to respond by adaptation, and to study the evolution at specific loci under selection (Hoffmann & Daborn 2007; Hoffmann & Willi 2008). The pros and cons of using molecular markers in eco-toxicology have been extensively reviewed (Hoffmann *et al.* 1995; Belfiore & Anderson 1998; Belfiore & Anderson 2001; Hoffmann & Daborn 2007; Monserrat *et al.* 2007; Hoffmann & Willi 2008).

Shifts in allele frequencies at particular loci are one of the most obvious consequences of directional selection (Hoffmann & Daborn 2007). The assessment of allozyme frequency differences between impacted and non-impacted populations was very common especially in the studies carried out between 1992 and 1997 (Fig. A.3). Their use was also common between 1998 and 2003 to follow a sharp decline between 2004 and 2014. Non-specific techniques such as RAPD (random amplified of polymorphic DNA) and RFLP (restriction fragment length polymorphism) surveys were represented especially in the period between 1998 and 2003 with a boom for RAPD studies and 2004-2009 for RFLP. The assessment of genetic diversity through the use of microsatellite markers increased through time thanks to their high levels of polymorphism and ease of application. Early attempts (2004-2009) in identifying genes underlying trait variation were done by the use of molecular tags such as amplified fragment length polymorphisms (AFLP) characterized by a large number of polymorphisms per samples, and single nucleotide polymorphisms (SNPs), distinguished for their high accuracy and large number (2004-2014). The investigation of regulatory changes under control and stressful conditions was another approach used to study adaptive alterations at the level of gene expression. The use of DNA microarrays in relation to pollution prevailed between 2004 and 2009 and the studies were differentiated into the ones that targeted a specific region of the genome for its known role in tolerance and studies that looked at the genome-wide gene expression in order to identify genes involved in responses to pollutants. The majority of the studies that targeted a specific region of the DNA looked at metallothionein genes which encode for cystein-rich proteins involved in the regulation of metal concentrations in the cell playing an important role of detoxification and protection against toxic substances. For example, Sterenborg

and Roelofs (2003) and Timmermans *et al.* (2005) looked at metallothionein expression on the springtail *Orchesella cincta* and found a higher level of expression of these genes in populations from metal contaminated environments. Several studies found evidence of selection on metallothionein genes by looking directly at changes in allele frequencies (Timmermans *et al.* 2007). Additionally, other studies directly measured metallothionein protein concentrations in different tissues of invertebrates and fish and found that the concentration of these proteins was correlated with contamination levels (Moraga *et al.* 2002) or higher in the tissue of individuals from contaminated habitats (Ross *et al.* 2002; Knapen *et al.* 2004). Similar progress in the understanding of the mechanistic basic of resistance has been made in relation to PCBs and dioxin-like contaminants in fish (Hahn *et al.* 2004; Wirgin *et al.* 2011). These types of toxicities are known to be mediated by the aryl hydrocarbon receptor (AHR) which is a ligand-activated transcription factor through which contaminants cause altered gene expression (Hahn *et al.* 2004). Several studies have compared the structure and expression of these molecules between impacted populations and non-impacted populations reviewed in Wirgin & Waldman (2004).

Overall, there was inconsistency among studies that used markers such as allozymes and microsatellites. Despite their relatively high polymorphism levels, they did not always succeed in providing results that reflected predictions (i.e. decreased genetic diversity in impacted populations, shifts in allele frequencies correlated to contamination). Even when predictions were confirmed, links between patterns and causes were not generally easy to demonstrate and remained unknown (Ford 2002). As argued by Belfiore & Anderson (2001) and Staton *et al.* (2001) many other factors may contribute to the genetic changes observed. Mutations, gene flow, genetic drift represent other sources of these changes whose interplay influences the phylo-geography of species' distribution. Population declines due to the detrimental effects of contaminants can also play a substantial role in determining the genetic structure of impacted populations leading to a remarkable random genetic drift.

The identification of candidate genes that influence the variation in particular traits and that undergo changes in allele frequency in response of environmental change is indispensable for a deep understanding of the mechanisms of selection and adaptation (Hoffmann & Daborn, 2007; Hoffmann & Willi, 2008) to pollution. Opportunities for the identification of genes under selection increased in the last years due to the recent advances in next generation sequencing

techniques. Genomics, transcriptomics, and proteomics technologies will finally revolutionize discovery-based research thanks to their efficiency and relatively low costs (Morozova & Marra 2008; Davey *et al.* 2011).

Table A.1. Summary table of the studies reviewed, with information about the species tested, the type of pollution studied, the type of the approach or response measured, the major findings, and whether the study took place in the field or under laboratory conditions. The type of pollution list can include metals that have been tested in the lab but also metals present in the sampling site. Field* means that the populations were sampled from the field and samples were sacrificed for molecular analyses. When the source is not specified, the findings refer to the populations from contaminated sites.

Organism	Type of pollution	Type of response measured	Main findings	Lab or field	Reference
PLANTS					
Angiosperms					
Acer pseudoplatanus	Heavy metals (Cu)	Growth of callus tissue	Persistence metal tolerance trait (also after growing in uncontaminated media)	Lab	Turner and Dickinson, 1993
Acer rubrum	Heavy metals (Cd, Co, Cu, Ni) and P	Life-history traits: above-ground size, base stem diameter, dry mass, total leaf area, stem diameter, rooting depth, dry mass, leaf dry matter content (LDMC), turgescent fresh mass, specific leaf area (SLA)	Decreased growth in uncontaminated substrates but small difference between controls	Outdoor with experimental sand	Kirkey *et al.*, 2012

Arabidopsis arenosa	Heavy metals (Cd, Pb, Zn)	Biometric measurements: # of leaves/plant, length and width of 5 leaves of each plant, height of the plants, # reproductive shoots and flowers/plant, length of 5 siliques/plant, # seed/silique, weight of seeds, root test, cytogenetic analysis	Significant morphological differences, higher tolerance in the pop from contaminated site	Field and lab	Przedpelska and Wierzbicka 2007
Arabidopsis sp.	Radiation (Chernobyl), other mutagens	Transient recombination assay, expression of radical scavenging and DNA-repair genes, genome methylation	Lower frequency of extrachromosomal homologous recombination, significant differences in radical scavenging and DNA-repair genes expression, higher level of methylation in the pop from contaminated site	Lab	Kovalchuk *et al.,* 2004
Arabidopsis halleri	Heavy metals (Zn)	Transcriptomics, real time RT-PCR	High constitutive expression of metal homeostasis genes in the shoots, differences between root and shoot transcript profile. Transcript abundance higher than *A. thaliana*	Lab	Becher *et al.,* 2004
Arabidopsis halleri	Heavy metals (Zn)	Quantitative trait loci (QTL) analysis	High broad sense heritability, 5/70 significant QTLs	Lab	Frérot *et al.,* 2010

Arabidopsis halleri	Heavy metals (Cd, Zn)	Sequencing of 13 DNA segments across the HMA4 region, quantitative PCR	Enhanced gene product dosage. Higher transcript level than *A. Thaliana*	Lab	Hanikenne *et al.*, 2013
Arabidopsis halleri	Heavy metals (Zn)	RNA interference to downregulate HMA4 (HeavyMETAL ATIPASE4) expression	Zn hyperaccumulation and full hypertolerance to Cd and Zn depends on the metal pump HMA4 (combination of modified cis-regulatory sequences and copy number expansion in comparison to *A. Thaliana)*	Lab	Hanikenne *et al.*, 2008
Arabidopsis halleri	Heavy metals (Cd, Pb, Zn)	Molecular markers (AFLP), neutrality tests	Four loci departed from neutrality	Lab and field*	Meyer *et al.*, 2009
Arabidopsis halleri	Heavy metals (Zn)	Molecular markers (RFLP on chloroplastDNA)	No founding effects, similarity between close pop instead of adapted or reference	Lab and field*	Pauwels *et al*., 2005
Arabidopsis halleri	Heavy metals (Cd, Pb, Zn)	Molecular markers (5 microsats)	High clonal diversity, limited seed dispersal. Clonal spread was more extensive in the lowly polluted zone but no evidence of genetic divergence due to heavy metal heterogeneity	Lab and field*	Van Rossum *et al.*, 2004
Arabidopsis halleri	Heavy metals (Zn)	QTL analysis, Effective concentration (EC) 100, molecular markers (65 sequence-based markers and 18 AFLP)	At all QTL positions Zn tolerance was enhanced by *A. halleri* alleles	Lab and greenhouse	Willems *et al.*, 2007

Arabidopsis thaliana	Heavy metals (Zn)	Transcriptomics, real time RT-PCR	Transcript abundances of several genes much higher in *A. Halleri* after 4 days of Zn exposure	Lab	Becher *et al.*, 2004
Arabidopsis thaliana	Heavy metals (Cs)	Selection exp, life-history traits (root elongation, dry weight), Cs content, molecular markers (nptII gene conferring kanamycin resistance)	Decreased root elongation in the absence of Cs; ability to develop aerial organs in the presence of Cs (control did not)	Lab	Marmiroli *et al.*, 2009
Betula papyrifera	Heavy metals (Cd, Co, Cu, Ni) and P	Life-history traits: above-ground size, base stem diameter, dry mass; total leaf area, stem diameter, rooting depth, dry mass, leaf dry matter content (LDMC), turgescent fresh mass, specific leaf area	Decreased growth in uncontaminated substrates	Outdoor with experimental sand	Kirkey *et al.*, 2012
Betula pubescens	Heavy metals (Cu, Ni)	Survival (seeds), life-history traits (seedling height, length, chlorophyll fluorescence)	Reduced performance in pristine conditions	Greenhouse	Eranen 2008
Biscutella laevigata	Heavy metals (calamine waste heap)	Metal uptake, mineral status, molecular markers (AFLP)	No significant change in metal uptake. Equal genetic variability	Lab and field*	Wasowicz *et al.* 2014
Biscutella laevigata	Heavy metals (Pb, Zn)	Life-history traits (leaf length, width and color, degree of coverage of hairs on leaf, thickness of palisade and spongy mesophyl cells), root measurement, tolerance index	Growth was stimulated by Pb and Zn in pop from contaminated site	Lab and field	Wierzbicka and Panufnik 2004

Calamagrostis epigejos	Heavy metals (Cd, Cu, Pb, Zn)	Life-history traits (root elongation, biomass of roots, rhizomes, shoots, and tb), tolerance index (TI)	Cu: significant higher TI, higher performance in high Cu in pop from contaminated site Tolerance: Cu > Cd > Zn > Pb	Lab (growth chamber)	Lehmann and Rebele 2004
Cynodon dactylon	Heavy metals (Cd)	Molecular markers (67 microsats), turf quality, transpiration rate, chlorophyll content, leaf water content and growth rate	Wide phenotypic variation. The majority of accessions from the same/adjacent region clustered into the same groups	Lab and field*	Xie *et al.* 2014
Dianthus carthusianorum	Waste heap (Pb, Zn)	Life-history traits (# of leaves, average leaf length and width, length of inflorescence), accumulation of proline, anthocyanins, and photosynthetic pigments. Molecular markers (17 RAPD and 4 ISSR)	Persistent significant differences in leaf size and shape and genetic differences	Lab and field*	Wójcik *et al.* 2013
Dianthus carthusianorum	Heavy metals (Pb, Zn)	Life-history traits (leaf length and width, # of flowers per inflorescence). Root tolerance test	Lower weight of aerial parts, shorter and narrower leaves, smaller # of leaves/plant	Lab	Zalęcka and Wierzbicka 2002
Elodea nuttallii	Heavy metals (Cd, Hg)	Gene expression with next generation sequencing (NGS)	Subset of metal responsive genes able to assess metal contamination	Lab	Regier *et al.* 2013

Elsholtzia haichowensis	Heavy metals (Cu)	Metal content, chlorophyll content, electrolyte leakage, antioxidant enzyme assay	Chlorophyll content and electrolyte leakage less affected by Cu and lower Cu content in pop from contaminated site. Antioxidant enzyme activity was induced	Lab	Liu and Xiong 2005
Elymus repens	Heavy metals (Cd, Cu, Pb, Zn)	Life-history traits (root elongation, biomass of roots, rhizomes and shoots), tolerance index (TI)	Zn: more tolerant than *C. Epigejos* Tolerance: Cu > Cd > Pb > Zn	Lab (growth chamber)	Lehmann and Rebele 2004
Eucalyptus calophylla	Metals (Al), acidification	Survival, molecular markers (10 allozymes)	No significant differences in allele frequencies and no differences in survival	Lab and field*	Egerton-Warburton 1995
Eucalyptus patens	Metals (Al), acidification	Survival, molecular markers (10 allozymes)	No significant differences in allele frequencies and no differences in survival	Lab and field*	Egerton-Warburton 1995
Eucalyptus rudis	Metals (Al), acidification	Survival, molecular markers (11 allozymes)	No significant differences in allele frequencies and no differences in survival	Lab and field*	Egerton-Warburton 1995
Hordeum vulgare	Heavy metals (Cd)	Enzyme activity	Induction of antioxidant enzymes and increase in the level of proteins	Lab	Patra and Panda 1998
Mimulus guttatus	Heavy metals (Cu)	Life-history traits (length of roots), selection exp (known tolerance gene present)	Variation in root length detected at higher levels of copper is heritable	Lab	Macnair *et al.*, 1993

Mimulus luteus	Heavy metals (Cu)	Life-history traits (root # and length)	Root # and length strongly inhibited in pop from reference sites	Lab	Ginocchio *et al.*, 2002
Plantago arenaria	Heavy metals (Cd, Cu, Ni, Zn)	Metal content, root bending assay	Presence of constitutive tolerance except for Cu (adaptive)	Lab	Remon *et al.*, 2007
Poa annua	Organic chemicals	Molecular markers (5 allozymes)	Higher # of heterozygous in the polluted sites	Lab and field*	Chen *et al.*, 2003
Prosopis sp.	Heavy metals (Cu)	Survival, metal uptake, individual growth	Higher survival, faster growth and metal content in pop from contaminated site	Lab	Haque *et al.*, 2009
Sedum alfredii	Heavy metals (Cd, Pb, Zn)	Metal accumulation, molecular markers (17 RAPD)	Reduced genetic diversity, high variation in metal accumulation	Lab and field*	Deng *et al.*, 2007
Silene dioica	Heavy metals (Cu)	Mineral nutrient concentration, metal content, phenolic metabolism-related parameters, oxidative stress-related proteins, estimation of free aminoacids	Highest tolerance in pop from the locality with the highest soil Cu content (soluble proteins least affected, enhanced enzyme activity)	Lab	Kováčik *et al.*, 2010
Silene paradoxa	Heavy metals (Cu)	Molecular markers (5 microsats)	Independent colonization events from serpentine populations, grouping of populations according to geographical location	Lab and field*	Mengoni *et al.*, 2001
Silene paradoxa	Heavy metals (Cu, Ni)	Molecular markers (8 RAPD)	Distribution of genetic polymorphism related to the tolerance to Cu and location. Two bands exclusive to Cu-tolerant populations	Lab and field*	Mengoni *et al.*, 2000

Silene vulgaris	Heavy metals (Cu)	Mineral nutrient concentration, metal content, phenolic metabolism-related parameters, oxidative stress-related proteins, estimation of free aminoacids	Higher tolerance in pop from contaminated sites	Lab	Kováčik *et al.*, 2010
Silene vulgaris	Heavy metals (Cd, Cu, Zn)	Root growth (qualitative), cross tests	Few specific major genes involved	Lab	Schat *et al.*, 1996
Silene vulgaris	Calamine waste heap	Life-history traits: leaf width, thickness, palisade and spongy mesophylls measurements, cell surface estimate, size of epidermal cells and # of stomata, individual growth rate. Fresh and dry weight of roots and shoots, metal content, root hairs	Faster growth, thicker and narrower leaves, trailing shoots of small diameters in pop from contaminated site	Lab and field	Wierzbicka and Panufnik 1998
Taraxacum officinale	Heavy metals (Cd, Fe, Ni, Pb), airborne particulate matter (PM10)	Molecular markers (variable-number-tandem-repeat loci), metal content	Negative correlation between the # of genotypes at a site and increasing amounts of PM10, concentrations of five soil metals (Cd, Cu, Fe, Ni and Pb), leaf tissue levels of Fe	Lab and field*	Keane *et al.*, 2005

T*hlaspi caerulescens*	Heavy metals (Cd, Zn)	Molecular markers (3CAPS and 7 microsats)	Genetic differentiation linked to heavy metal concentrations at some candidate loci (gene encoding metal transporter)	Lab and field*	Besnard *et al.,* 2009
T*hlaspi caerulescens*	Heavy metals (Zn)	Molecular markers (4 isozymes)	Tolerance might have evolved twice in populations from different areas	Lab and field*	Koch *et al.,* 1998
Typha latifolia	Organic and inorganic chemicals	Variable-number-tandem-repeat (VNTR)	Higher genetic diversity in the two most polluted sites	Lab and field*	Keane *et al.,* 1999
Viola tricolor	Heavy metals (Cd, Pb, Zn)	ISSR PCR fingerprinting	Higher genetic polymorphism and gene diversity in pop from contaminated sites	Lab and field*	Slomka *et al.,* 2011
Bryophyta					
Ceratodon purpureus	Heavy metals (Cd, Cu, Pb, Zn)	Gametophytic growth, reproductive expression	Higher protonemal growth in pop near a smelter	Lab	Jules and Shaw 1994
Pynophyta					
Picea abies	Heavy metals (Pb, Zn)	Survival, molecular markers (8 isozymes)	Heterozygote frequencies at four loci increased in the surviving seedling	Lab and field	Bergmann and Hosius 1996
Picea abies	Sulphur (S)	Molecular markers (10 isoenzymes)	Higher heterozygosity and genotypic polymorphism index in the tolerant pop	Lab and field*	Prus-Glowacki and Godzik 1995

Picea rubens	Sulphate pollution (and climate change)	Molecular markers (33 SNPs and 9 microsats)	Three out of seven SNPs strongly associated with pollution class	Lab and field*	Bashalkhanov *et al.*, 2013
Pinus sylvestris	Radionuclide	Aberrant cells in the root meristem of germinated seeds, reproductive ability (frequency of abortive seeds)	No consistent difference in reproductive ability	Lab and field*	Gera's kin *et al.*, 2011
Pinus sylvestris	Radionuclide	Molecular markers (5 isozymes)	All indices of genetic variability increased with the dose absorbed	Lab and field*	Gera's kin *et al.*, 2010
Pinus sylvestris	Fertilizers, organic dyes	Molecular markers (9 allozymes)	Lower genetic diversity close to the source of pollution	Lab and field*	Korshikov *et al.*, 2002
Pinus sylvestris	Radiation (Chernobyl)	Molecular markers (222 AFLP)	6% of loci (15/222) identified as candidates for selective responses	Lab and field*	Kuchma and Finkeldey 2011
Pinus sylvestris	Heavy metals (Cd, Pb)	Molecular markers (18 isoenzymes), cytogenetic analysis	Higher # of genotypes in the sensitive pop but lower heterozygosity	Lab and field*	Prus-Glowacki *et al.*,2006
Pinus sylvestris	Heavy metals (Cu)	Molecular markers (allozymes), metal content	Higher genetic variation than control pop	Lab and field*	Prus-Glowacki *et al.*,1999
Pinus sylvestris	Oxides of Sulphur (S)	Molecular markers (9 isoenzymes)	Populations with a greater heterozygosity were more tolerant to pollution	Lab and field*	Wojnicka-Póltorak 1997

INVERTEBRATES

Annelida

Aporectodea caliginosa	Pesticides	Weight, detoxification enzymes analysis, energy resources analysis	Activities of two enzymes increased with contamination. Pre-exposure accelerated activation of detoxification activities	Lab and field	Givaudan *et al.*, 2014
Aporectodea chlorotica	Pesticides	Weight, detoxification enzymes analysis, energy resources analysis	Stress was reflected in depletion of energy reserves. Pre-exposure accelerated activation of the detoxification enzyme sGST towards epoxiconazole	Lab and field	Givaudan *et al.*, 2014
Aporectodea tuberculata	Heavy metals (Cu, Zn)	Biomarkers: metallothionein (MT), Cytochrome P4501A (CYP1A) and glutathione-S-transferase (GST)	MT concentration and the other protein activities decreased with increasing distance from the smelter	Lab	Lukkari *et al.*, 2004
Cognettia sphagnetorum	Heavy metals (Cu)	Survival, body size at reproduction, # fragments produced, individual growth rate, age of reproduction, age of death), pop growth, CO_2 production, Molecular markers (9 allozymes)	Slower growth rate, fewer fragments of larger size, slower pop growth rate, reduced genetic diversity in the pop from contaminated site	Lab and field*	Haimi *et al.*, 2006
Cognettia sphagnetorum	Heavy metals (Cu)	Survival, segments of worms, body size, density of worms	Higher survival in pop from contaminated site	Lab	Salminen and Haimi 2001

Dendrobaena octaedra	Heavy metals (Cu, Zn)	Survival, weight, cocoon production, metallothionein level	No significant differences between contaminated and reference sites	Lab	Bengtsson *et al.*, 1992
Dendrobaena octaedra	Heavy metals (Cu)	Gene expression of 6 genes potentially involved in resistance	Up-regulation in pop from contaminated sites	Lab	Fisker *et al.*, 2013
Dendrobaena octaedra	Heavy metals (Cu)	Survival, life-history traits (mean weight, mean cocoon production per adult), estimates of growth rate, hatchability	Higher individual growth rate, reduced time to maturity, increased reproduction, and increased mortality of pop from polluted area in both control and polluted environment	Lab	Fisker *et al.*, 2011
Dendrobaena octaedra	Heavy metals (Cd)	Hatching rate, Cd content, body mass changes, life-history traits (maturation and 1st repro, cocoon production, cocoon mass), survival analysis (longevity, survival time of F1, hazard rate, median life expectancy)	Heritable higher reproduction and higher survival	Lab	Rozen 2006
Dendrodrilus rubidus	Heavy metals (Cu)	Survival, weight, earthworm condition index (Langdon *et al*. 1999; health assessment), metal content	Higher tolerance, higher metal content and less change in weight in pop from contaminated sites	Lab	Arnold *et al.*, 2008

Dendrodrilus rubidus	Arsenic (As)	Comet assay (measurement of DNA damage), internal content and speciation	Very high levels of As observed in the treatments	Lab	Button *et al.*, 2012
Eisenia fetida	Heavy metals (Cd, Pb, Zn)	Metal content. Gene expression of four genes	Response of Cd-mt gene and accumulation of Cd in worms consistent with [Cd]	Lab	Brulle *et al.*, 2011
Eisenia fetida	Heavy metals (Cd, Pb, Zn)	Gene expression	Three candidate genes strongly induced in the treatments	Lab	Brulle *et al.*, 2008
Hediste diversicolor	Heavy metals (Cu)	Survival, molecular markers (6 allozymes)	Specific alleles linked to lower mortalities	Lab	Virgilio and Abbiati 2004
Limnodrilus hoffmeisteri	Heavy metals (Cd, Co, Ni)	Estimates of extra genetic variance, survival time	Single segregating genetic factor underlies the resistance to heavy metals	Lab	Martinez and Levinton 1996
Lumbricus castaneous	Arsenic (As)	Comet assay (measurement of DNA damage), internal content and speciation	Very high levels of As observed in the treatments	Lab	Button *et al.*, 2012
Lumbricus rubellus	Arsenic (As)	Survival, weight, food consumption, cocoon production rate, metal content, pop growth rate estimates, molecular marker (COI)	Three clades. Sensitivities changed based on life-history stages but not among clades. Risk of extinction at environmentally relevant concentration	Lab	Anderson *et al.*, 2013
Lumbricus rubellus	Heavy metals (Pb, Zn)	Metal content and partitioning profile, molecular markers (COII)	Fewer haplotypes in the lineages from mine sites	Lab and field*	Andre *et al.*, 2010

Lumbricus rubellus	Arsenic (As) and heavy metals (Cu)	Molecular markers (COI and AFLP), DNA methylation analysis	The association between metylation sensitive AFLP(me-AFLP) and soil As levels differed in the two lineages. Epigenetic mechanisms in lineage B and genetic processes in lineage A (no strong association between me-AFLP and As levels in soil)	Lab and field*	Kille *et al.*, 2013
Lumbricus rubellus	Heavy metals (As)	Survival, cocoon viability, x-ray absorption spectra	Higher resistance and cocoon viability in offspring from As-contaminated sites	Lab	Langdon *et al.*, 2009
Lumbricus rubellus	Heavy metals (Zn)	Survival, weight, cocoon production, metal content	No substantial differences	Lab	Spurgeon and Hopkin 1999
Lumbricus rubellus	Heavy metals (Cd, Cu, Pb, Zn)	Survival, weight, maturation time, accumulation and excretion of Zn	Mortality of smelter worms was higher than reference strains	Lab	Spurgeon and Hopkin 2000
Lumbricus rubellus	Heavy metals (Pb, Zn)	Fingerprinting amplified messenger RNA (mRNA)	DNA fragments specific to the induction of metal-chelating gene products	Lab and field*	Stürzenbaun *et al.*, 1998
Lumbricus rubellus	Heavy metals (Cd, Pb, Zn)	Expression profile of TCTP (translationally controlled tumour protein)	Expression of TCTP 14 times higher than control	Lab	Stürzenbaun *et al.*, 1998
Tubifex tubifex	Metals (Hg)	Survival, crosses	Worms raised in Hg had a higher LC50 even after 3 generations	Lab	Vidal and Horne 2003

Arthropoda

Agelena labyrinthica	Heavy metals (Cd, Cu, Pb, Zn)	Size, weight, analysis of detoxifying enzymes, metal content	CarE activity was higher in pop from most contaminated site	Lab and field*	Wilczek *et al.*, 2003
Amphibalanus variegatus	Heavy metals (Cu)	Toxicity test (immobilization), metal content, molecular markers (8 AFLP)	Higher tolerance in pop from contaminated sites but no evidence of selection	Lab and field*	Gall *et al.*, 2013
Anopheles gambiae	Heavy metals (Cd, Cu, Pb)	Survival, selection exp, life-history traits (eggs viability, larval and pupal survival, adult emergence, fecundity, net reproductive rate	Lower magnitude of egg viability, larval and pupal survivorship, adult emergence, fecundity and net reproductive rate than the control strain	Lab	Mireji *et al.*, 2010
Attheyella crassa	Heavy metals (Cu, Hg, Pb, Zn), hydrocarbons, antifouling paint	Molecular markers (AFLP), pop size estimates, RNA content analysis, cephalothorax length	Significant decrease in genetic diversity in the treatment, decrease in total abundance but one recovery in one treatment	Lab	Gardeström *et al.*, 2008
Balanus glandula	Heavy metals, pesticides, PAHs	Molecular markers (6 RAPD)	Reduced genetic diversity in impacted sites	Lab and field*	Ma *et al.*, 2000
Bathycletopsyllus sp.	Oil-drilling site	Molecular markers (COI)	Genetic diversity was in the range seen for species both from contaminated and uncontaminated sites	Lab and field*	Gregg *et al.*, 2010

Ceriodaphnia pulchella	Heavy metals (acid effluent with many metals like Al, Fe, Cu, Zn, etc.)	Survival, life-history traits (# of released neonates)	Strong genetically-determined increase in resistance	Lab	Lopes *et al.*, 2005
Chironomus februarius	Heavy metals (Cr, Cu, Ni, Pb, Zn), TPH	Emergence of different species, # individual each species, % total abundance, life-history traits (fecundity)	More flies emerged from the reference site and the reverse pattern occurred at the polluted site	Microcosms in the field	Bahrndorff *et al.*, 2006
Chironomus riparius	Heavy metals (Cd)	Effect of crossbreeding, larval survival and growth rate, length of larvaes	High control mortality, lower larval growth in clean conditions, increased EC50	Lab	Groenendijk et al., 2002
Chironomus riparius	Model pollutant tributyltin (TBT)	Larval mortality, mean emergence time, produced egg masses/female, hatchability of egg masses, pop growth rate, molecular markers (5 microsats)	TBT-exposed strains showed increased larval mortality, slightly reduced reproductive output, and delayed larval development. Reduced genetic diversity in treatments	Lab	Nowak *et al.*, 2009
Chironomus riparius	Heavy metals (Cd)	Survival, life-history traits (#eggs/female, tot # fertile eggs/initial 3 larvae), emergence time, molecular markers (5 microsats)	Genetic variation inversely proportional to tolerance, directly proportional to fitness in Cd conditions	Lab	Nowak *et al.*, 2008
Chironomus riparius	Heavy metals (Cd)	Metal content, Cd excretion, weight	Increased elimination rate	Lab	Postma *et al.*, 1996

Chironomus riparius	Heavy metals (Cd)	Survival, # of emerged males and females. Total # males and females, mortality, dry weight, metal content, # of eggs, pop growth rate	High control mortality, increased larval developmental time but no differences in larval mortality	Lab	Postma *et al.*, 1995
Chironomus riparius	Heavy metals (Cd, Fe, Zn)	Larval survival, growth rate, body length	Slower growth of larvae from contaminated sites in control conditions, different growth responses except for Zn	Lab	Postma *et al.*, 1995
Chironomus riparius	Heavy metals (Cd)	Survival, metal content, life-history traits (mean larval development, # males and females midges in the cage, # deposited egg-ropes, body growth of 1st instar larvae), pop growth rate, emergence time	High mortality in only one generation when NOEC values exceeded	Lab	Postma and Davids 1995
Chironomus riparius	Heavy metals (Cu)	Survival, molecular markers (4 microsats)	Twice LC50 value in pop from contaminated sites. Polluted sites in panmixis	Lab and field*	Soeter *et al.*, 2010
Chironomus riparius	Model pollutant tributyltin (TBT)	Emergence time, dry weight, sex ratio, life-history traits (#eggs/female, #eggs/eggs mass), hatchability, survival, molecular markers (5 microsats)	Larvae with significant tolerance, reproductive output increased in later generation. Non-random alteration in allele distribution	Lab	Vogt *et al.*, 2007

Daphnia longispina	Heavy metals (Cu, Zn), acid mine	Survival, feeding rate	Higher survival but no other differences between populations from contaminated and reference sites	Lab	Agra *et al.*, 2010
Daphnia longispina	Heavy metals (Cu), acid mine drainage	Survival, body length, life-history traits (# neonates/female)	Persistence tolerance (acclimated & not acclimated) genetically determined responses converge from lethal to sublethal toxicant exposures	Lab	Lopes *et al.*, 2006
Daphnia longispina	Heavy metals (Cu), acid mine drainage	Survival, feeding inhibition, life-history traits (time to 1st brood, # neonates/brood, time inter-broods, body length females after 4th brood)	Disappearance of the most sensitive lineages, no directional change in life-cycle traits	Lab	Lopes *et al.*, 2004
Daphnia longispina	Acid mine drainage	Survival, molecular markers (AFLP, contaminant indicative bands)	Significant correlation between individual genetic distance and tolerance	Lab and field*	Martins *et al.*, 2009
Daphnia longispina	Heavy metals (Cu), acid mine drainage	Survival, molecular markers (20 allozymes)	Allozymes not associated with increased resistance	Lab	Martins *et al.*, 2007
Daphnia longispina	Acid mine drainage (AMD)	Molecular markers (8 microsats)	Generally low diversity but higher diversity in the impacted population	Lab and field*	Silva *et al.*, 2010
Daphnia magna	Pesticides	Survival, molecular markers (allozymes)	Differences in susceptibilities and correlation between tolerance levels and land use intensity	Lab and field*	Coors *et al.*, 2009

Daphnia magna	Heavy metals (Cd)	Survival, hsp70 expression, Cd accumulation	Highest EC50 in the clone displaying the lowest hsp70 expression	Lab	Haap and Köhler 2009
Daphnia magna	Heavy metals (Cd)	EC10, reproductive performance, heritability	Very variable heritability among different clones	Lab	Messiaen *et al.*, 2013
Daphnia magna	Heavy metals (Cd) + temperature	Net reproductive rate, animal model (broad and narrow sense heritability, additive genetic variance)	Significant heritability of net reproductive rate under sub-lethal concentration	Lab	Messiaen *et al.*, 2012
Daphnia magna	Heavy metals (Cd)	Survival, life-history traits (time to 1st brood, length of parents at first brood, # of offspring at 1st brood, length of parents at day 21 and total reproduction). Pop growth rate estimates	Genetic correlation between traits was affected by Cd	Lab	Messiaen *et al.*, 2010
Daphnia magna	Heavy metals (Cd)	Survival, selection exp, life-history traits (day first culture brood, # of young produced in each culture), estimates of mean life span, mean # young/female, intrinsic rate of increase), molecular markers (AFLP). Cu, Pb, phenol tolerance test after selection exp	After selection exp: increase in Cd resistance in few generations but reduced size, lower genetic variability and sensitivity to another toxicant	Lab	Ward and Robinson 2005

Daphnia pulex	Heavy metals (Cd)	Microarray, body length, lipid-ovary index, # of clutches, per capita birth rate	Identified genes were associated with Cd-induced phenotypes and pop-level outcomes. Three genes coding for metallothionein	Lab	Shaw *et al.* 2007
Drosophila melanogaster	Heavy metals (Cd)	Survival, selection exp, life-history traits (developmental time, fecundity, emergence weight) crosses and backcrosses	Increased fitness in polluted environment. Crosses: evolved resistance due to a single sex-linked gene	Lab	Shirley and Sibly 1999
Drosophila subobscura	Heavy metals (Pb)	Fecundity, egg-to-adult viability and developmental time	Higher increase in variation in fecundity and developmental time in pop from uncontaminated site	Lab	Kenig *et al.*, 2014
Drosophila subobscura	Heavy metals (Pb)	Fecundity, developmental time, egg-to-adult viability	Higher fecundity, viability, faster egg-to-adult development in pop from most polluted site	Lab	Kenig *et al.*, 2013
Drosophila subobscura	Heavy metals (Pb)	Developmental stability, wings size	Genotypes reared on the highest Pb concentration were in developmental homeostasis	Lab	Kurbalija *et al.*, 2010
Folsomia candida	Heavy metals (Cd)	EC50, Metallothionein-like gene expression	Expression was induced by Cd exposure but not by an oxidative stress	Lab	Nakamori *et al.*,2010

Folsomia candida	Heavy metals (Cd)	Life-history traits (# of juveniles produced), transcriptome analysis	Divergent fitness responses and significant differences between the Cd-affected transcriptomes	Lab	Nota *et al.*, 2013
Folsomia candida	Heavy metals (Ba, Cd, Co, Cr, Pb, Zn), phenanthrene	Methallothionein gene expression	Metallothionein expression induced after exposure to all metals (except Cr)	Lab	Nota *et al.*, 2011
Folsomia candida	Heavy metals (Ba, Cd, Co, Cr, Pb, Zn)	Gene expression analysis (classifier analysis), life-history traits (# of juveniles), EC10 and EC50, filed soil effect on reproduction	188 genes could discriminate between 6 different metals (83% of accuracy in predicting the correct classes for samples)	Lab	Nota *et al.*, 2010
Gammarus fossarum	Heavy metals (Cd)	Survival, sib analysis	No heritability, no additive genetic components	Lab	Chaumot *et al.*, 2009
Helisoma trivolvis	Heavy metals	Morphometric data (body size), molecular markers (5 isozymes)	Body size associated with a particular genotype. Allele selected in the contaminated habitat may be related to contaminant tolerance and body size plasticity	Lab and field*	Benton *et al.*, 1994
Hyalella azteca	Heavy metals (Cd, Zn), acidification	Survival, molecular markers (3 allozymes)	Conflicting data, inconsistent results	Lab	Duan *et al.*, 2001
Hyalella azteca	PAH, fluoranthene contaminants	Survival, molecular markers (3 allozymes)	Alteration of the frequencies of several genotypes	Lab	Duan *et al.*, 2000 II

Hyalella azteca	Pesticides	Molecular markers (COI and 28S), sequencing of voltage-gated sodium channel, gene expression analysis	Point mutations were responsible for differences in sensitivities	Lab	Weston *et al.*, 2013
Isonychia bicolor	Metals (Hg)	Survival, morphometric data (body length), molecular markers (29 allozymes)	Fitness differences to Hg exposure among allozyme variants	Lab and field*	Snyder and Hendricks 1997
Isotoma notabilis	Heavy metals (Cu, Zn)	Survival, growth rate, life-history traits (cumulated # of eggs/replicate, cumulated # of eggs/treatment and mean # of reproductive day), mean maximum length	Produced more juveniles and became more abundant than the reference population in all treatments	Lab	Tranvik *et al.*, 1993
Kiefferulus intertinctus	Heavy metals (Cr, Cu, Ni, Pb, Zn), other TPH	Emergence of different species, # individual each species, % total abundance, life-history traits (fecundity)	Absence of significant site by treatment interaction terms for some species	Microcosms in the field	Bahrndorff *et al.*, 2006
Leander intermedius	Heavy metals (Cd, Cu, Mn, Pb, Zn)	Survival, molecular markers (45 RAPD), metallothionein level	Reduced genetic diversity. All individuals of the species may possess mechanisms to cope with elevated concentrations of metals in their environment	Lab and field*	Ross *et al.*, 2002

Leptodiaptomus minutus	Acidification	Survival, molecular markers (COI)	Reduced variation in pH tolerance compared to the recovery period (strong selection). No variance differences of survival between pre-industrial and acid time period	Lab and field*	Derry *et al.*, 2010
Microarthridion littorale	Pesiticides	Survival	The most common haplotype in contaminated sites was the most tolerant in lab	Lab	Schizas *et al.*, 2001
Nectopsyche albida	Metals (Hg)	Survival, molecular markers (6 allozymes)	Genotypes showing differential sensitivity	Lab	Benton *et al.*, 1992
Nitocra lacustris	Xenobiotics	Adult survival, life-history traits (# of offsprings surviving), molecular markers (RFLP on mtDNA), pop size effect on diversity (on dataset)	Loss of haplotype diversity (when reproductive output decreased) was due to an increase in the most common haplotype	Lab	Street *et al.*, 1998
Onychiurus armatus	Heavy metals (Cu, Zn)	Survival, growth rate, reproduction (cumulated # of eggs/replicate, cumulated # of eggs/treatment and mean # of reproductive day), mean maximum length	Exposed population reached reproductive size faster and laid more eggs than the reference population at all treatment	Lab	Tranvik *et al.*, 1993
Orchesella bifasciata	Heavy metals (Cu, Zn)	Survival, grazing activity, biomarker (70 kDa stress proteins)	High variability even within replicates	Lab	Köhler *et al.*, 1999

Orchesella cincta	Heavy metals (Cd)	Molecular markers (AFLP, microsats, functional MT promoter)	No reduced genetic diversity in impacted sites in neither markers	Lab	Costa *et al.*, 2012
Orchesella cincta	Heavy metals (Cd, Zn, Pb, Cu)	Molecular markers (22 allozymes)	No decreased variation, frequency of Got alleles correlated with metal tolerance	Lab and field*	Frati *et al.*, 1992
Orchesella cincta	Heavy metals (Cd, Cu, Fe, Ni, Pb, Zn)	Metallothionein alleles	One allele was higher in pop from polluted sites. Association between allele frequencies and specific metals	Lab and field*	Janssens *et al.*, 2008
Orchesella cincta	Heavy metals (Cd)	Methallotionein coding region sequencing, oxidative stress inducer, moulting hormone analysis	Deviation from neutral expectation in tolerant pop, promoter allele frequencies differed significantly from reference	Lab and field*	Janssens *et al.*, 2007
Orchesella cincta	Heavy metals (Cd)	Excretion efficiency, metal content, gut pellets analysis, body growth rate	Milder growth reduction upon exposure, probably caused by decreased body concentrations of Cd in pop from contaminated sites	Lab	Posthuma *et al.*, 1992
Orchesella cincta	Heavy metals (Cd)	Survival, excretion efficiency, offspring-parent regression, half-sib analysis	Offspring-parent regressions showed that additive genetic variation for Cd excretion efficiency was present in the population from the reference site	Lab	Posthuma *et al.*, 1993

Orchesella cincta	Heavy metals (Cd)	Survival, life-history traits (body growth, age and weight at 1st reproduction, clutch size)	High control mortality in pop from contaminated site, lower age at 1st reproduction	Lab	Posthuma *et al.*, 1993
Orchesella cincta	Heavy metals (Cd)	Transcriptomics: cDNAs gene expression	Reference population showed a strong signature of stress-induced genome-wide perturbation of gene expression while tolerant ones maintained normal gene expression	Lab	Roelofs *et al.*, 2009
Orchesella cincta	Heavy metals (Cd)	Metallothionein expression and gene expression pattern	Tolerant animals maintained normal gene expression while reference animals showed stress induced gene expression	Lab	Roelofs *et al.*, 2007
Orchesella cincta	Heavy metals (Cd)	Molecular markers (RFLP), mt gene expression (real time RT-PCR) on parents and offspring RNA and subjected to regression analysis	Significant heritability, 8 promoter alleles showed structural variation, 3 alleles showed increased frequencies in families with high mt expression. Another gene involved in stress response	Lab	Roelofs *et al.*, 2006
Orchesella cincta	Heavy metals (Cd)	*Mt* mRNA	*Mt* expression levels higher in pop originating in a polluted site compared to reference pop	Lab	Sterenborg and Roelofs 2003

Orchesella cincta	Heavy metals	Metallothionein gene, molecular markers (SSCP, RFLP)	Selection on metallothionein gene. Analysis of molecular variance assigned a small, but significant amount of the total variance to differences between metal-stressed and non-stressed populations	Lab and field*	Timmermans *et al.*, 2007
Orchesella cincta	Heavy metals (Cd)	Survival, *mt* mRNA expression (RT-PCR)	Five out of eight pop evolved increased Cd tolerance, MT mRNA expression of populations from polluted sites was higher than reference sites but not correlation with [Cd] in the field and with Survival rates (other mechanisms must be involved in prolonged tolerance)	Lab	Timmermans *et al.*, 2005
Orchesella cincta	Heavy metals (Cd)	Metallothionein locus genotyping (RFLP–PCR) and metallothionein induction	higher expression of metallothionein gene in pop from contaminated site and large degree of polymorphism of its promoter	Lab and field*	Van Straalen *et al.*, 2011
Pardosa lugubris	Heavy metals (Cd, Cu, Pb, Zn)	Size, weight, analysis of detoxifying enzymes, metal content	CarE activity was higher in pop from most contaminated site. Better adaptation than *Agelena labyrinthica*	Lab and field*	Wilczek *et al.*, 2003

Pardrosa saltans	Heavy metals (Cd, Pb, Zn)	Metal content, metallothionein level	MT concentration did not increase in exposed pop. Adult size and conditions correlated negatively and egg mass positively with [Cd]	Lab	Eraly *et al.*, 2011
Pardrosa saltans	Heavy metals (Cd)	Survival, individual growth rate, metallothionein level, metal content	Increased protein level in both treatments and control but no significant correlation with [Cd]	Lab	Eraly *et al.*, 2010
Peramphithoe parmerong	Heavy metals (Cu)	Survival, broad sense heritability of survival and growth, body size, feeding rate, full-sib, split family design	Significant genotype-by-environment interaction in offspring survival between treatments and controls revealed variation in tolerance. Smaller size and lower feeding rate in treatments	Lab	Pease *et al.*, 2010
Pirata piraticus	Heavy metals (Cd)	Life-history traits (initial weight, growth rate and egg size), crosses, animal model	Reduced growth rate and increased egg size in the contaminated site, low heritability	Lab	Hendrickx *et al.*, 2008
Pirata piraticus	Heavy metals (Cu, Cd, Zn)	Life-history traits (reproductive output, fecundity, egg size)	Reduced reproductive output and fecundity, increased egg size in pop from contaminated sites	Lab	Hendrickx *et al.*, 2003
Platynympha longicaudata	Heavy metals (Zn, Pb, Cd, Cu, Mn)	Survival, molecular markers (45 RAPD), metallothionein level	Reduced gen diversity. Lower survival in controls. Clear genetic diversity differences	Lab and field*	Ross *et al.*, 2002
Porcellio scaber	Heavy metals (Zn)	Feeding rate, food and Zn assimilation, metal content	Higher growth efficiency, lower increase in Zn body burden	Lab	Donker *et al.*, 1996

Porcellio scaber	Heavy metals (Cd, Cu, Fe, Zn)	Life-history traits (body growth, sex ratio, reproduction at 1st and 2nd generations), metal content	Earlier reproduction, increased reproduction allocation, lower weight	Lab	Donker *et al.*, 1993
Spodoptera exigua	Heavy metals (Cd, Zn)	Survival rate, metal content, Catalase, Superoxidase dismutase and glutathione transferase activity	Pre-exposure control and Zn- pre-exposed organisms had lower survival than control animals. Metal content increased with concentration and Cd-pre-exposure had a significant effect in metal accumulation in larvae	Lab	Kafel *et al.*, 2014
Spodoptera exigua	Heavy metals (Cd)	Glutathione, protein thiols, total anti-oxidant capacity level, glutathione transferase activity. Metal content. Larval survival, larval duration time and last instar body weight	Higher metal content, higher mortality and longer duration of the larval stage in one-generation exposed insects in comparison with those exposed for many generations. Positive relation between higher metal content and glutathione oxidation	Lab	Kafel *et al.*, 2012
Tetrix tenuicornis	Heavy metals	Molecular markers (20 RAPD), metal content	Reduced genetic diversity in pop from contaminated sites. Significant changes in elemental concentrations	Lab and field*	Grzywacz *et al.*,2012

Thamnocephalusplatyurus	pesticides	Survival	Higher survival in populations from contaminated sites	Lab	Brausch and Smith 2009
Tigriopus angulatus	Heavy metals (Cu)	Growth rate, juvenile survival, life-history traits (age specific survival, intrinsic rate of natural increase)	Juvenile survival affected but unaffected intrinsic rate of natural increase	Lab	Medina *et al.*, 2009
Bryozoa					
Bugula neritina	Heavy metals (Cu)	Larval attachment success, post-metamorphic survival and growth of recruits. Field: post-exposure survival, growth of colonies	Resistance to Cu closely related to the relative levels of pollution experienced by the source populations	Lab and field	Piola and Johnston 2006
Celleporella hyalina	Acidification	Life-history traits (estimates of specific growth rate, growth efficiency, colony conditions, reproductive investment, gender allocation), SEM analysis	Significant effect of different clone on growth rate, reproductive investment and sex ratio, with clones showing contrasting responses to the various temperature and pH combinations	Lab	Pistevos *et al.*, 2011
Watersipora subtorquata	Heavy metals (Cu)	Survival, larval settlement and metamorphosis, full-sib split family design, larval size	No difference in tolerance between sites. Larval size significantly different	Lab	McKenzie *et al.*, 2011
Chordata					

Styela plicata	Heavy metals (Cu)	Hatching success, quantitative genetic breeding design	Difference in genetic basis of resistance between high and low concentration	Lab	Galletly *et al.,* 2007
Cnidaria					
Nematostella vectensis	Heavy metals (Cd, Cu, Hg, Zn)	Transcriptomics (RNA-seq)	Hg greatest impact followed by Cu, Zn and Cd. Co-up-regulation of immediate-early transcription factors such as Egr1, AP1 and NF-jB	Lab	Elran *et al.,* 2014
Echinodermata					
Centrostephanus rodgersii	Acidification	Crosses, life-history traits (fertilization success, cleavage success, sire success, normal gastrulae)	Presence of tolerant genotypes, early development not constrained in adapting	Lab	Foo *et al.,* 2012
Strongylocentrotus franciscanus	Acidification	Full-factorial breeding design, larvae size, heritability quantification	Greater levels of phenotypic and genetic variation for larval size in future CO_2 conditions, greater differential in mean trait values before and after selection		Sunday *et al.,* 2011
Strongylocentrotus purpuratus	Acidification	Growth rate, estimates of additive genetic variance for larval size with breeding exp	Abundant genetic variation for body size under elevated pCO_2	Lab	Kelly *et al.,* 2013
Strongylocentrotus purpuratus	Acidification	Molecular markers (SNPs), genomics	Allelic change in 40 functional classes of proteins involving hundreds of loci	Lab	Pespeni *et al.,* 2013

Mollusca

Biomphalaria glabrata	Heavy metals (Cd)	Shell length, time-to-death, hatching success, # eggs/snail/day, time to maturity, eggs per mass	Higher tolerance to chronic and lethal [Cd] in resistant strain	Lab	Salice *et al.*, 2010
Cantareus aspersus	Heavy metals (Cd, Pb, Zn)	Metal content, reciprocal transplant	Heavier shell than *C. Nemoralis*, no significant differences in metal accumulation	Field	Fritsch *et al.*, 2011
Cassostrea gigas	Heavy metals (Cd, Cu, Zn), pesticides	Metal content, gene expression	Genetic differentiation in the pop from contaminated site, two specific alleles were associated with metal sensitivity	Lab and field*	David *et al.*, 2012
Cassostrea gigas	Mixed chemicals	Microarray	Expression increased with pollution level. Potential selective effect on heterozygote frequency	Lab and field*	David *et al.*, 2007
Cassostrea gigas	Tribultyltin (TBT)	Survival, molecular markers (6 allozymes)	Allele frequencies varied significantly between resistant and sensitive pop	Lab	Tanguy *et al.*, 1999
Cassostrea angulata	Heavy metals (Cu, Zn)	Enzymatic activities (antioxidant defences, oxidative damage), metal content	More sensitive to pollution than *Oyster*, absent in the most contaminated site	Lab	Funes *et al.*, 2006

Cepaea nemoralis	Heavy metals (Cd, Pb, Zn)	Metal content, reciprocal transplant	Greater internal metal concentration than *C. Aspersus*, no significant differences in metal accumulation	Field	Fritsch *et al.*, 2011
Dreissena polymorpha	Heavy metals	Molecular markers (COI and 1 microsat), transcriptome analysis	Expression level correlated with variation in fitness and loads of heavy metals	Lab and field*	Navarro *et al.*, 2013
Lymanea stagnalis	Pesticides	Life-history traits (individual growth, female reproduction, hatching success), molecular markers (12 microsats)	Pesticide and other human pressures had little correspondence with evolutionary patterns	Lab and field*	Bouétard *et al.*, 2014
Macoma balthica	Heavy metals (Cu)	Survival, molecular markers (7 isozymes)	Differences in sensitivity and genetic diversity between pop sampled from the distribution limits of the species	Lab	Hummel *et al.*, 1997
Macoma balthica	Heavy metals (Ag, As, Cd, Cu, Mn, Pb, Se, V and Zn)	Metal content, molecular markers (DALP)	Individual with irregular shell shape exhibited higher concentrations of all metals	Lab and field*	Sokolowski *et al.*, 2002
Mytilus edulis	Heavy metals (Cu)	Survival, embryo development, crosses	Development less affected in pop from contaminated site, maternal effect revealed by crosses	Lab	Hoare *et al.*, 1995

Mytilus galloprovincialis	Heavy metals (Cu, Zn)	Enzymatic activities (antioxidant defences, oxidative damage), metal content	Combined increase of antioxidant defences and metal stabilization by complexation protect them	Lab	Funes *et al.*, 2006
Mytilus galloprovincialis	Heavy metals, pesticides, PAHs	Molecular markers (6 RAPD)	Same haplotype shared in individuals at impacted sites	Lab and field*	Ma *et al.*, 2000
Mytilus galloprovincialis	Municipal, industrial and shipyard wastewaters	Comet assay, micronucleus test, oxidative stress parameters, molecular markers (8 microsats)	Higher levels of genetic diversity in the pop from contaminated site	Lab and field*	Štambuk *et al.*, 2013
Mytilus galloprovincialis	Heavy metals (Cd, Cu, Hg), organic contaminants	Gene expression, shell length	Some gene markers traced organic contaminants more than heavy metals	Lab	Venier *et al.*, 2006
Mytilus trossulus	Acidification	Full-factorial breeding design, larvae size, heritability quantification	Less level of phenotypic variation for larval size in future CO_2 conditions than *Strongylocentrotus franciscanus*	Lab	Sunday *et al.*, 2011
Nucella lapillus	Tributyltin (TBT)	Molecular markers (18 allozymes), shell size	No differences in genetic diversity but higher variation in shell size in pop from contaminated site	Lab and field*	Plejdrup *et al.*, 2006
Perna viridis	Heavy metals (Cd, Cu, Pb, Zn)	Metal content, shell size, molecular markers (19 microsats)	Two clusters based on location and heavy metal contamination	Lab and field*	Yap *et al.*, 2013

Perna viridis	Heavy metals (Cd, Cu, Hg, Pb, Zn)	Metal content, molecular markers (10 allozymes), metallothionein level	Increased genetic variation and altered allozyme frequencies in the pop from contaminated site	Lab and field*	Yap *et al.*, 2004
Ruditapes decussatus	Heavy metals	Two molecular markers, protein marker (metallothionein)	Presence of a relationship between metallothionein concentrations and the level of metal pollution. Differential metallothionein induction between species	Lab and field*	Moraga *et al.*, 2002
Ruditapes philippinarum	Heavy metals	Two molecular markers, protein marker (metallothionein)	Relationship between metallothionein concentrations and the level of metal pollution. Differential metallothionein induction between species	Lab and field*	Moraga *et al.*, 2002
Saccostrea glomerata	Acidification	Oxygen consumption at gravid stage, standard metabolic rate, larval percentage survival, larval life-history traits (mean shell length, stage of development)	Larvae spawned from adults exposed to elevated Pco2 were larger and developed faster, but similar survival compared with larvae spawned from adults exposed to ambient Pco2	Lab	Parker *et al.*, 2012
Sphaerium novaezelandiae	Heavy metals (Zn)	Survival, reburial rate, molecular markers (allozymes)	No differences in mortality. Significant difference in reburial rates	Lab	Phillips and Hickey 2010
Nematoda					

Caenorhabditis elegans	Uranium (U)	Survival, fecundity, early and late growth (body length), heritability	Heritability decreases for fecundity and early growth in polluted environments. Decrease in heritability not proportional to the pop fitness reduction	Lab	Dutilleul *et al.*, 2015
Caenorhabditis elegans	Uranium (U)	Life-history traits (brood size, index of fertility, male body length and body bend frequency)	Reduced stability of trait structure and higher capacity to respond by acclimation. Lower evolutionary responses compared to salt treatment. Higher pop rate of increase	Lab	Dutilleul *et al.*, 2014
Caenorhabditis elegans	Uranium (U)	Survival, generation time, brood size, body length, body bend	At low concentrations negative effects reduced in the 2nd and 3rd generation (acclimation) while at high negative effects increased across generation	Lab	Dutilleul *et al.*, 2013
Platyhelminthes					
Polycelis tenuis	Heavy metals (Cd)	Survival, EC50, LC50, body size, reproduction, metal content	One of the pop had higher LC50 and eliminated Cd at a higher rate than reference pop	Lab	Indeherberg *et al.*, 1999
FISH					
Chordata					
Ameiurus nebulosus	Toxic chemicals (PAHs)	Molecular markers (RFLP on mtDNA)	Reduced genetic diversity in impacted sites	Lab and field*	Murdoch and Hebert 1994

Anguilla anguilla	Heavy metals (As, Cd, Cr, Cu, Hg, Ni, Pb, Zn and Se)	Metal content, molecular markers (12 allozymes and 8 microsats)	Negative correlation between pollution load and fitness. Reduced genetic variability in strongly polluted eels	Lab and field*	Maes *et al.*, 2005
Anguilla anguilla	PCBs	Transcriptomic platform for global gene expression	High expression of detoxification genes, lowered expression of genes involved in metabolism	Lab and field*	Pujolar *et al.*, 2012
Campostoma anomalum	Heavy metals, low water quality	Molecular markers (10 allozymes)	Reduced genetic diversity in impacted sites (higher)	Lab and field*	Heithaus and Laushman 1997
Catostomus occidentalis	Pesticides	Molecular markers (AFLP and microsats)	No correlation with pesticide exposure history and genetic structure	Lab and field*	Whitehead *et al.*, 2003
Coregonus lavaretus	Industrial pollution	Body size and weight, sex, gonad maturity, fatness, stomach fullness	Smaller body size and more variable sexual maturation time, frequency of spawning and life span decreased in treatments	Lab	Moiseenko 2002
Cyprinodon variegatus	Heavy metals (Zn), phenantrene	Survival, heritability	Low heritability, negative relationship between heritability of resistance and # of contaminants	Lab	Klerks and Moreau 2001
Danio rerio	Heavy metals (Cd, Cu)	Gene expression of ABCB10 gene	ABCB10 gene up-regulated as a result of metallic contamination	Lab	Sabri *et al.*,2012
Etheostoma blennioides	Heavy metals, low water quality	Molecular markers (10 allozymes)	Reduced genetic diversity in impacted sites (intermediated)	Lab and field*	Heithaus and Laushman 1997

Etheostoma caeruleum	Heavy metals, low water quality	Molecular markers (10 allozymes)	Reduced genetic diversity in impacted sites (lowest)	Lab and field*	Heithaus and Laushman 1997
Fundulus heteroclitus	PAHs	Survival (embryos), developmental delays, heart rate, morphology, microarray	Significant differences in each trait except in the microarray	Lab	Bozinovic and Oklesiak 2010
Fundulus heteroclitus	PCBs	Hatching success, larval survival, larval growth, CYp1A expression	No induction of CYp1A in fish from contaminated sites	Lab	Elskus *et al.*, 1999
Fundulus heteroclitus	PCBs, PAH	cDNA arrays	Lack of gene expression variation (common mechanisms in different pop), 2 genes have a common response	Lab and field*	Fisher and Oklesiak 2007
Fundulus heteroclitus	Dioxin	Molecular markers (25 SNPs)	AHR1 locus is highly polymorphic, allele frequencies differ between some dioxin-sensitive and dioxin-resistant populations. But the proteins encoded do not differ functionally	Lab and field*	Hahn *et al.*, 2004
Fundulus heteroclitus	Organic chemicals (bleached kraft mill effluent)	Molecular markers (15 allozymes)	Increased temporal variability in the pop closest to the source of pollution	Lab and field*	Kirchhoff *et al.*, 1999
Fundulus heteroclitus	PCBs	Molecular markers (232 AFLP)	No differences in genetic diversity	Lab and field*	McMillan *et al.*, 2006

Fundulus heteroclitus	PAHs	Aryl hydrocarbon receptor gene expression	Lack of inducibility of genes that are normally inducible by AHR agonists in pop from contaminated site	Lab	Meyer *et al.*, 2003
Fundulus heteroclitus	PAHs, menadione, *t*-butyl hydroperoxide	Antioxidant parameters	Upregulated antioxidant defenses	Lab	Meyer *et al.*, 2003
Fundulus heteroclitus	Xenobiotics, PAH	Survival, developmental abnormalities, developmental rate, resistance of the larvae to phototoxicity	Increased ability of F1, F2 to develop normally and to survive than reference individuals. Fitness cost	Lab	Meyer and Di Giulio 2003
Fundulus heteroclitus	PCBs/dioxin	Egg deposition, hatching success, and larval growth and survival	No site-related differences	Lab	Monosson *et al.*, 1995
Fundulus heteroclitus	PAHs	Molecular markers (14 allozymes and one general protein)	Significant correlation between individual genetic distance and differences among site in [PAH]	Lab and field*	Mulvey *et al.*, 2002
Fundulus heteroclitus	PCBs	Hatching and survival of embryos, embryonic urinary bladder fluorescence (correlated to EROD), EROD activity. LC20, EC50	Variation in the magnitude of heritability but similarities among biochemical mechanisms across impacted pop	Lab	Nacci *et al.*, 2010
Fundulus heteroclitus	Dioxin-like contaminants	Survival, EROD activity, changes in the activity of cytochrome P450 enzyme	Inherited resistance, lethal to reference individuals	Lab	Nacci *et al.*, 1999

Fundulus heteroclitus	Dioxin-like contaminants	Larval survival, incorporation of PCB 126 and radioactive content	Increased tolerance in population from contaminated site	Lab	Nacci *et al.*, 2002
Fundulus heteroclitus	PAHs, fluoranthene contaminants	Cardiovascular abnormalities in embryos	Uncontaminated pop higher abnormalities, heritable differences	Lab	Ownby *et al.*, 2002
Fundulus heteroclitus	PCB-126	cDNA arrays, RNA-seq to confirm results	Striking differences between pop. In one pop other genes involved, not only CYP1A	Lab	Oleksiak *et al.*, 2011
Fundulus heteroclitus	Dioxin-like contaminants	Molecular markers (98 SNPs), 3 AHR-related loci	Strong pop genetic structure at AHR-related loci, non-neutral change at the AHR2 locus	Lab and field*	Reitzel *et al.*, 2014
Fundulus heteroclitus	PCBs	Selection exp, survival, molecular markers (10 allozymes)	Allele frequencies reflect a pattern of isolation by distance but nothing else	Lab and field*	Roark *et al.*, 2005
Fundulus heteroclitus	PCB 126	Survival, developmental effects, transcriptomics	Desensitization of aryl-hydrocarbon receptor-mediated transcriptional activation, which is associated with extreme tolerance	Lab	Whitehead *et al.*, 2012

Fundulus heteroclitus	PCBs	Transcriptomics, hatching, survival, developmental abnormalities	Dramatic effects in the sensitive pop (expression associated with toxicity), genome-wide expression was comparatively refractory to PCB induction in the tolerant pop. Global blockade of AHR signalling pathway in tolerant pop (but leaky with extreme concentrations)	Lab	Whitehead *et al.*, 2010
Fundulus heteroclitus	Chemical pollutants	Molecular markers (300 AFLP)	1-6% loci under selection or linked to areas of the genome in polluted pop. Shared loci among polluted sites	Lab and field*	Williams and Oleksiak 2008
Fundulus heteroclitus	Not specified	Molecular markers (458 SNPs)	Non-neutral patterns, one SNP in the gene identified as refractory to induction	Lab and field*	Williams and Oleksiak 2011
Gambusia affinis	Radionuclide	Molecular markers (RAPD), DNA strand breakage	Specific alleles more abundant in pop from contaminated site, higher DNA integrity	Lab and field*	Theodorakis *et al.*, 1999
Gambusia affinis	Radionuclide	Molecular markers (RAPD)	The frequency of three markers was greater in the contaminated than the reference sites. Same pattern of band frequency shift of another species of the genus	Lab	Theodorakis *et al.*, 1998

Gambusia affinis	Radionuclide	Molecular markers (allozymes and 40 RAPD), fecundity (brood size/body length)	Allozymes: higher % of polymorphism and heterozygosity in the pop from contaminated site RAPD: increased genetic diversity and 17 out of 142 bands occurred at higher frequency in the pop from contaminated site	Lab and field*	Theodorakis *et al.*, 1997
Gambusia holbrooki	Heavy metals	Morphometric data (body size), molecular markers (5 isozymes)	Body size associated with a particular genotype. Genotype associated with small body size favored	Lab and field*	Benton *et al.*, 1994
Gambusia holbrooki	Uranium (U)	Survival, molecular markers (8 allozymes)	2nd-generation fish from polluted sites more tolerant, lower genetic variation	lab and field*	Keklak *et al.*, 1994
Gambusia holbrooki	Heavy metals (Hg)	Size-at-age data, otoliths analysis, weight and length, # eggs and developing embryos/gravid female, sex ratio, molecular markers (8 allozymes)	Associations between genotypes (or different metabolism) and responses to stress	Lab	Mulvey *et al.*, 1995
Gambusia holbrooki	Radionuclide	Molecular markers (RAPD)	The frequency of three markers was greater in the contaminated than the reference sites. Same pattern of band frequency shift of another species of the genus	Lab and field*	Theodorakis *et al.*, 1998

Gasterosteus aculeatus	Pulp mill	Molecular markers (AFLP)	F_{ST}-outlier analysis: non-neutral distribution in polluted sites (21 loci)	Lab and field*	Lind and Grahn 2011
Gillichthys mirabilis	Mixed chemicals	Individual growth rate, size-distribution, reciprocal transplant	No significant differences in growth	Field	Forrester *et al.*, 2003
Gobio gobio	Heavy metals (Cd, Zn)	Molecular markers (11 allozymes, 7 microsats)	Differences at 2 allozyme loci, 2 microsats appeared to be under selection, direct relationship between fish conditions and one allozyme allele which showed a large difference in allele frequency	Lab and field*	Knapen *et al.*, 2009
Gobio gobio	Heavy metals (Cd, Zn)	Molecular markers (metallothionein concentrations mRNA levels)	Ratio of the long mRNA variant relative to total MT mRNA was surprisingly constant, independent of exposure history	Lab and field*	Knapen *et al.*, 2007
Gobio gobio	Heavy metals (Cd)	Survival, Cd uptake, metallothionein analysis in liver and gill tissues	Higher survival of polluted pop, faster production and higher levels of MTLD	Lab	Knapen *et al.*, 2004
Gobionellus boleosoma	PAHs	Survival, molecular markers (13 isozymes)	No differences between polluted and unpolluted sites	Lab and field*	Klerks *et al.*, 1997
Heterandria formosa	Heavy metals (Cd)	Molecular markers (7 microsats)	Lower heterozygosity in selection pop	Lab	Athrey *et al.*, 2007

Heterandria formosa	Heavy metals (Cd)	Selection exp, survival	Fast response to selection, higher survival than non-selection individuals. Heritability 0.50	Lab	Xie and Klerks 2003
Lepomis auritus	Heavy metals, organic chemicals, ammonia	Molecular markers (13 RAPD)	Polluted pop less genetically distant from each other than they were from each of the reference sites. Frequency of unique genotypes correlated to pollutant gradient	Lab and field*	Nadig *et al.*, 1998
Leuciscus cephalus	PAHs, PCBs, benzene, Heavy metals	Molecular markers (28 allozymes), biochemical markers (EROD + DNA damage)	Higher frequency of one particular allele in two contaminated sites. Lower DNA damage level in the impacted pop	Lab and field*	Larno *et al.*, 2001
Microgadus tomcod	PCBs	Ligand-binding assay, gene expression assay of AHR	Six-base deletion in AHR2 as the basis of resistance	Lab and field*	Wirgin *et al.*, 2011
Perca flavescens	Heavy metals (Cd, Cu)	Transcriptomics, molecular markers (87 SNPs, 454 sequencing of mtDNA)	AA 204 substitution (dissimilar amminoacids around an outlier) involve it in a growth enhancement that would lead to a younger age of reproduction	Lab and field*	Bélanger-Deschênes *et al.*, 2013
Pimephales promelas	Heavy metals (Cu)	Survival, molecular markers (5 allozymes), weight	Small size in adapted individuals in which certain alleles associated to high survivorship in Cu	Lab	Schlueter *et al.*, 1995

Salmo trutta	Heavy metals (Cu, Zn)	Molecular markers (7 microsats)	No isolation due to metal contamination. High differentiation between two close populations	Lab and field*	Durrant *et al.,* 2011
Salmo trutta	Heavy metals (Cd, Zn)	Metallothionein level, hematocrit, condition factors, plasma chloride, molecular markers (26 allozymes)	Negative correlation between MT content and condition factor, lower heterozigosity in population from contaminated site	Lab and field*	Olsvik *et al.,* 2001
Solea Solea	Heavy metals, pesticides, organic chemicals	Body size, body mass, molecular markers (15 microsats and MT gene)	Two loci under directional selection, no genetic differentiation in MT gene	Lab and field*	Guinand *et al.,* 2013
Umbra limi	Acidification	Survival, molecular markers (16 allozymes)	Stressed site: higher frequencies of one particular allozyme, most tolerant fish were significantly more genetically variable	Lab and field*	Kopp *et al.,* 1992
AMPHIBIANS					
Chordata					
Rana arvalis	Acidification	Embryonic and larval fitness traits (embryonic survival, larval growth, age and size at metamorphosis)	Higher embryonic and larval acid tolerance, higher larval growth but slower larval development rate and bigger size at metamorphosis	Lab	Hangartner *et al.,* 2011

Rana arvalis	Acidification	Survival, developmental anomalies, life-history traits (length of larvae, development rate, egg size, individual growth rate), additive genetic variance	Increased tolerance, strong maternal effect, small heritability, little additive genetic variation	Lab	Merilä *et al.*, 2004
Rana arvalis	Acidification	Embryonic survival, life-history traits (hatchling size, age), estimated rates of divergence	Higher survival and less impaired growth performance under acid conditions. High rate of divergence	Lab	Räsänen *et al.*, 2003
Rana temporaria	PAHs, benzo[a]pyrene (BaP)	# of micronucleated erythrocytes	No correlation between PAHs level and tolerance in the lab	Lab	Marquis *et al.*, 2009
Rana temporaria	Acidification	Survival, body size, body shape, heritability	Low additive genetic variation independent of pH treatment	Lab	Pakkasmaa *et al.*, 2003
MICROALGAE					
Chlorophyta					
Chlamydomonas cf. fonticola	Acidification	Growth rate, fluctuation analysis, resistant cell count	Large variation in the number of resistant cells observed in the set 1 experiment, in contrast to the low variation in set 2 controls. Sex was crucial	Lab	Garcia-Balboa *et al.*, 2013
Chlamydomonas reinhardtii	Acidification	Growth rate, fluctuation analysis, resistant cell count	Large variation in the number of resistant cells observed in the set 1 experiment, in contrast to the low variation in set 2 controls. Sex was crucial	Lab	Garcia-Balboa *et al.*, 2013

Dictyosphaerium chlorelloides	Herbicides	Dose-effect response, fluctuation analysis, pop growth rate	Recurrent mutation for resistance but detrimental in terms of fitness in the absence of herbicides	Lab	Costas *et al.,* 2001
Dictyosphaerium chlorelloides	Acidification	Growth rate, fluctuation analysis, resistant cell count	Large variation in the # of resistant cells observed in the set 1 experiment, in contrast to the low variation in set 2 controls. Resistant mutant isolated retained resistance through generations	Lab	Garcia-Balboa *et al.,* 2013
Dictyosphaerium chlorelloides	Formaldehyde	Growth rate, photosynthetic performance, fluctuation analysis	After 50-d in inhibiting concentration rare formaldehyde-resistant cells occurred. Estimates of the frequency of formaldehyde-resistant alleles in non-extreme environment, (selection-mutation balance)	Lab	Lopez-Rodas *et al.,* 2008
Dictyosphaerium chlorelloides	Herbicides	Growth rate, demography, fluctuation analysis	High fluctuation in # of herbicide-resistant cells observed in set 1 cultures, in contrast with low fluctuation of set 2 controls	Lab	Marv *et al.,* 2010
Scenedesmus intermedius	Heavy metal (mixture)	Growth rate, fluctuation analysis	Resistant cells driven to extinction in the absence of metals	Lab	Baos *et al.,* 2002

Scenedesmus intermedius	Herbicides	Growth rate, demography, fluctuation analysis	High fluctuation in # of herbicide-resistant cells observed in set 1 cultures, in contrast with low fluctuation of set 2 controls	Lab	Marvá *et al.*, 2010
Cyanobacteria					
Microcystis aeruginosa	Heavy metals (Cu)	Fluctuation analysis, resistant cell count, morphometric data (cell size)	After 4-w in Cu: rare Cu-resistant cells recovered. Diminished fitness in the absence of copper sulphate but small size	Lab	Garcia-Villada 2004
Microcystis aeruginosa	Heavy metals (Cu, Ni, Zn)	Growth rate, chlorophyll *a*, total carotenoid, phycobiliprotein concentration, cell permeability, toxin concentration, morphological changes	Retention of cell viability, increased toxin concentration	Lab	Polyak *et al.*, 2013
Dinophyta					
Alexandrium minutum	Acidification	Selection exp, growth rate, toxin cell quota	Toxin cell quota pattern attributable to neutral mutations (final variances were significantly higher than those measured at the start of the exp)	Lab	Flores-Moya *et al.*, 2012
Haptophyta					

Emiliania huxleyi	Acidification	Molecular markers (minimum 1 microsat), selection exp, growth rate, cell diameter, PIC, POC/cell (and production rate)	Higher growth rates, in both the single- and multiclone exp, calcification partly restored	Lab	Lohbeck *et al.*, 2012
Emiliania huxleyi	Acidification	Selection exp, growth rate, morphometric data (size), calcite and biomass production	Growth rates were up to 16% higher in populations adapted for 1 year to warming when assayed at their upper thermal tolerance limit. Particulate inorganic (PIC) and organic (POC) carbon production was restored to values under present-day ocean conditions (higher than control)	lab	Schlüter *et al.*, 2014
Gephyrocapsa oceanica	Acidification	Selection exp, photosynthetic carbon fixation, growth rate, cell size, POC, PON production, C:N ratio	Enhanced growth rate and assimilations of C and N but decreased C:N ratios	Lab	Jin *et al.*, 2013
Ochrophyta					
Gomphonema parvulum	Heavy metals (Cu, Zn)	Algal pigment concentration, photon yeld, metal content, dry weight, % of viable cells, EC50	EC50 higher in the strain from the polluted site, persistence tolerance to Zn after 2 y	Lab	Ivorra *et al.*, 2002
MACROALGAE					
Hetokonta					

Ectocarpus siliculosus	Heavy metals (Cu)	Max quantum yield of PSII, loss of chlorophyll autofluorescence, percentage of fluorescent cells, proteomics	Differential soluble proteome profiling: identification of the induction of proteins related to processes such as energy production, glutathione metabolism as well as accumulation of HSPs. Striking expression of a stabilizing protein and a binding protein	Lab	Ritter *et al.*, 2010
Fucus serratus	Heavy metals (Cu)	Adult and embryo growth, accumulation of Cu^{2+} by adults	Metal exclusion mechanisms involved, the Cu^{2+} resistance of the photosynthetic apparatus may also be a significant factor	Lab	Nielsen *et al.*, 2003

Table A.2. Synopsis of studies testing for pollution-driven phenotypic responses, the presence of suitable genetic variation and responses to selection. Also indicated is whether the pollutant studied has been established as the casual factor of the observed selection. The numbers in parentheses refer to methods listed in Table 1. For the source population, "C vs. R" = population from contaminated sites compared to population from reference sites, "N" = population from the wild that do not have a known history of contamination, "Lab pop" = populations grown in the lab for an undetermined amount of time. "Y" (yes) = evidence provided, "N" (no) = evidence not provided, "NA" (not applicable) = not investigated. In some cases, when the results were not easy to interpret, words like "likely" or "contrasting" are also present. Experimental selection (27) is considered both "Response to selection" and "Selection due to pollution" given that, under laboratory conditions, the selection is highly controlled and very likely due to the pollutant used. Data in bold is obtained by the combination of articles focusing on the same population (geographical coordinates are given under Source population).

Species	Source population	Phenotypic response	Presence of genetic variation	Response to selection	Selection due to pollution	Pop fitness measurements	Reference
PLANTS							
Angiosperms							
Acer pseudoplatanus	C vs. R	Y (2)	NA	Y (27)	Y (27)	NA	Turner and Dickinson 1993
Acer rubrum	C vs. R	Y (3, 5)	NA	NA	NA	NA	Kirkey *et al.*, 2012
Arabidopsis arenosa	C vs. R	Y (5)	NA	NA	NA	NA	Przedpelska and Wierzbicka 2007
Arabidopsis sp.	C vs. R	Y (2)	Y (16)	NA	NA	NA	Kovalchuk *et al.*, 2004
Arabidopsis halleri	Lab pop	Y (bps)	Y (17)	NA	NA	NA	Becher *et al.*, 2004

Arabidopsis halleri	C vs. R (*A.lyrata petraea*)	Y (bps)	Y partially (10, 11)	NA	NA	NA	Frérot *et al.*, 2010
Arabidopsis halleri	C vs. R	Y (bps)	Y (18)	Y (24)	NA	NA	Hanikenne *et al.*, 2013
Arabidopsis halleri	Lab pop	Y (bps)	Y (16)	NA	NA	NA	Hanikenne *et al.*, 2008
Arabidopsis halleri	C vs. R	Y (bps)	NA	Y (23, 26, 27)	Y (27)	NA	Meyer *et al.*, 2009
Arabidopsis halleri	C vs. R	Y (bps)	NA	N (23)	NA	NA	Pauwels *et al.*, 2005
Arabidopsis halleri	C vs c	Y (bps)	NA	N (23)	NA	NA	Van Rossum *et al.*, 2004
Arabidopsis halleri	C & R	Y (1)	Y (8)	Y (27)	Y (27)	NA	Willems *et al.*, 2007
Arabidopsis halleri	**51.92 10.26 (Europe)**	**Y (bps, 1)**	**Y (8,10,11,16, 17, 18)**	**Y (23, 24, 26, 27)**	**Y (27)**	**NA**	**8 studies**
Arabidopsis thaliana	Lab pop	Y (bps)	Y (17)	NA	NA	NA	Becher *et al.*, 2004
Arabidopsis thaliana	Lab pop	Y (2, 3)	Y (18)	NA	NA	NA	Marmiroli *et al.*, 2009
Betula papyrifera	C vs. R	Y (3, 5)	NA	NA	NA	NA	Kirkey *et al.*, 2012
Betula pubescens	C vs. R	Y (1, 3, 5)	NA	Y (27)	Y (27)	NA	Eranen 2008
Biscutella laevigata	C vs. R	N (3)	NA	N (23)	NA	NA	Wasowicz *et al.*, 2014

Biscutella laevigata	C vs. R	Y (5)	NA	NA	NA	NA	Wierzbicka and Panufnik 2004
Calamagrostis epigejos	C vs. R	N (2)	NA	NA	NA	NA	Lehmann and Rebele 2004
Cynodon dactylon	C vs. R	Y (2, 3)	Y (9)	Y (23, 27)	Y (27)	NA	Xie *et al.*, 2014
Dianthus carthusianorum	C vs. R	Y (3, 5)	NA	Y (23)	NA	NA	Wójcik *et al.*, 2013
Dianthus carthusianorum	C vs. R	Y (5)	NA	NA	NA	NA	Zalęcka and Wierzbicka 2002
Elodea nuttallii	N	NA	Y (17)	NA	NA	NA	Regier *et al.*, 2013
Elsholtzia haichowensis	C vs. R	Y (3)	NA	NA	NA	NA	Liu and Xiong 2005
Elymus repens	C vs. R	N (2)	NA	NA	NA	NA	Lehmann and Rebele 2004
Eucalyptus calophylla	C vs. R	N (1)	NA	N (23)	NA	NA	Egerton-Warburton 1995
Eucalyptus patens	C vs. R	N (1)	NA	N (23)	NA	NA	Egerton-Warburton 1995
Eucalyptus rudis	C vs. R	N (1)	NA	N (23)	NA	NA	Egerton-Warburton 1995
Hordeum vulgare	Lab pop	Y (3)	NA	NA	NA	NA	Patra and Panda 1998
Mimulus guttatus	C vs. R	Y (1, 5)	Y (10)	Y (27)	Not clear (27, 28)	NA	Macnair *et al.*, 1993

Mimulus luteus	C vs. R	Y (5)	NA	NA	NA	NA	Ginocchio *et al.*, 2002
Mimulus guttatus	**37.94 -120.69 (USA)**	**Y (1, 5)**	**Y (10)**	**Y (27)**	**Y (27, ~28, 29)**	**NA**	**2 studies**
Plantago arenaria	C vs. R + other species	Y for Cu (2, 3)	NA	NA	NA	NA	Remon *et al.*, 2007
Poa annua	C vs. R	NA	NA	Y (23, 27)	Y (27)	NA	Chen *et al.*, 2003
Prosopis sp.	C vs. R and vendor seeds	Y (1, 3, 5)	NA	NA	NA	NA	Haque *et al.*, 2009
Sedum alfredii	C vs. R	Y (3)	NA	Y (23)	NA	NA	Deng *et al.*, 2007
Silene dioica	C vs. R	Y (3)	NA	NA	NA	NA	Kováčik *et al.*, 2010
Silene paradoxa	C vs. R	NA	NA	Y (23)	NA	NA	Mengoni *et al.*, 2001
Silene paradoxa	C vs. R	NA	NA	Y (23)	Y (29)	NA	Mengoni *et al.*, 2000
Silene paradoxa	**43.35 9.91 (Europe)**	**NA**	**NA**	**Y (23)**	**Y (29)**	**NA**	**2 studies**
Silene vulgaris	C vs. R	Y (3)	NA	NA	NA	NA	Kováčik *et al.*, 2010
Silene vulgaris	C vs. R	Y (1)	Y (8)	NA	NA	NA	Schat *et al.*, 1996

Silene vulgaris	C vs. R	Y (3, 5)	NA	NA	NA	NA	Wierzbicka and Panufnik 1998
Taraxacum officinale	C vs. R	NA	NA	Likely (23)	Likely (29)	NA	Keane *et al.*, 2005
T*hlaspi caerulescens*	C vs. R	Y (bps)	NA	Y (23)	Y (29)	NA	Besnard *et al.*, 2009
T*hlaspi caerulescens*	C vs. R	Y (bps)	NA	N (23)	NA	NA	Koch *et al.*, 1998
Typha latifolia	C vs. R	NA	NA	Y (23)	Y (29)	NA	Keane *et al.*, 1999
Viola tricolor	C vs. R	NA	NA	Y (23, 27)	Y (27)	NA	Slomka *et al.*, 2011
Bryophyta							
Ceratodon purpureus	C vs. R	Y (2, 6)	NA	NA	NA	NA	Jules and Shaw 1994
Pynophyta							
Picea abies	C vs. R	Y (1)	NA	Y (23, 27)	Y (27)	NA	Bergmann and Hosius 1996
Picea abies	C vs. R	NA	NA	Y (23)	NA	NA	Prus-Glowacki and Godzik 1995
Picea rubens	C vs. R	NA	NA	Y (23)	Y (29)	NA	Bashalkhanov *et al.*, 2013
Pinus sylvestris	C vs. R	N (6)	NA	NA	NA	NA	Gera's kin *et al.*, 2011

Pinus sylvestris	C vs. R	NA	NA	Y (23)	NA	NA	Gera's kin *et al.*, 2010
Pinus sylvestris	**52.9 33.5 (Europe)**	**N (6)**	**NA**	**Y (23)**	**NA**	**NA**	**2 studies**
Pinus sylvestris	C vs. R	NA	NA	Y (23)	NA	NA	Korshikov *et al.*, 2002
Pinus sylvestris	C vs. R	NA	NA	Y (23, 27)	Y (27)	NA	Kuchma and Finkeldey 2011
Pinus sylvestris	C vs. R	NA	NA	Y (23)	NA	NA	Prus-Glowacki *et al.*,2006
Pinus sylvestris	C vs. R	NA	NA	Likely (23, 27)	Y (27)	NA	Prus-Glowacki *et al.*, 1999
Pinus sylvestris	C vs. R	Y (bps)	NA	Y (23)	NA	NA	Wojnicka-Póltorak 1997
Pinus sylvestris	**50.49 28.88 (Europe)**	**Y (bps)**	**NA**	**Y (23, 27)**	**Y (27)**	**NA**	**3 studies**
INVERTEBRATES							
Anellida							
Aporectodea caliginosa	C vs. R	Y (3,5)	Y (13)	NA	NA	NA	Givaudan *et al.*, 2014
Aporectodea chlorotica	C vs. R	N (3)	N (13)	NA	NA	NA	Givaudan *et al.*, 2014
Aporectodea tuberculata	C vs. R	NA	Y (13)	NA	NA	NA	Lukkari *et al.*, 2004

Cognettia sphagnetorum	C vs. R	Y (1, 2, 5, 6)	NA	Y (23)	N (29)	Y	Haimi *et al.*, 2006
Cognettia sphagnetorum	C vs. R	Y (1,5)	NA	NA	NA	Y	Salminen and Haimi 2001
Cognettia sphagnetorum	**61.31 22.13 (Europe)**	**Y (1, 2, 5, 6)**	**NA**	**Y (23)**	**N (29)**	**Y**	**2 studies**
Dendrobaena octahedra	C vs. R	N (1, 2, 6)	Y (13)	NA	NA	NA	Bengtsson *et al.*, 1992
Dendrobaena octahedra	C vs. R	Y (bps)	Y (16)	NA	NA	NA	Fisker *et al.*, 2013
Dendrobaena octahedra	C vs. R	Y (1, 2, 4, 6)	NA	NA	NA	Y	Fisker *et al.*, 2011
Dendrobaena octahedra	**58.27 16.5 (Europe)**	**Y (bps, 1, 2, 4, 6)**	**Y (16)**	**NA**	**NA**	**Y**	**2 studies**
Dendrobaena octahedra	C vs. R	Y (1, 2, 3, 6)	NA	NA	NA	NA	Rozen 2006
Dendrodrilus rubidus	C vs. R	Y (1, 2, 3, 4, 5, 6)	NA	NA	Y (28)	NA	Arnold *et al.*, 2008
Dendrodrilus rubidus	C vs. R	Y (3)	NA	NA	NA	NA	Button *et al.*, 2012
Eisenia fetida	Lab pop	N (3)	Y and N (16)	NA	NA	NA	Brulle *et al.*, 2011
Eisenia fetida	Lab pop	NA	Y (17)	NA	NA	NA	Brulle *et al.*, 2008
Eisenia fetida	C vs. R	N (1, 2, 3)	NA	NA	NA	NA	Spurgeon and Hopkin 2000

Hediste diversicolor	C	Y (1)	NA	Y (23, 27)	Y (27)	NA	Virgilio and Abbiati 2004
Limnodrilus hoffmeisteri	C vs. R	Y (1)	Y (10)	NA	NA	NA	Martinez and Levinton 1996
Lumbricus castaneous	C vs. R	Y (3)	NA	NA	NA	NA	Button *et al.,* 2012
Lumbricus rubellus	N	N (1, 2, 3)	NA	Y (23)	NA	Y	Anderson *et al.,* 2013
Lumbricus rubellus	C vs. R	Y (3)	NA	Y (23)	NA	NA	Andre *et al.,* 2010
Lumbricus rubellus	C vs. R	Y (bps)	NA	Y (23)	Y and N (29)	NA	Kille *et al.,* 2013
Lumbricus rubellus	C vs. R	Y (1)	NA	NA	NA	NA	Langdon *et al.,* 2009
Lumbricus rubellus	C vs. R	N (1, 2, 3, 6)	NA	NA	NA	NA	Spurgeon and Hopkin 1999
Lumbricus rubellus	C vs. R	NA	Y (17)	NA	NA	NA	Stürzenbaun *et al.,* 1998
Lumbricus rubellus	C vs. R	NA	Y (16)	NA	NA	NA	Stürzenbaun *et al.,* 1998
Lumbricus rubellus	**50.8 -3.77 (Europe)**	**NY (bps, 1, 2, 3, 6)**	**Y (16, 17)**	**Y (23)**	**YN (29)**	**Y**	**6 studies**
Tubifex tubifex	Lab pop	Y (1)	NA	NA	NA	NA	Vidal and Horne 2003

Arthropoda

Agelena labyrinthica	Gradient	Y (3)	Y (13)	NA	NA	NA	Wilczek *et al.*, 2003
Amphibalanus variegatus	C vs. R	Y (1, 3)	NA	N (23)	NA	NA	Gall *et al.*, 2013
Anopheles gambiae	Lab pop	Y (1, 4, 6)	NA	Y (27)	Y (27)	NA	Mireji *et al.*, 2010
Attheyella crassa	Lab pop	N (5)	NA	Y (23)	NA	Y	Gardeström *et al.*, 2008
Balanus glandula	C vs. R	NA	NA	Y (23, 27)	Y (27)	NA	Ma *et al.*, 2000
Bathycletopsyllus sp.	C	NA	NA	N (23)	NA	NA	Gregg *et al.*, 2010
Ceriodaphnia pulchella	C vs. R	Y (1, 6)	NA	NA	NA	NA	Lopes *et al.*, 2005
Chironomus februarius	C vs. R	Y (6)	N (12)	NA	NA	Y	Bahrndorff *et al.*, 2006
Chironomus riparius	Lab pop	N (1, 4, 6)	NA	Y (23, 27)	Y (27)	Y	Nowak *et al.*, 2009
Chironomus riparius	Lab pop	Y (1, 6)	NA	Y (23)	NA	N	Vogt *et al.*, 2007
Chironomus riparius	**Lab pop**	**YN (1, 4, 6)**	**NA**	**Y (23, 27)**	**Y (27)**	**YN**	**2 studies**
Chironomus riparius	Lab pop	N (1, 4, 6)	Y (9)	Y (23)	NA	NA	Nowak *et al.*, 2008
Chironomus riparius	C vs. R	Y (1, 4)	Y (8)	NA	NA	NA	Groenendijk *et al.*, 2002

Chironomus riparius	C vs. R	Y (2,3)	NA	NA	NA	NA	Postma *et al.*, 1996
Chironomus riparius	C vs. R	Y, N (1, 3, 4, 5, 6)	NA	NA	NA	Y	Postma *et al.*, 1995
Chironomus riparius	C vs. R	Y (1, 2, 4, 5)	NA	NA	NA	NA	Postma *et al.*, 1995
Chironomus riparius	Not provided	Y (1, 3, 4, 6)	NA	NA	NA	Y	Postma and Davids 1995
Chironomus riparius	**51.61 5.33 (Europe)**	**Y (1, 2, 3, 4)**	**Y (8)**	**NA**	**NA**	**Y**	**5 studies**
Chironomus riparius	C vs. R	Y (1)	NA	Y (23, 27)	Y (27)	NA	Soeter *et al.*, 2010
Daphnia longispina	C vs. R	Y (1, 3)	Y (11)	NA	NA	NA	Agra *et al.*, 2010
Daphnia longispina	C vs. R	Y (1, 5, 6)	NA	NA	NA	NA	Lopes *et al.*, 2006
Daphnia longispina	C vs. R	Y (1, 3, 5, 6)	NA	NA	NA	NA	Lopes *et al.*, 2004
Daphnia longispina	C vs. R	Y (1)	Y (9)	Likely (23, 27)	Y (27)	NA	Martins *et al.*, 2009
Daphnia longispina	C vs. R	Y (1)	N (9)	N (23)	NA	NA	Martins *et al.*, 2007
Daphnia longispina	C vs. R	Y (bps)	NA	Y (23, 27)	Y (27)	NA	Silva *et al.*, 2010

Daphnia longispina	**37.7 -7.50 (Europe)**	**Y (bps, 1, 3, 5, 6)**	**YN (9)**	**YN (23, 27)**	**Y (27)**	**NA**	**5 studies**
Daphnia magna	Landscape with anthropogenic impact	Y (1)	NA	Y (23)	Y (28)	NA	Coors *et al.*, 2009
Daphnia magna	Lab pop	Y (1, 3)	Y (13)	NA	NA	NA	Haap and Köhler 2009
Daphnia magna	N vs. lab pop	N (1, 6)	Y (10)	NA	NA	NA	Messiaen *et al.*, 2013
Daphnia magna	N	Y (6)	Y (10)	Y (22, 27)	Y (27)	NA	Messiaen *et al.*, 2012
Daphnia magna	N	Y (1, 6)	Y (10)	NA	NA	Y	Messiaen *et al.*, 2010
Daphnia magna	**51.05 2.71 (Europe)**	**YN (1, 6)**	**Y (10)**	**Y (22, 27)**	**Y (27)**	**Y**	**3 studies**
Daphnia magna	Lab pop	Y (1, 6)	NA	Y (23, 27)	Y (27)	NA	Ward and Robinson 2005
Daphnia pulex	Lab pop	Y (3, 5, 6)	Y (16)	NA	NA	NA	Shaw *et al.*, 2007
Drosophila melanogaster	Lab pop	Y (1, 4, 5, 6)	Y (8)	Y (27)	Y (27)	NA	Shirley and Sibly 1999
Drosophila subobscura	C vs. R	Y (4, 6)	NA	NA	NA	NA	Kenig *et al.*, 2014

Drosophila subobscura	C vs. R	Y (4, 6)	NA	NA	NA	NA	Kenig *et al.*, 2013
Drosophila subobscura	**44.19 18.68 (Europe)**	**Y (4, 6)**	**NA**	**NA**	**NA**	**NA**	**2 studies**
Drosophila subobscura	N	Y (4, 5)	NA	Y (27)	Y (27)	NA	Kurbalija *et al.*, 2010
Folsomia candida	Lab pop	Y (1)	Y (16)	NA	NA	NA	Nakamori *et al.*, 2010
Folsomia candida	Lab pop	Y (6)	Y (16)	NA	NA	NA	Nota *et al.*, 2013
Folsomia candida	Lab pop	Y (1,6)	Y (17)	NA	NA	NA	Nota *et al.*, 2010
Folsomia candida	**Lab pop**	**Y (1, 6)**	**Y (16, 17)**	**NA**	**NA**	**NA**	**2 studies**
Folsomia candida	C vs. R	NA	Y (16)	NA	NA	NA	Nota *et al.*, 2011
Gammarus fossarum	N	Y (1)	N (10)	NA	NA	NA	Chaumot *et al.*, 2009
Hyalella azteca	Lab pop	Y (1)	Y (9)	Y (23, 27)	Y (27)	NA	Duan *et al.*, 2001
Hyalella azteca	Lab pop	Y (1)	Y (9)	Y (23, 27)	Y (27)	NA	Duan *et al.*, 2000 II
Hyalella azteca	**Lab pop**	**Y (1)**	**Y (9)**	**Y (23, 27)**	**Y (27)**	**NA**	**2 studies**

Hyalella azteca	Lab pop vs. N	NA	Y (17, 18)	NA	NA	NA	Weston *et al.*, 2013
Isonychia bicolor	C vs. R	Y (1, 5)	Y (9)	N (23)	NA	NA	Snyder and Hendricks 1997
Isotoma notabilis	C vs. R	Contrasting (1, 2, 5, 6)	NA	NA	NA	Y	Tranvik *et al.*,1993
Kiefferulus intertinctus	C vs. R	Y (6)	N (12)	NA	NA	Y	Bahrndorff *et al.*, 2006
Leander intermedius	C vs. R	N (1)	Y (13)	N (23)	NA	NA	Ross *et al.*, 2002
Leptodiaptomus minutus	C vs. R	N (1)	NA	Y (20, 27)	Y (27)	NA	Derry *et al.*, 2010
Microarthridion littorale	N	Y (1)	NA	NA	Y (28)	NA	Schizas *et al.*, 2001
Nectopsyche albida	N	Y (1)	NA	Y (23, 27)	Y (27)	NA	Benton *et al.*, 1992
Nitocra lacustris	N	Not clear (1, 6)	NA	Y (23, 27)	Y (27)	Y	Street *et al.*, 1998
Onychiurus armatus	C vs. R	Contrasting (1, 2, 5, 6)	NA	NA	NA	Y	Tranvik *et al.*, 1993
Orchesella bifasciata	C vs. R	N (1, 3)	N (13)	NA	NA	NA	Köhler *et al.*, 1999
Orchesella cincta	C vs. R	Y (1)	Y (9, 18)	Y (23)	NA	NA	Costa *et al.*, 2012
Orchesella cincta	C vs. R	NA	NA	Y (23)	Y for one allele and Cu (29)	NA	Frati *et al.*, 1992

Orchesella cincta	C	NA	Y (18)	Y (23)	Y (29)	NA	Janssens *et al.*, 2008
Orchesella cincta	C vs. R	Y (bps)	Y (16, 18)	NA	NA	NA	Janssens *et al.*, 2007
Orchesella cincta	C vs. R	Y (2, 3)	NA	NA	NA	NA	Posthuma *et al.*, 1992
Orchesella cincta	C vs. R	Y (3)	Y (10)	NA	Y (28)	NA	Posthuma *et al.*, 1993
Orchesella cincta	C vs. R	Y (1, 2, 3, 5, 6)	NA	NA	NA	NA	Posthuma *et al.*, 1993
Orchesella cincta	C vs. lab pop	Y (bps)	Y (17)	NA	NA	NA	Roelofs *et al.*, 2009
Orchesella cincta	C vs. R	NA	Y (17)	NA	NA	NA	Roelofs *et al.*, 2007
Orchesella cincta	Lab pop	NA	Y (10, 16)	Y (23, 27)	Y (27)	NA	Roelofs *et al.*, 2006
Orchesella cincta	C vs. lab pop	Y (bps)	Y (16)	Y (27)	Y (27)	NA	Sterenborg and Roelofs 2003
Orchesella cincta	C vs. R	Y (bps)	NA	Y (24, 26)	Y (29)	NA	Timmermans *et al.*, 2007
Orchesella cincta	C vs. R	Y (1)	Y (16)	NA	N (29)	NA	Timmermans *et al.*, 2005
Orchesella cincta	C vs. R	NA	Y (16, 18)	NA	NA	NA	Van Straalen *et al.*, 2011
Orchesella cincta	**51.57 10.93 (Europe)**	**Y (bps, 1, 2, 3, 5)**	**Y (9, 10, 16, 17, 18)**	**Y (23, 26, 27)**	**Y (27, 28, 29)**	**NA**	**14 studies**

Pardosa lugubris	Gradient	Y (3)	Y (13)	NA	NA	NA	Wilczek *et al.*, 2003
Pardosa saltans	C vs. R	N (3, 5, 6)	N (13)	NA	NA	NA	Eraly *et al.*, 2011
Pardosa saltans	C vs. R	N (1, 2, 3, 6)	Y (13)	NA	NA	NA	Eraly *et al.*, 2010
Pardosa saltans	**50.63 5.46 (Europe)**	**N (1, 2, 3, 5, 6)**	**YN (13)**	**NA**	**NA**	**NA**	**2 studies**
Peramphithoe parmerong	C vs. R	Y (1, 3, 5)	Y (8, 11)	NA	NA	NA	Pease *et al.*, 2010
Pirata piraticus	C vs. R	Y (5, 6)	Y (8, 10)	NA	NA	NA	Hendrickx *et al.*, 2008
Pirata piraticus	C vs. R	Y (6)	NA	NA	NA	NA	Hendrickx *et al.*, 2003
Pirata piraticus	**50.68 3.77 (Europe)**	**Y (5, 6)**	**Y (8, 10)**	**NA**	**NA**	**NA**	**2 studies**
Platynympha longicaudata	C vs. R	Y (1)	Y (13)	N (23)	NA	NA	Ross *et al.*, 2002
Porcellio scaber	C vs. R	Y (2, 3)	NA	NA	NA	NA	Donker *et al.*, 1996
Porcellio scaber	C vs. R	Y (2, 3, 6)	NA	Y (27)	Y (27)	NA	Donker *et al.*, 1993
Porcellio scaber	**51.27 5.55 (Europe)**	**Y (2, 3, 6)**	**NA**	**Y (27)**	**Y (27)**	**NA**	**2 studies**

Spodoptera exigua	Lab pop	Y (1,3)	NA	NA	NA	NA	Kafel *et al.*, 2014
Spodoptera exigua	Lab pop	Y (1, 3, 4, 5)	NA	NA	NA	NA	Kafel *et al.*, 2012
Spodoptera exigua	**Lab pop**	**Y (1,3, 4, 5)**	**NA**	**NA**	**NA**	**NA**	**2 studies**
Tetrix tenuicornis	C vs. R	Y (3)	NA	Y (23)	NA	NA	Grzywacz *et al.*,2012
Thamnocephalusplatyurus	C vs. R	Y (1)	NA	NA	NA	NA	Brausch and Smith 2009
Tigriopus angulatus	Lab pop	Y and N (1, 6)	NA	Y (27)	Y (27)	Y	Medina *et al.*, 2009
Bryozoa							
Bugula neritina	C vs. R	Y (1, 4)	Y (12)	NA	NA	Y	Piola and Johnston 2006
Celleporella hyalina	N	Y (1, 2, 6)	NA	NA	NA	Y	Pistevos *et al.*, 2011
Watersipora subtorquata	C vs. R	N (1, 4)	Y (11)	NA	NA	NA	McKenzie *et al.*, 2011
Chordata							
Styela plicata	N	Y and N (4)	It depends on the degree of stress (10)	NA	NA	NA	Galletly *et al.*, 2007
Cnidaria							

Nematostella vectensis	Lab pop	NA	Y (17)	NA	NA	NA	Elran *et al.*, 2014
Echinodermata							
Centrostephanus rodgersii	N	Y (1, 4, 6)	Y (10)	Y (21)	Y (29)	NA	Foo *et al.*, 2012
Strongylocentrotus franciscanus	N	Y (4)	Y (10)	Y (27)	Y (27)	NA	Sunday *et al.*, 2011
Strongylocentrotus purpuratus	N	Y (4)	Y (10)	Y (27)	Y (27)	Y	Kelly *et al.*, 2013
Strongylocentrotus purpuratus	N	NA	NA	Y (23, 26, 27)	Y (27)	NA	Pespeni *et al.*, 2013
Mollusca							
Biomphalaria glabrata	Lab pop	Y (1, 4, 6)	NA	NA	NA	N	Salice *et al.*, 2010
Cantareus aspersus	C vs. R	Y (3)	N (12)	NA	NA	NA	Fritsch *et al.*, 2011
Cassostrea gigas	C vs. R	Y (3)	Y (16)	NA	Y (29)	NA	David *et al.*, 2012
Cassostrea gigas	C vs. R	NA	Y (17)	NA	NA	NA	David *et al.*, 2007
Cassostrea gigas	N	N (1)	NA	Y (23)	Y (29)	NA	Tanguy *et al.*, 1999
Cassostrea gigas	**44.88 -1.59 (Europe)**	**YN (1, 3)**	**Y (16, 17)**	**Y (23)**	**Y (29)**	**NA**	**3 studies**

Cassostrea angulata	C vs. R	Y (3)	Y (13)	Y (27)	Y (27)	NA	Funes *et al.*, 2006
Cepaea nemoralis	C vs. R	N (3)	N (12)	NA	NA	NA	Fritsch *et al.*, 2011
Dreissena polymorpha	C vs. R	NA	Y (17)	N (23)	Y (28)	NA	Navarro *et al.*, 2013
Helisoma trivolvis	C vs. R	Y (5)	Y (9)	Likely (23, 27)	Y (27)	NA	Benton *et al.*, 1994
Lymanea stagnalis	C vs. R	N (2, 6)	N (18)	N (27)	N (27)	NA	Bouétard *et al.*, 2014
Macoma balthica	N	Y (1)	NA	Y (23)	NA	NA	Hummel *et al.*, 1997
Macoma balthica	C vs. R	Y (3, 5)	NA	N (23)	NA	NA	Sokolowski *et al.*, 2002
Mytilus edulis	C vs. R	Y (1, 4)	N (8)	NA	NA	NA	Hoare *et al.*, 1995
Mytilus galloprovincialis	C vs. R	N (3)	Y (13)	Y (27)	Y (27)	NA	Funes *et al.*, 2006
Mytilus galloprovincialis	C vs. R	NA	NA	Y (23, 27)	Y (27)	NA	Ma *et al.*, 2000
Mytilus galloprovincialis	C vs. R	NA	NA	Y (23)	N (28)	NA	Štambuk *et al.*, 2013
Mytilus galloprovincialis	N	Y (5)	Y (17)	NA	NA	NA	Venier *et al.*, 2006
Mytilus trossulus	N	N (4)	N (10)	Y (27)	Y (27)	NA	Sunday *et al.*, 2011

Nucella lapillus	C vs. R	Y (5)	NA	N (23)	NA	NA	Plejdrup *et al.*, 2006
Perna viridis	C vs. R	Y (3, 5)	NA	Y (23)	Y (28)	NA	Yap *et al.*, 2013
Perna viridis	C vs. R	Y (3)	NA	Y (23)	Y (29)	NA	Yap *et al.*, 2004
Perna viridis	**2.008 102.61 Asia)**	**Y (3, 5)**	**NA**	**Y (23)**	**Y (28, 29)**	**NA**	**2 studies**
Ruditapes decussatus	C vs. R	NA	Y (13, 18)	Y (23)	Y (28)	NA	Moraga *et al.*, 2002
Ruditapes philippinarum	C vs. R	NA	Y (13, 18)	Y (23)	Y (28)	NA	Moraga *et al.*, 2002
Saccostrea glomerata	N and lab pop (selection pop)	(1, 2, 4)	NA	NA	NA	NA	Parker *et al.*, 2012
Sphaerium novaezelandiae	N	Y and N (1, 3)	NA	N (23)	NA	NA	Phillips and Hickey 2010
Nematoda							
Caenorhabditis elegans	Lab pop	N (1, 5, 6)	N (10)	N (27)	N (27)	NA	Dutilleul *et al.*, 2015
Caenorhabditis elegans	Lab pop	Y (5, 6)	NA	Y (27)	Y (27)	Y	Dutilleul *et al.*, 2014
Caenorhabditis elegans	Lab pop	N (1, 5, 6)	NA	N (27)	N (27)	NA	Dutilleul *et al.*, 2013
Caenorhabditis elegans	**Lab pop**	**N (1, 5, 6)**	**N (10)**	**YN (27)**	**YN (27)**	**Y**	**3 studies**

Platyhelminthes							
Polycelis tenuis	C vs. R	Y (1, 3, 5, 6)	NA	NA	Y (28)	NA	Indeherberg *et al.,* 1999
FISH							
Chordata							
Ameiurus nebulosus	C vs. R	NA	NA	Maybe (23)	Y (27)	NA	Murdoch and Hebert 1994
Anguilla anguilla	N	NA	NA	Y (23)	Y (32)	NA	Maes *et al.,* 2005
Anguilla anguilla	Reads	NA	Y (17)	NA	NA	NA	Pujolar *et al.,* 2012
Anguilla anguilla	**42.02 11.62 (Europe)**	**NA**	**Y (17)**	**Y (23)**	**Y (32)**	**NA**	**2 studies**
Campostoma anomalum	Varying H_2O quality	NA	NA	Maybe (23, 27)	Y (27)	NA	Heithaus and Laushman 1997
Catostomus occidentalis	C vs. R	NA	NA	N (23)	N (29)	NA	Whitehead *et al.,* 2003
Coregonus lavaretus	C vs. R	Y (3, 5, 6)	NA	NA	NA	NA	Moiseenko 2002
Cyprinodon variegatus	N	N (1)	N (10)	NA	NA	NA	Klerks and Moreau 2001
Danio rerio	Pet farm	NA	Y (18)	NA	NA	NA	Sabri *et al.,* 2012

Etheostoma blennioides	Varying H_2O quality	NA	NA	Maybe (23, 27)	Y (27)	NA	Heithaus and Laushman 1997
Etheostoma caeruleum	Varying H_2O quality	NA	NA	Maybe (23, 27)	Y (27)	NA	Heithaus and Laushman 1997
Fundulus heteroclitus	C vs. R	Y (1, 3, 4, 5)	N (17)	NA	NA	NA	Bozinovic and Oleksiak 2010
Fundulus heteroclitus	C vs. R	Y (bps)	Y (16)	NA	NA	NA	Fisher and Oklesiak 2007
Fundulus heteroclitus	C vs. R	Y (bps)	Y (18)	Y (23, 27)	Y (27)	NA	Hahn *et al.*, 2004
Fundulus heteroclitus	C vs. R	NA	NA	Y (23)	NA	NA	Kirchhoff *et al.*, 1999
Fundulus heteroclitus	C vs. R	Y (bps)	NA	N (23)	NA	NA	McMillan *et al.*, 2006
Fundulus heteroclitus	C vs. R	NA	N (16)	NA	NA	NA	Meyer *et al.*, 2003
Fundulus heteroclitus	C vs. R	Y (3)	Y (13)	NA	NA	NA	Meyer *et al.*, 2003
Fundulus heteroclitus	C vs. R	Y (1, 4)	NA	NA	NA	NA	Meyer and Di Giulio 2003
Fundulus heteroclitus	C vs. R	N (1, 4, 6)	NA	NA	NA	NA	Monosson *et al.*, 1995
Fundulus heteroclitus	C vs. R	NA	NA	Y (23)	Y (29)	NA	Mulvey *et al.*, 2002
Fundulus heteroclitus	C vs. R	Y (1, 3, 4)	NA	NA	NA	NA	Nacci *et al.*, 2010

Fundulus heteroclitus	C vs. R	Y (1)	NA	NA	NA	NA	Nacci *et al.*, 2002
Fundulus heteroclitus	C vs. R	Y (1, 3)	NA	NA	NA	NA	Nacci *et al.*, 1999
Fundulus heteroclitus	C vs. R	Y (bps)	Y (17)	NA	NA	NA	Oleksiak *et al.*, 2011
Fundulus heteroclitus	C vs. R	Y (3)	NA	NA	NA	NA	Ownby *et al.*, 2002
Fundulus heteroclitus	C vs. R	Y (bps)	NA	Y (23, 27)	Y (27)	NA	Reitzel *et al.*, 2014
Fundulus heteroclitus	C vs R	Y (bps)	NA	N (23, 27)	Y (27)	NA	Roark *et al.*, 2005
Fundulus heteroclitus	C vs. R	Y (1)	Y (17)	NA	NA	NA	Whitehead *et al.*, 2012
Fundulus heteroclitus	C vs. R	Y (1, 4)	Y (16)	NA	NA	NA	Whitehead *et al.*, 2010
Fundulus heteroclitus	C vs. R	NA	NA	Y (23)	Y (29)	NA	Williams and Oleksiak 2011
Fundulus heteroclitus	C vs. R	Y (bps)	NA	Y (23, 27)	Y (27)	NA	Williams and Oleksiak 2008
Fundulus heteroclitus	**36.8 -76.44 (USA)**	**Y (bps, 1, 3, 4, 5, 6)**	**YN (13, 16, 17, 18)**	**YN (23, 27)**	**Y (27, 29)**	**NA**	**21 studies**
Fundulus heteroclitus	C vs. R	Y (4)	NA	NA	NA	NA	Elskus *et al.*, 1999
Gambusia affinis	C vs. R	Y (bps)	NA	Y (23, 27)	Y (27, 30)	NA	Theodorakis *et al.*, 1999

Gambusia affinis	C vs. R	NA	NA	Y (23 27)	Y (27)	NA	Theodorakis *et al.*, 1998
Gambusia affinis	C vs. R	Y (6)	NA	Y (23)	Y (30)	NA	Theodorakis *et al.*, 1997
Gambusia affinis	35.93 -84.31 (USA)	Y (bps, 6)	NA	Y (**23, 27**)	Y (**27, 30**)	NA	3 studies
Gambusia holbrooki	C vs. R	Y (5)	Y (9)	Likely (23)	Y (28)	NA	Benton *et al.*, 1994
Gambusia holbrooki	C vs. R	Y (1)	NA	Y (23, 27)	Maybe (27)	NA	Keklak *et al.*, 1994
Gambusia holbrooki	N	Y (5, 6)	Y (9)	Y (23, 27)	Y (27)	NA	Mulvey *et al.*, 1995
Gambusia holbrooki	C vs. R	NA	NA	Y (23, 27)	Y (27)	NA	Theodorakis *et al.*, 1998
Gasterosteus aculeatus	C vs. R	NA	NA	Y (23, 27)	Y (27)	NA	Lind and Grahn 2011
Gillichthys mirabilis	C vs. C	NA	N (12)	NA	NA	NA	Forrester *et al.*, 2003
Gobio gobio	C vs. R	Y (bps)	NA	Y (23)	N (31)	NA	Knapen *et al.*, 2009
Gobio gobio	C vs. R	Y (bps)	Y (13, 16)	NA	NA	NA	Knapen *et al.*, 2007
Gobio gobio	C vs. R	Y (1, 3)	Y (13)	NA	NA	NA	Knapen *et al.*, 2004
Gobio gobio	**51.13 4.59 (Europe)**	**Y (bps, 1, 3)**	**Y (13, 16)**	**Y (23)**	**N (31)**	**NA**	**3 studies**

Gobionellus boleosoma	C vs. R	N (1)	NA	N (23)	NA	NA	Klerks *et al.,* 1997
Heterandria formosa	Selection pop vs. lab pop	NA	NA	Y (23, 27)	Y (27)	NA	Athrey *et al.,* 2007
Heterandria formosa	Selection pop	Y (1)	Y (10)	Y (27)	Y (27)	NA	Xie and Klerks 2003
Lepomis auritus	C vs. R	NA	NA	Y (23)	Y (29)	NA	Nadig *et al.,* 1998
Leuciscus cephalus	C vs. R	NA	NA	Y (23, 27)	Y (27)	NA	Larno *et al.,* 2001
Microgadus tomcod	C vs. R	NA	Y (16)	Y (27)	Y (27)	NA	Wirgin *et al.,* 2011
Perca flavescens	C vs. R	NA	Y (17)	NA	Y (29)	NA	Bélanger-Deschênes *et al.,* 2013
Pimephales promelas	N	Y (1, 5)	Y (9)	Y (23)	Y (28)	NA	Schlueter *et al.,* 1995
Salmo trutta	C vs. C-R	NA	NA	Y (23)	NA	NA	Durrant *et al.,* 2011
Salmo trutta	C vs. R	NA	NA	Y (23)	NA	NA	Olsvik *et al.,* 2001
Solea Solea	C vs. R	Y (5)	N (18)	Y (23)	Y (30)	NA	Guinand *et al.,* 2013
Umbra limi	C vs. R	Y (1)	NA	Y (23, 27)	Y (27)	NA	Kopp *et al.,* 1992

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Chordata							
Rana arvalis	C vs. R	Y (1, 4)	NA	Y (19)	Y, it depends on the trait (28)	NA	Hangartner *et al.*, 2011
Rana arvalis	C vs. R	Y (1, 2, 4)	Y (8, 10)	NA	NA	NA	Merilä *et al.*, 2004
Rana arvalis	C vs. R	Y (1, 4)	NA	Y (21, 27)	Y (27)	NA	Räsänen *et al.*, 2003
Rana temporaria	N	N (1, 5)	Y but low (10)	NA	NA	NA	Pakkasmaa *et al.*, 2003
Rana arvalis	**59.02 11.91 (Europe)**	**YN (1, 2, 4, 5)**	**Y (8, 10)**	**Y (19, 21, 27)**	**Y (27, 28)**	**NA**	**4 studies**
Rana temporaria	C vs. R	N (3)	NA	NA	NA	NA	Marquis *et al.*, 2009
MICROALGAE							
Chlorophyta							
Chlamydomonas cf. fonticola	C vs. R	Y (3)	NA	Y (27)	Y (27)	Y	Garcia-Balboa *et al.*, 2013
Chlamydomonas reinhardtii	C vs. R	Y (3)	NA	Y (27)	Y (27)	Y	Garcia-Balboa *et al.*, 2013
Dictyosphaerium chlorelloides	Lab pop	Y (1)	NA	Y (27)	Y (27)	Y	Costas *et al.*, 2001
Dictyosphaerium chlorelloides	C vs. R	Y (3)	NA	Y (27)	Y (27)	Y	Garcia-Balboa *et al.*, 2013

Dictyosphaerium chlorelloides	N	Y (3)	NA	Y (27)	Y (27)	Y	Lopez-Rodas *et al.*, 2008
Dictyosphaerium chlorelloides	C vs. R	NA	NA	Y (27)	Y (27)	Y	Marvá *et al.*, 2010
Scenedesmus intermedius	Lab pop	NA	NA	Y (27)	Y (27)	Y	Baos *et al.*, 2002
Rana arvalis	**59.02 11.91 (Europe)**	**YN (1, 2, 4, 5)**	**Y (8, 10)**	**Y (19, 21, 27)**	**Y (27, 28)**	**NA**	**5 studies**
Scenedesmus intermedius	C vs. R	NA	NA	Y (27)	Y (27)	Y	Marvá *et al.*, 2010
Cyanobacteria							
Microcystis aeruginosa	Lab pop	Y (5)	NA	Y (27)	Y (27)	Y	Garcia-Villada 2004
Microcystis aeruginosa	Lab pop	Y (3, 5)	NA	NA	NA	Y	Polyak *et al.*, 2013
Dinophyta							
Alexandrium minutum	Lab pop	Y (3)	NA	Y (27)	Y (27)	Y	Flores-Moya *et al.*, 2012
Haptophyta							
Emiliania huxleyi	N	Y (3)	NA	Y (27)	Y (27)	Y	Lohbeck *et al.*, 2012
Emiliania huxleyi	Lab pop	Y (3, 5)	NA	Y (27)	Y (27)	Y	Schlüter *et al.*, 2014

Gephyrocapsa oceanica	Lab pop	Y (3, 5)	NA	Y (27)	Y (27)	Y	Jin *et al.*, 2013
Ocrophyta							
Gomphonema parvulum	C vs. R	Y (3)	NA	NA	NA	Y	Ivorra *et al.*, 2002
MACROALGAE							
Hetokonta							
Ectocarpus siliculosus	C vs. R	Y (3)	Y (14)	NA	NA	NA	Ritter *et al.*, 2010
Fucus serratus	C vs. R	Y (2, 3, 4)	NA	NA	NA	NA	Nielsen *et al.*, 2003

Table A.3. Response variable and fixed factors included in the mixed-model analysis. The dataset included a large variety of experimental designs that were mainly classified as either "field" and "experiment" studies. The "field" studies involved direct measurements on traits from populations sampled from contaminated or reference sites. In this case we used the metal concentration measured in the contaminated site to calculate the effect sizes and we considered as control the population(s) from the reference site and as treatment the population(s) from contaminated habitats. The experiment studies involved manipulations under laboratory conditions where populations from contaminated and reference sites could be studied for their responses in both pristine and contaminated environments. In this case we used the response at concentration 0 as the control and the response at higher concentrations as the treatments for the calculations of effect sizes.

Response variable	Hedge's d effect size
Metal concentration (ppm)	Continuous: [metal]/threshold
Metal	type of metal (Cd, Cu, Pb, Zn)
Subclass	subclass name
Phylum	phylum name
Habitat	terrestrial-freshwater-marine
Other metals	yes-possible-no
Exp or field	exp-field
Exp length	Number of days

Table A.4. Selection table with second-order Akaike information criterion (AICc) and log-likelihoods (LL). Fixed terms: metal concentration (C), experiment or field factor (E), habitat (H), metal (M), subclass (S), phylum (P), presence of other metals (O). Metal concentration is ln([ppm]/threshold specific to habitat) and "na" stands for not applicable. ΔAICc values refer to the AICc-best models (in bold).

ID	Fixed terms	Random terms	LL	AICc	ΔAICc
Weight					
Null model	na	na	-38.22	78.67	46.26
Random model	na	Study ID	-12.41	**32.41**	0
Mixed model (one factor)	C	Study ID	-12.49	32.57	0.16
Mixed model (2 factors)	C + H	Study ID	-10.91	37.28	4.87
Mixed model (3 factors)	C + H + E	Study ID	-10.37	42.08	9.67
No. of neonates					
Null model	na	na	-211.21	424.54	306.04
Random model	na	Study ID	-62.52	131.77	13.27
Mixed model (one factor)	C	Study ID	-60.27	127.27	8.77
Mixed model (2 factors)	C + O	Study ID	-52.51	120.13	1.63
Mixed model (3 factors)	C + O + M	Study ID	-45.96	**118.50**	0
Body metal content					
Null model	na	na	-180.25	362.56	187.51
Random model	na	Study ID	-89.34	185.09	10.04
Mixed model (one factor)	C	Study ID	-93.61	193.63	18.58
Mixed model (2 factors)	C + S	Study ID	-74.25	176.65	1.60
Mixed model (3 factors)	C + S + M	Study ID	-67.85	**175.05**	0

Table A.5. Model-averaged coefficients from AICc-best models for weight, number of neonates and body metal content. Heterogeneity (Tau-squared), ICC (rho) and residual heterogeneity are also shown. Study ID was the random term in all models and random effects for the different metal concentrations tested within the same study were correlated through a multivariate parameterization. Fixed terms: metal concentration (C), habitat (H), metal (M), subclass (S), presence of other metals (O).

Weight (Random model)			**No. of neonates** (C+O+M)			**Body metal content** (C+S+M)		
	Estimate	**SE**	**Fixed terms**	**Estimate**	**SE**	**Fixed terms**	**Estimate**	**SE**
Intercept	0.12	0.16	ln([Metal]/threshold)	-0.26	0.15	ln([Metal]/threshold)	0.19	0.08
			Other metals (no)	-3.11	1.53	Subclass (Collembola)	0.58	0.60
			Other metals (possible presence)	-1.93	1.49	Subclass (Eumalacostraca)	1.89	0.52
			Other metals (yes)	0.32	0.98	Subclass (Oligochaeta)	1.94	0.42
			Metal (Cd)	1.68	1.63	Subclass (Orthogastropoda)	1.91	0.74
			Metal (Cu)	2.43	1.41	Subclass (Pteriomorphia)	-0.07	0.94
			Metal (Zn)	2.03	1.77	Subclass (Pterygota)	1.82	0.63
						Subclass (Trepaxonemata)	-0.92	0.63
						Metal (Cd)	-0.25	0.46
						Metal (Cu)	-1.71	0.61
						Metal (Pb)	-1.58	0.60
						Metal (Zn)	-1.19	0.51
ICC	0.89		*ICC (rho)*	0.52		*ICC (rho)*	-0.11	
Heterogeneity	0.23		*Heterogeneity*	1.40		*Heterogeneity*	0.44	
			Residual heterogeneity	Qe= 291.15	Df= 31	*Residual heterogeneity*	Qe= 168.15	Df= 52

Figure A.1 All the typologies of pollution and their relative prevalence found in the studies and grouped as "other".

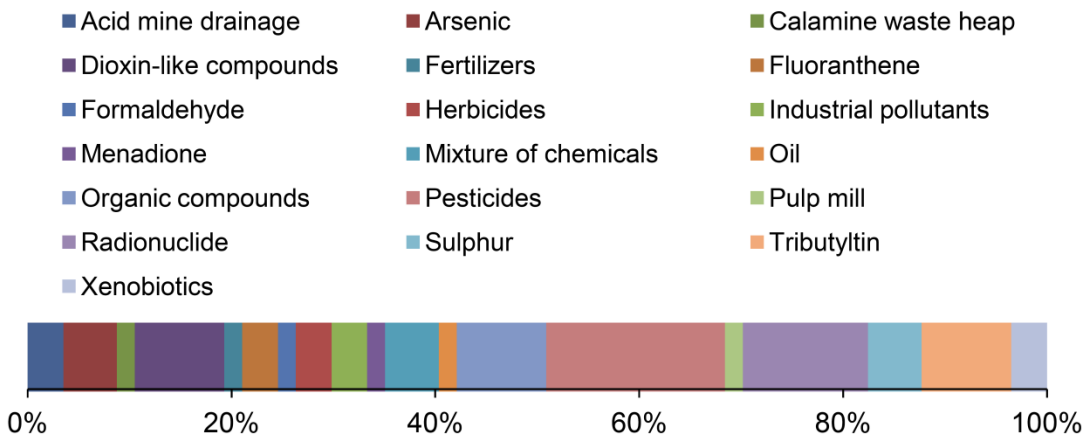


Figure A.2 Number of species and number of articled reviewed per phylum.

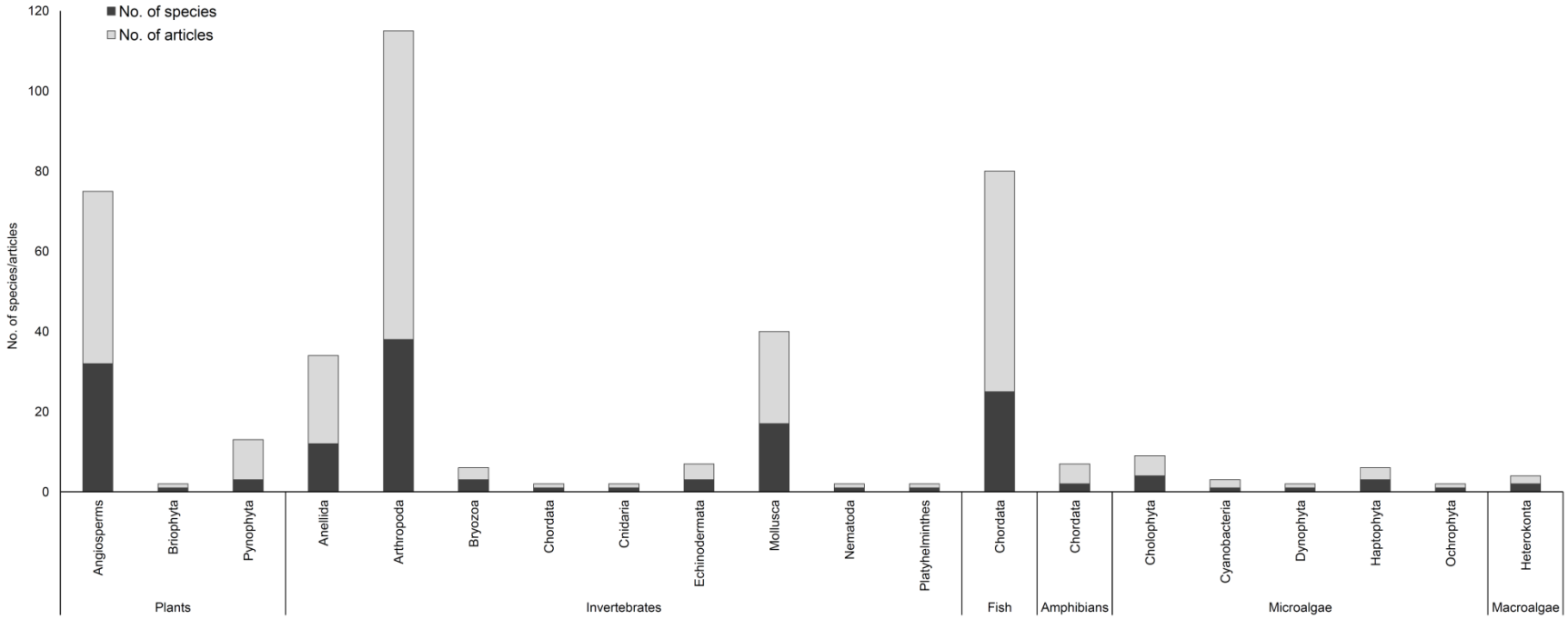


Figure A.3 Percentage of studies published between 1992 and 2014 that used molecular markers, genomics, transcriptomics and proteomics approaches to assess population genetic diversity, find evidence of selection and identify candidate functional genes under selection in the presence of pollution.

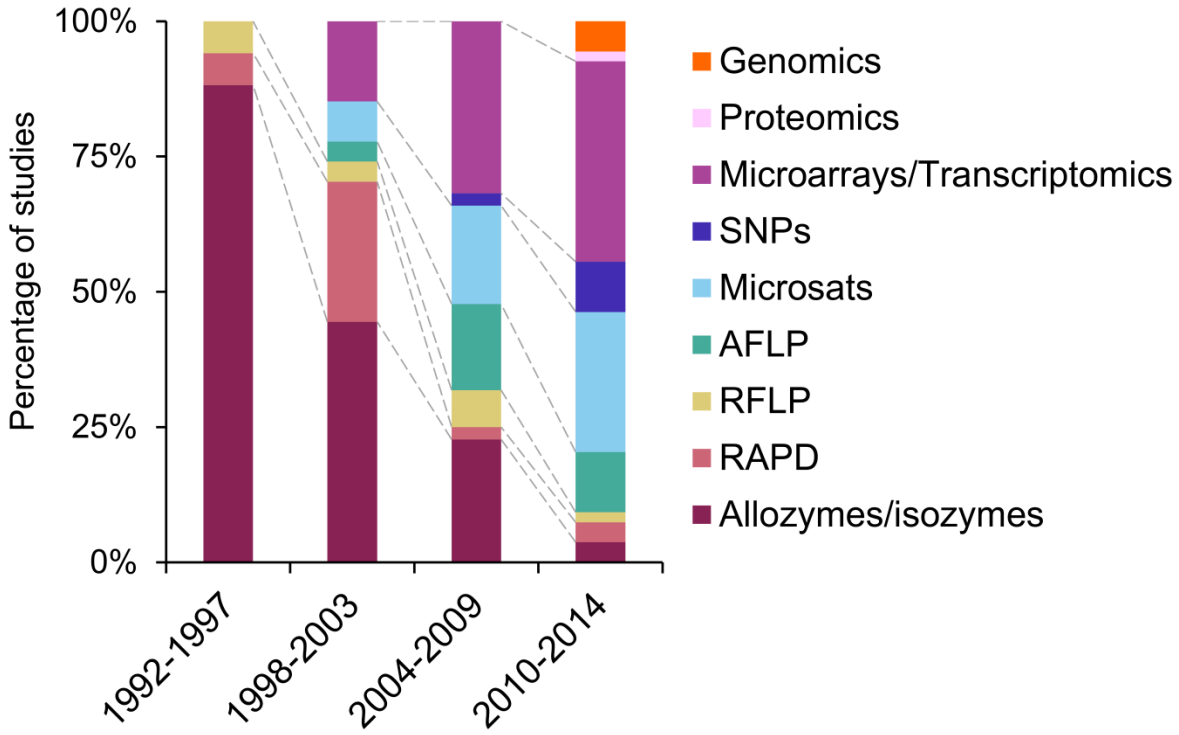
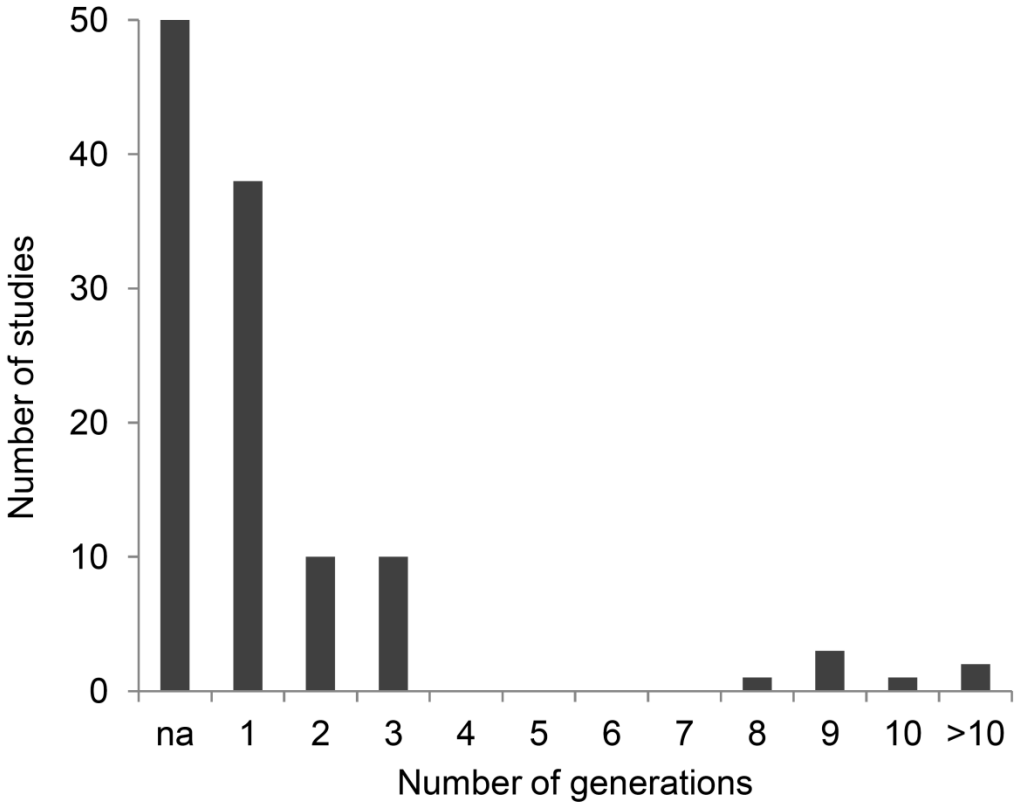


Figure A.4 Number of generations investigated by the reviewed studies.



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Appendix B

Supplementary material for Chapter 2

Establishment and maintenance of *Daphnia* lineages before the experiment

Prior to the experiment, organisms were cultured separately in plastic *Drosophila* vials (diameter 0.98 in. and height 3.74 in.) in approximately 25 mL of medium until they reached the required number of individuals to start the experiment. The medium used was a soft water combo (FLAMES) which mimics the water chemistry of Chalk Lake, a natural habitat for *D. pulex* (Celis-Salgado *et al.* 2008). Each individual was fed *ad libitum* with a 1:1:1 mixture of three species of algae (*Ankistrodesmus* sp., *Pseudokirchneriella* sp., *Scenedesmus* sp.). The cultures were kept at 18°C with a photoperiod of twelve hours of light and twelve hours of dark.

Population size estimation protocol

We sampled individuals with 24 ml scintillation vials. by gently stirring the tanks and taking ten samples, two from each quadrant and two others from the center of the tank at the bottom and at the surface. A validation of the protocol was iteratively verified for increments of 100 up to 1,300 individuals, prior to the experiment. Averaged estimates between two people (a colleague and myself) using the above protocol were calculated after adding to a 9-L tank, first, 50 individuals and, then, additional 100 individuals each time up to 1300 individuals (Fig. B.2).

Microsatellite genotyping

Total genomic DNA was extracted with the gSYNCT DNA extraction Kit (FroggaBio) after the whole adult individuals were crushed and homogenized with a plastic pestle inside a microcentrifuge tube. DNA was assayed at 10 previously mapped microsatellite loci (Table B.1) in order to determine allelic richness in the populations and its change over time (Cristescu *et al.* 2006). We employed the M13(-21) primer genotyping protocol (Schuelke 2000). The forward sequence-specific primer were 5` extended with the M13(-21) oligonucleotide. The polymerase chain reactions (PCR) were performed in 12-µL reactions with 10 ng of DNA template, 1XPCR buffer with 25 nmol of Mg^{2+} , 0.5 units of

Taq polymerase, 2.5 nmol of each dNTP, 1 pmol of the forward primer, 2 pmol of the reverse primer, and 2 pmol of the universal fluorescence-labeled M13(-21) primer. To reduce non-specific amplification, we used a touchdown PCR. Thermal cycle programs included an initial denaturation step of 3 min at 95°C followed by 10 cycles of 35 s denaturation at 94°C, 35 s at final annealing temperature + 10°C (the annealing temperature was decreased by 1°C every cycle during each of the 9 following cycles), 45 s extension at 72°C followed by 30 cycles of 35 s denaturation at 94°C, 35 s annealing temperature at 48°C, and 45 s extension at 72°C, with a final extension at 72°C for 10 min. The amplified products were diluted 20-fold and combined in groups of four according to their size and fluorescent labels (NED, PET, FAM, VIC). Two microliters of the diluted PCR product were then mixed with 8.35 µl of HiDi formamide (Life Technologies) and 0.15 µl of GeneScan–500 LIZ size standard (Applied Biosystems, Foster City, CA, USA). Samples were genotyped using an ABI 3730XL Analyzer and chromatographs were evaluated using GeneMapper Software v3.0 (Applied Biosystems). The microsatellite analysis was carried out using a pooling approach assuming that the detected pattern of fluorescence peaks reflected the composite pattern of the individual alleles (Eschbach & Schöning 2013). Using GeneMapper, we set up microsatellite allele bins for manual allele identification based on the fragment sizes of the individually amplified clones. The intensity of the signal peak was annotated and allelic richness was measured for each tank by counting the number of alleles per locus at each time point.

Table B.1. List of microsatellite markers used in the analyses with their expected size and linkage group (Cristescu *et al.* 2006).

Primer code	Primer name	M13 dye	Exp size	Linkage group
d078	Dp616	VIC	189/199	III
d153	Dp1350	VIC	239/247	VI
d006	Dp70	PET	272/278	XI
d087	Dp648	PET	328/337	V
d088	Dp660	FAM	124/129	IX
d105	Dp779	FAM	145/149	IV
d111	Dp907	FAM	268/288	VI
d005	Dp463	NED	302/307	X
d042	Dp208	NED	341/358	V
d070	Dp325	NED	184/192	II

Table B.2. Clonal lines' identity with their number of alleles across the 10 microsatellite markers and the number of unique alleles characterizing each clonal line. Clones' ID in bold represent the clonal lines used to create the MNC populations.

Clone ID	No. of alleles	Unique alleles
Long Lake		
LL1	14	0
LL3	14	0
LL10	14	0
LL11	15	2
LL13	14	0
LL16	**14**	**0**
Sportsman Lake		
SP8	**14**	**0**
SP12	16	0
SP14	14	0
SP18	12	0
SP19	14	0
SP21	15	0
Clear Lake		
CL3	13	0
CL4	13	0
CL11	15	0
CL12	15	0
CL13	**16**	**0**
CL28	14	0
Average Lakes	14.2	
Clone ID	No. of alleles	Unique alleles
Dump Pond		
DP1	19	2
DP6	17	4
DP8	19	3
DP9	**16**	**0**
DP21	19	2
DP25	19	0
Bridge N. Pond		
BN2	**17**	**1**
BN9	17	2
BN11	18	2

BN13	16	2
BN20	15	2
BN27	13	1
Center Pond		
CT10	17	0
CT11	16	1
CT16	15	0
CT18	15	0
CT24	**17**	**0**
CT26	17	0
Average ponds	16.7	
Total average	15.5	

Table B.3. Number of unique alleles per each microsatellite marker. Symbol * represents the presence of alleles characterizing only a particular population. Symbol ** depicts the presence of two diagnostic alleles (not shared with any other lake or pond habitat). The total # of alleles represents the number of alleles observed in the lake and pond populations; some alleles were shared between the two groups (lakes and ponds).

	Lakes			Ponds		
Primer code	Long	Clear	Sports.	Dump	Bridge	Center
d078				2	1	
d153				* 2	* 2	* 1
d006	1	1			2	1
d087				3	1	
d088				3	1	
d105				1		1
d111				** 2	* 3	* 2
d005				* 2	* 2	
d042				1	2	
d070				1	* 2	
Total # of unique alleles	1	1	0	17	16	5
Total # of alleles		27			68	

Table B.4. (a) Mean and standard deviation (SD) of growth rates [$Ln(N_{t+1}-N_t)$] in control populations. These values were used to obtain values in table (b). **(b)** Quantitative information regarding population growth rates and their relative differences comparing to control populations. Specifically, mean growth rate of each population ("Mean"), its standard deviation ("SD"), extinction day ("Ex.day"), number of positive and negative picks exceeding the standard deviation of control groups (N+ and N-), and detailed information about these picks in population growth rates: the natural logarithm of population size, day they occurred, copper concentration (expected), and ratio between the pick value and the standard deviation of all control populations (C), high diversity populations (MTC), pooled monoclonal populations (MNC), and monoclonal-by-habitat (# SD C, # SD MTC c, etc.). Numbers in bold represent the ratios between corresponding treatment and control (e.g., MTC treatment population pick value over the standard deviation of MTC control group; Long treatment population pick value over the standard deviation of MNC control group or Long lake control, etc.). Number in gray represent ratios between non-corresponding treatment and control (e.g., MTC treatment pick value over the standard deviation of MNC control group; Long treatment population pick value over the standard deviation of MTC control group or Dump pond control, etc.).

(a)

Population	Mean	SD
Controls (C)	-0.07	0.76
MTC controls (MTC c)	0.01	0.32
MNC controls (MNC c)	0.00	0.35
Long lake control (c)	0.00	0.30
Sportsman lake control (c)	-0.02	0.35
Clear lake control (c)	-0.01	0.30
Dump pond control (c)	0.01	0.36
Bridge pond control (c)	0.00	0.35
Center pond control (c)	0.00	0.42

(b)

Population	Mean	SD	Ex.day	N+	N-	Value	Day	[Cu] µg/L	+ \ -	# SD C	# SD MTC c	# SD MNC c	# SD Long c	# SD Sportsman c	# SD Clear c	# SD Dump c	# SD Bridge c	# SD Center c
MTC t1	-0.12	0.98	194	5	6	0.87	59	175	+	**1.15**	**2.69**	2.52	2.92	2.49	2.87	2.43	2.50	2.07
						0.51	73	178	+	**0.67**	**1.58**	1.48	1.71	1.46	1.68	1.42	1.46	1.21
						-0.51	86	182	-	**-0.67**	**-1.58**	-1.48	-1.71	-1.46	-1.68	-1.42	-1.46	-1.21
						-0.95	96	184	-	**-1.25**	**-2.94**	-2.75	-3.19	-2.72	-3.14	-2.65	-2.73	-2.26
						-0.4	103	186	-	**-0.53**	**-1.24**	-1.16	-1.34	-1.15	-1.32	-1.12	-1.15	-0.95
						0.78	110	186	+	**1.03**	**2.41**	2.26	2.62	2.23	2.58	2.18	2.24	1.85
						-1.69	115	186	-	**-2.23**	**-5.23**	-4.89	-5.67	-4.84	-5.58	-4.72	-4.85	-4.02
						0.28	118	186	+	**0.37**	**0.87**	0.81	0.94	0.80	0.93	0.78	0.80	0.67
						-4.28	124	186	-	**-5.65**	**-13.25**	-12.39	-14.36	-12.26	-14.14	-11.94	-12.28	-10.17
						2.92	139	186	+	**3.86**	**9.04**	8.45	9.79	8.37	9.65	8.15	8.38	6.94
						-2.92	145	186	-	**-3.86**	**-9.04**	-8.45	-9.79	-8.37	-9.65	-8.15	-8.38	-6.94
						-0.69	194	186	-	**-0.91**	**-2.14**	-2.00	-2.31	-1.98	-2.28	-1.93	-1.98	-1.64
MTC t2	-0.13	1.42	173	5	9	-0.69	59	175	-	**-0.91**	**-2.14**	-2.00	-2.31	-1.98	-2.28	-1.93	-1.98	-1.64
						1.09	66	176	+	**1.44**	**3.37**	3.16	3.66	3.12	3.60	3.04	3.13	2.59
						-0.51	73	178	-	**-0.67**	**-1.58**	-1.48	-1.71	-1.46	-1.68	-1.42	-1.46	-1.21
						0.69	79	180	+	**0.91**	**2.14**	2.00	2.31	1.98	2.28	1.93	1.98	1.64
						-0.59	86	182	-	**-0.78**	**-1.83**	-1.71	-1.98	-1.69	-1.95	-1.65	-1.69	-1.40
						-2.06	100	186	-	**-2.72**	**-6.38**	-5.96	-6.91	-5.90	-6.81	-5.75	-5.91	-4.90
						1.59	103	186	+	**2.10**	**4.92**	4.60	5.33	4.56	5.25	4.44	4.56	3.78
						-0.51	118	186	-	**-0.67**	**-1.58**	-1.48	-1.71	-1.46	-1.68	-1.42	-1.46	-1.21
						-4	121	186	-	**-5.28**	**-12.38**	-11.58	-13.42	-11.46	-13.22	-11.16	-11.48	-9.51
						4.69	124	186	+	**6.19**	**14.52**	13.58	15.73	13.44	15.49	13.09	13.46	11.15
						-4.69	129	186	-	**-6.19**	**-14.52**	-13.58	-15.73	-13.44	-15.49	-13.09	-13.46	-11.15
						2.92	136	186	+	**3.86**	**9.04**	8.45	9.79	8.37	9.65	8.15	8.38	6.94
						-2.92	145	186	-	**-3.86**	**-9.04**	-8.45	-9.79	-8.37	-9.65	-8.15	-8.38	-6.94
						-0.69	173	186	-	**-0.91**	**-2.14**	-2.00	-2.31	-1.98	-2.28	-1.93	-1.98	-1.64

Population	Mean	SD	Ex.day	N+	N-	Value	Day	[Cu] µg/L	+ \ -	# SD C	# SD MTC c	# SD MNC c	# SD Long c	# SD Sportsman c	# SD Clear c	# SD Dump c	# SD Bridge c	# SD Center c
						1.02	79	180	+	**1.35**	**3.16**	2.95	3.42	2.92	3.37	2.85	2.93	2.42
						-0.91	86	182	-	**-1.20**	**-2.82**	-2.63	-3.05	-2.61	-3.01	-2.54	-2.61	-2.16
						-0.35	107	186	-	**-0.46**	**-1.08**	-1.01	-1.17	-1.00	-1.16	-0.98	-1.00	-0.83
						1.09	110	186	+	**1.44**	**3.37**	3.16	3.66	3.12	3.60	3.04	3.13	2.59
						-0.54	115	186	-	**-0.71**	**-1.67**	-1.56	-1.81	-1.55	-1.78	-1.51	-1.55	-1.28
						-0.56	118	186	-	**-0.74**	**-1.73**	-1.62	-1.88	-1.60	-1.85	-1.56	-1.61	-1.33
						-0.69	121	186	-	**-0.91**	**-2.14**	-2.00	-2.31	-1.98	-2.28	-1.93	-1.98	-1.64
MTC t3	-0.14	1.31	226	5	10	-3.6	124	186	-	**-4.75**	**-11.14**	-10.42	-12.07	-10.32	-11.89	-10.05	-10.33	-8.55
						2.92	166	186	+	**3.86**	**9.04**	8.45	9.79	8.37	9.65	8.15	8.38	6.94
						-2.92	177	186	-	**-3.86**	**-9.04**	-8.45	-9.79	-8.37	-9.65	-8.15	-8.38	-6.94
						2.92	180	186	+	**3.86**	**9.04**	8.45	9.79	8.37	9.65	8.15	8.38	6.94
						3.60	208	186	+	**4.75**	**11.14**	10.42	12.07	10.32	11.89	10.05	10.33	8.55
						-0.68	212	186	-	**-0.90**	**-2.10**	-1.97	-2.28	-1.95	-2.25	-1.90	-1.95	-1.62
						-2.92	215	186	-	**-3.86**	**-9.04**	-8.45	-9.79	-8.37	-9.65	-8.15	-8.38	-6.94
						-0.69	226	186	-	**-0.91**	**-2.14**	-2.00	-2.31	-1.98	-2.28	-1.93	-1.98	-1.64
						-0.47	59	175	-	**-0.62**	**-1.45**	-1.36	-1.58	-1.35	-1.55	-1.31	-1.35	-1.12
						0.69	66	176	+	**0.91**	**2.14**	2.00	2.31	1.98	2.28	1.93	1.98	1.64
						0.53	73	178	+	**0.70**	**1.64**	1.53	1.78	1.52	1.75	1.48	1.52	1.26
						-0.63	86	182	-	**-0.83**	**-1.95**	-1.82	-2.11	-1.81	-2.08	-1.76	-1.81	-1.50
						-0.98	103	186	-	**-1.29**	**-3.03**	-2.84	-3.29	-2.81	-3.24	-2.73	-2.81	-2.33
						0.51	107	186	+	**0.67**	**1.58**	1.48	1.71	1.46	1.68	1.42	1.46	1.21
						0.47	110	186	+	**0.62**	**1.45**	1.36	1.58	1.35	1.55	1.31	1.35	1.12
MTC t4	-0.13	0.89	177	6	9	-0.51	118	186	-	**-0.67**	**-1.58**	-1.48	-1.71	-1.46	-1.68	-1.42	-1.46	-1.21
						-0.41	124	186	-	**-0.54**	**-1.27**	-1.19	-1.38	-1.17	-1.35	-1.14	-1.18	-0.97
						-0.68	132	186	-	**-0.90**	**-2.10**	-1.97	-2.28	-1.95	-2.25	-1.90	-1.95	-1.62
						0.68	136	186	+	**0.90**	**2.10**	1.97	2.28	1.95	2.25	1.90	1.95	1.62
						-4	139	186	-	**-5.28**	**-12.38**	-11.58	-13.42	-11.46	-13.22	-11.16	-11.48	-9.51
						2.92	145	186	+	**3.86**	**9.04**	8.45	9.79	8.37	9.65	8.15	8.38	6.94
						-2.92	149	186	-	**-3.86**	**-9.04**	-8.45	-9.79	-8.37	-9.65	-8.15	-8.38	-6.94
						-0.69	177	186	-	**-0.91**	**-2.14**	-2.00	-2.31	-1.98	-2.28	-1.93	-1.98	-1.64

Population	Mean	SD	Ex.day	N+	N-	Value	Day	[Cu] µg/L	+ \ -	# SD C	# SD MTC c	# SD MNC c	# SD Long c	# SD Sportsman c	# SD Clear c	# SD Dump c	# SD Bridge c	# SD Center c
MTC t5	-0.13	1.08	191	4	9	-0.47	59	175	-	**-0.62**	**-1.45**	-1.36	-1.58	-1.35	-1.55	-1.31	-1.35	-1.12
						1.09	66	176	+	**1.44**	**3.37**	3.16	3.66	3.12	3.60	3.04	3.13	2.59
						0.43	79	180	+	**0.57**	**1.33**	1.24	1.44	1.23	1.42	1.20	1.23	1.02
						-0.51	86	182	-	**-0.67**	**-1.58**	-1.48	-1.71	-1.46	-1.68	-1.42	-1.46	-1.21
						-0.69	93	184	-	**-0.91**	**-2.14**	-2.00	-2.31	-1.98	-2.28	-1.93	-1.98	-1.64
						-0.47	100	186	-	**-0.62**	**-1.45**	-1.36	-1.58	-1.35	-1.55	-1.31	-1.35	-1.12
						-0.69	115	186	-	**-0.91**	**-2.14**	-2.00	-2.31	-1.98	-2.28	-1.93	-1.98	-1.64
						-3.6	121	186	-	**-4.75**	**-11.14**	-10.42	-12.07	-10.32	-11.89	-10.05	-10.33	-8.55
						2.92	124	186	+	**3.86**	**9.04**	8.45	9.79	8.37	9.65	8.15	8.38	6.94
						-2.92	129	186	-	**-3.86**	**-9.04**	-8.45	-9.79	-8.37	-9.65	-8.15	-8.38	-6.94
						2.92	173	186	+	**3.86**	**9.04**	8.45	9.79	8.37	9.65	8.15	8.38	6.94
						-2.92	177	186	-	**-3.86**	**-9.04**	-8.45	-9.79	-8.37	-9.65	-8.15	-8.38	-6.94
						-0.69	191	186	-	**-0.91**	**-2.14**	-2.00	-2.31	-1.98	-2.28	-1.93	-1.98	-1.64
MTC t6	-0.14	0.69	180	4	8	-0.85	86	182	-	**-1.12**	**-2.63**	-2.46	-2.85	-2.44	-2.81	-2.37	-2.44	-2.02
						-0.69	96	184	-	**-0.91**	**-2.14**	-2.00	-2.31	-1.98	-2.28	-1.93	-1.98	-1.64
						-0.51	100	186	-	**-0.67**	**-1.58**	-1.48	-1.71	-1.46	-1.68	-1.42	-1.46	-1.21
						-1.08	103	186	-	**-1.43**	**-3.34**	-3.13	-3.62	-3.09	-3.57	-3.01	-3.10	-2.57
						1.77	107	186	+	**2.34**	**5.48**	5.12	5.94	5.07	5.85	4.94	5.08	4.21
						-0.4	110	186	-	**-0.53**	**-1.24**	-1.16	-1.34	-1.15	-1.32	-1.12	-1.15	-0.95
						0.40	115	186	+	**0.53**	**1.24**	1.16	1.34	1.15	1.32	1.12	1.15	0.95
						-0.91	121	186	-	**-1.20**	**-2.82**	-2.63	-3.05	-2.61	-3.01	-2.54	-2.61	-2.16
						-0.68	124	186	-	**-0.90**	**-2.10**	-1.97	-2.28	-1.95	-2.25	-1.90	-1.95	-1.62
						0.68	132	186	+	**0.90**	**2.10**	1.97	2.28	1.95	2.25	1.90	1.95	1.62
						-3.6	136	186	+	**-4.75**	**-11.14**	-10.42	-12.07	-10.32	-11.89	-10.05	-10.33	-8.55
						-0.69	180	186	-	**-0.91**	**-2.14**	-2.00	-2.31	-1.98	-2.28	-1.93	-1.98	-1.64

Population	Mean	SD	Ex.day	N+	N-	Value	Day	[Cu] µg/L	+ \ -	# SD C	# SD MTC c	# SD MNC c	# SD Long c	# SD Sportsman c	# SD Clear c	# SD Dump c	# SD Bridge c	# SD Center c
MNC t13 Long	0.02	0.35	166	5	9	0.43	66	176	+	**0.57**	1.33	**1.24**	**1.44**	1.23	1.42	1.20	1.23	1.02
						-1.32	93	184	-	**-1.74**	-4.09	**-3.82**	**-4.43**	-3.78	-4.36	-3.68	-3.79	-3.14
						-0.69	96	184	-	**-0.91**	-2.14	**-2.00**	**-2.31**	-1.98	-2.28	-1.93	-1.98	-1.64
						1.09	100	186	+	**1.44**	3.37	**3.16**	**3.66**	3.12	3.60	3.04	3.13	2.59
						-1.09	103	186	-	**-1.44**	-3.37	**-3.16**	**-3.66**	-3.12	-3.60	-3.04	-3.13	-2.59
						1.09	107	186	+	**1.44**	3.37	**3.16**	**3.66**	3.12	3.60	3.04	3.13	2.59
						-1.09	110	186	-	**-1.44**	-3.37	**-3.16**	**-3.66**	-3.12	-3.60	-3.04	-3.13	-2.59
						1.20	115	186	+	**1.58**	3.71	**3.47**	**4.02**	3.44	3.96	3.35	3.44	2.85
						-1.24	118	186	-	**-1.64**	-3.84	**-3.59**	**-4.16**	-3.55	-4.10	-3.46	-3.56	-2.95
						-3.6	121	186	-	**-4.75**	-11.14	**-10.42**	**-12.07**	-10.32	-11.89	-10.05	-10.33	-8.55
						3.60	132	186	+	**4.75**	11.14	**10.42**	**12.07**	10.32	11.89	10.05	10.33	8.55
						-3.6	136	186	-	**-4.75**	-11.14	**-10.42**	**-12.07**	-10.32	-11.89	-10.05	-10.33	-8.55
						2.92	139	186	+	**3.86**	9.04	**8.45**	**9.79**	8.37	9.65	8.15	8.38	6.94
						-2.92	142	186	-	**-3.86**	-9.04	**-8.45**	**-9.79**	-8.37	-9.65	-8.15	-8.38	-6.94
						-0.69	166	186	-	**-0.91**	-2.14	**-2.00**	**-2.31**	-1.98	-2.28	-1.93	-1.98	-1.64
MNC t14 Sportsman	0.01	0.35	177	3	6	-0.86	66	176	-	**-1.14**	-2.66	**-2.49**	-2.88	**-2.46**	-2.84	-2.40	-2.47	-2.04
						0.75	73	178	+	**0.99**	2.32	**2.17**	2.52	**2.15**	2.48	2.09	2.15	1.78
						-0.63	79	180	-	**-0.83**	-1.95	**-1.82**	-2.11	**-1.81**	-2.08	-1.76	-1.81	-1.50
						-1.78	100	186	-	**-2.35**	-5.51	**-5.15**	-5.97	**-5.10**	-5.88	-4.97	-5.11	-4.23
						1.08	103	186	+	**1.43**	3.34	**3.13**	3.62	**3.09**	3.57	3.01	3.10	2.57
						-4	115	186	-	**-5.28**	-12.38	**-11.58**	-13.42	**-11.46**	-13.22	-11.16	-11.48	-9.51
						2.92	129	186	+	**3.86**	9.04	**8.45**	9.79	**8.37**	9.65	8.15	8.38	6.94
						-2.92	132	186	-	**-3.86**	-9.04	**-8.45**	-9.79	**-8.37**	-9.65	-8.15	-8.38	-6.94
						-0.69	177	177	-	**-0.91**	-2.14	**-2.00**	-2.31	**-1.98**	-2.28	-1.93	-1.98	-1.64
MNC t15 Clear	0.01	0.32	142	0	6	-0.44	86	182	-	**-0.58**	-1.36	**-1.27**	-1.48	-1.26	**-1.45**	-1.23	-1.26	-1.05
						-0.59	93	184	-	**-0.78**	-1.83	**-1.71**	-1.98	-1.69	**-1.95**	-1.65	-1.69	-1.40
						-0.51	96	184	-	**-0.67**	-1.58	**-1.48**	-1.71	-1.46	**-1.68**	-1.42	-1.46	-1.21
						-1.36	107	186	-	**-1.80**	-4.21	**-3.94**	-4.56	-3.90	**-4.49**	-3.80	-3.90	-3.23
						-2.92	110	186	-	**-3.86**	-9.04	**-8.45**	-9.79	-8.37	**-9.65**	-8.15	-8.38	-6.94
						-0.69	142	185	-	**-0.91**	-2.14	**-2.00**	-2.31	-1.98	**-2.28**	-1.93	-1.98	-1.64

Population	Mean	SD	Ex.day	N+	N-	Value	Day	[Cu] µg/L	+ \ -	# SD C	# SD MTC c	# SD MNC c	# SD Long c	# SD Sportsman c	# SD Clear c	# SD Dump c	# SD Bridge c	# SD Center c
MNC t16 Dump	0.01	0.30	187	6	11	-1.78	103	186	-	**-2.35**	-5.51	**-5.15**	-5.97	-5.10	-5.88	**-4.97**	-5.11	-4.23
						2.00	110	186	+	**2.64**	6.19	**5.79**	6.71	5.73	6.61	**5.58**	5.74	4.75
						-0.48	118	186	-	**-0.63**	-1.49	**-1.39**	-1.61	-1.38	-1.59	**-1.34**	-1.38	-1.14
						-0.69	121	186	-	**-0.91**	-2.14	**-2.00**	-2.31	-1.98	-2.28	**-1.93**	-1.98	-1.64
						0.81	124	186	+	**1.07**	2.51	**2.34**	2.72	2.32	2.68	**2.26**	2.32	1.92
						-0.69	132	186	-	**-0.91**	-2.14	**-2.00**	-2.31	-1.98	-2.28	**-1.93**	-1.98	-1.64
						-1.36	136	186	-	**-1.80**	-4.21	**-3.94**	-4.56	-3.90	-4.49	**-3.80**	-3.90	-3.23
						0.68	139	186	+	**0.90**	2.10	**1.97**	2.28	1.95	2.25	**1.90**	1.95	1.62
						-0.68	142	186	-	**-0.90**	-2.10	**-1.97**	-2.28	-1.95	-2.25	**-1.90**	-1.95	-1.62
						-2.92	145	186	-	**-3.86**	-9.04	**-8.45**	-9.79	-8.37	-9.65	**-8.15**	-8.38	-6.94
						4.00	149	186	+	**5.28**	12.38	**11.58**	13.42	11.46	13.22	**11.16**	11.48	9.51
						-4	152	186	-	**-5.28**	-12.38	**-11.58**	-13.42	-11.46	-13.22	**-11.16**	-11.48	-9.51
						2.92	159	186	+	**3.86**	9.04	**8.45**	9.79	8.37	9.65	**8.15**	8.38	6.94
						-2.92	163	186	-	**-3.86**	-9.04	**-8.45**	-9.79	-8.37	-9.65	**-8.15**	-8.38	-6.94
						2.92	166	186	+	**3.86**	9.04	**8.45**	9.79	8.37	9.65	**8.15**	8.38	6.94
						-2.92	170	186	-	**-3.86**	-9.04	**-8.45**	-9.79	-8.37	-9.65	**-8.15**	-8.38	-6.94
						-0.69	187	186	-	**-0.91**	-2.14	**-2.00**	-2.31	-1.98	-2.28	**-1.93**	-1.98	-1.64
MNC t17 Bridge	0.00	0.30	139	0	3	-0.53	79	180	-	**-0.70**	-1.64	**-1.53**	-1.78	-1.52	-1.75	-1.48	**-1.52**	-1.26
						-0.51	86	182	-	**-0.67**	-1.58	**-1.48**	-1.71	-1.46	-1.68	-1.42	**-1.46**	-1.21
						-0.69	139	186	-	**-0.91**	-2.14	**-2.00**	-2.31	-1.98	-2.28	-1.93	**-1.98**	-1.64
MNC t18 Center	0.01	0.33	103	0	4	-0.79	79	180	-	**-1.04**	-2.45	**-2.29**	-2.65	-2.26	-2.61	-2.20	-2.27	**-1.88**
						-0.81	93	184	-	**-1.07**	-2.51	**-2.34**	-2.72	-2.32	-2.68	-2.26	-2.32	**-1.92**
						-1.36	96	184	-	**-1.80**	-4.21	**-3.94**	-4.56	-3.90	-4.49	-3.80	-3.90	**-3.23**
						-3.6	103	186		**-4.75**	-11.14	**-10.42**	-12.07	-10.32	-11.89	-10.05	-10.33	**-8.55**

Table B.5. pH values measured throughout the experiment. MTC t (multiclonal treatment), MTC c (multiclonal control), MNC t (monoclonal treatment), and MNC c (monoclonal control).

Day	Sep-12	Oct-03	Nov-14	Nov-28	Dec-05	Dec-12	Dec-19	Dec-26	Jan-02	Jan-09	Feb-13	Feb-27	Mar-26
Tank	**34**	**56**	**97**	**111**	**119**	**125**	**133**	**140**	**146**	**153**	**188**	**202**	**226**
MTC t1	6.17	6.31	6.16	6.25	6.2	5.8	6.16	6.07	6.31		6.39	6.42	NA
MTC t2	6.17	6.25	6.11	6.08	6.16	5.88	6	6.06	6.2	6.25	6.37	NA	NA
MTC t3	6.18	6.31	6.1	6.23	6.13	6.02	6.05	6.11	6.23	6.37	6.28	6.4	NA
MTC t4	6.19	6.29	6.15	6.23	6.14	6.14	5.99	6.07	6.21	6.28	6.39	NA	NA
MTC t5	6.23	6.28	6.16	6.21	6.15	5.86	6.16	6.03	6.23	6.18	6.4	NA	NA
MTC t6	6.24	6.35	6.13	6.16	6.17	6.11	6.07	6.06	6.19	6.27	6.4	NA	NA
MTC c7	6.11	6.45	6.19	6.25	6.26	6.25	6.05	5.98	6.17	6.26	6.31	6.23	6.19
MTC c8	6.24	6.42	6.2	6.27	6.23	6.22	6.01	5.95	6.17	6.11	6.3	6.18	6.25
MTC c9	6.19	6.38	6.19	6.22	6.2	6.18	6	5.95	6.12	6.13	6.26	6.22	6.21
MTC c10	6.19	6.37	6.19	6.22	6.25	6.21	6.01	5.96	6.16	6.14	6.27	6.23	6.23
MTC c11	6.21	6.36	6.17	6.24	6.23	6.25	6.02	5.94	6.17	6.15	6.26	6.25	6.25
MTC c12	6.21	6.39	6.18	6.24	6.25	6.22	6.03	5.98	6.18	6.12	6.27	6.24	6.19
MNC t13 Long	6.2	6.37	6.13	6.26	6.16	5.8	6.14	6.1	6.22	6.29	6.31	NA	NA
MNC t14 Sportsman	6.17	6.56	6.06	6.29	6.29	6.16	6.06	6.07	6.29	6.26	6.43	NA	NA
MNC t15 Clear	6.2	6.54	6.06	6.3	6.26	6.02	6.17	6.07	6.27	6.29	NA	NA	NA
MNC t16 Dump	6.19	6.4	6.1	6.22	6.17	6.06	5.93	6.02	6.16	6.28	6.32	NA	NA
MNC t17 Bridge	6.24	6.35	6.13	6.3	6.25	6.14	6.22	6.19	6.32	6.33	NA	NA	NA
MNC t18 Center	6.19	6.33	6.02	6.35	6.22	5.95	6.19	NA	NA	NA	NA	NA	NA
MNC c19 Long	6.27	6.65	6.18	6.25	6.17	6.2	6.03	5.99	6.15	6.11	6.26	6.22	6.22
MNC c20 Clear	6.21	6.66	6.19	6.23	6.22	6.17	6.02	5.96	6.21	6.11	6.22	6.2	6.21
MNC c21 Sportsman	6.14	6.45	6.18	6.21	6.26	6.23	6.03	5.96	6.18	6.13	6.23	6.19	6.2
MNC c22 Dump	6.24	6.42	6.19	6.18	6.16	6.17	6.06	5.97	6.12	6.16	6.31	6.24	6.26
MNC c23 Bridge	6.2	6.39	6.14	6.13	6.26	6.19	6.01	5.96	6.16	6.12	6.27	6.22	6.28
MNC c24 Center	6.19	6.6	6.16	6.21	6.22	6.17	5.98	5.95	6.12	6.14	6.28	6.23	6.26

Table B.6. Copper concentrations at day 38, 101, 164, and 227 (tank MTC t3, MTC c9, MTC c11, MNC c21, MNC c22). Measurements were performed by inductively coupled plasma optical emission spectroscopy (ISP-OES). On the left (different calibration curves) there are the values measured following the calibration curve fitting, the day of the measurement. On the right (one unique calibration) there are values calculated through a pooled calibration curve obtained from averaged absorbance values measured at each measurement time point (day 38, 101, 164, and 227).

Time-point specific calibration curves					Calibration curve from pooled absorbance values				
Date	16-Sep	17-Nov	20-Jan	26-Mar	**Date**	16-Sep	17-Nov	20-Jan	26-Mar
Day	38	101	164	227	**Day**	38	101	164	227
Expected Cu	160	186	186	186	**Expected Cu**	160	186	186	186
MTCt1	174.4	159.7	197.8	na	**MTCt1**	173.7	168.5	193.5	na
MTCt2	174	160.8	197.4	na	**MTCt2**	173.2	169.5	193.1	na
MTCt3	173.1	161.8	195.2	168.0	**MTCt3**	172.3	170.4	190.8	170.8
MTCt4	170.2	161.4	197	na	**MTCt4**	169.1	170.1	192.6	na
MTCt5	171.8	162.1	196.2	na	**MTCt5**	170.8	170.6	191.8	na
MTCt6	170.2	162.4	197.5	na	**MTCt6**	169.1	170.9	193.2	na
MTCc7	0.0	0.0	0.0	na	**MTCc7**	0.0	0.0	0.0	na
MTCc8	0.0	0.0	0.0	na	**MTCc8**	0.0	0.0	0.0	na
MTCc9	0.0	0.0	0.0	0.0	**MTCc9**	0.0	0.0	0.0	0.0
MTCc10	0.0	0.0	0.0	na	**MTCc10**	0.0	0.0	0.0	na
MTCc11	0.0	0.0	0.0	0.0	**MTCc11**	0.0	0.0	0.0	0.0
MTCc12	0.0	0.0	0.0	na	**MTCc12**	0.0	0.0	0.0	na
MNCt13 Long	164.7	160.8	196.2	na	**MNCt13 Long**	163.2	169.5	191.8	na
MNCt14 Spor.	164.1	158.8	194.8	na	**MNCt14 Sports.**	162.5	167.7	190.3	na
MNCt15 Clear	164.9	159.9	194.8	na	**MNCt15 Clear**	163.4	168.7	190.3	na
MNCt16 Dump	176.5	158.3	195.1	na	**MNCt16 Dump**	163.4	167.2	190.7	na
MNCt17 Bridge	176	158.1	194	na	**MNCt17 Bridge**	162.9	167.0	189.5	na
MNCt18 Center	175.3	157.4	193.5	na	**MNCt18 Center**	162.2	166.4	188.9	na
MNCc19 Long	0.0	0.0	0.0	na	**MNCc19 Long**	0.0	0.0	0.0	na
MNCc20 Clear	0.0	0.0	0.0	na	**MNCc20 Clear**	0.0	0.0	0.0	na
MNCc21 Sports.	0.0	0.0	0.0	0.0	**MNCc21 Sports.**	0.0	0.0	0.0	0.0
MNCc22 Dump	0.0	0.0	0.0	0.0	**MNCc22 Dump**	0.0	0.0	0.0	0.0
MNCc23 Bridge	0.0	0.0	0.0	na	**MNCc23 Bridge**	0.0	0.0	0.0	na
MNCc24 Center	0.0	0.0	0.0	na	**MNCc24 Center**	0.0	0.0	0.0	na

Figure B.1 Location of the habitats from which original populations were sampled (Illinois, USA). Three populations of *Daphnia pulex* were sampled in ponds: Dump (40.2428 N, 87.7795 W), Bridge North (40.1221 N, 87.7367 W) and Center (40.13291 N, 88.14004 W) and three populations of *Daphnia pulicaria* were sampled in lakes: Long (40.13254 N, 87.73641 W), Clear (40.1419 N, 87.7378 W) and Sportsman's (40.14 N, 87.44 W).

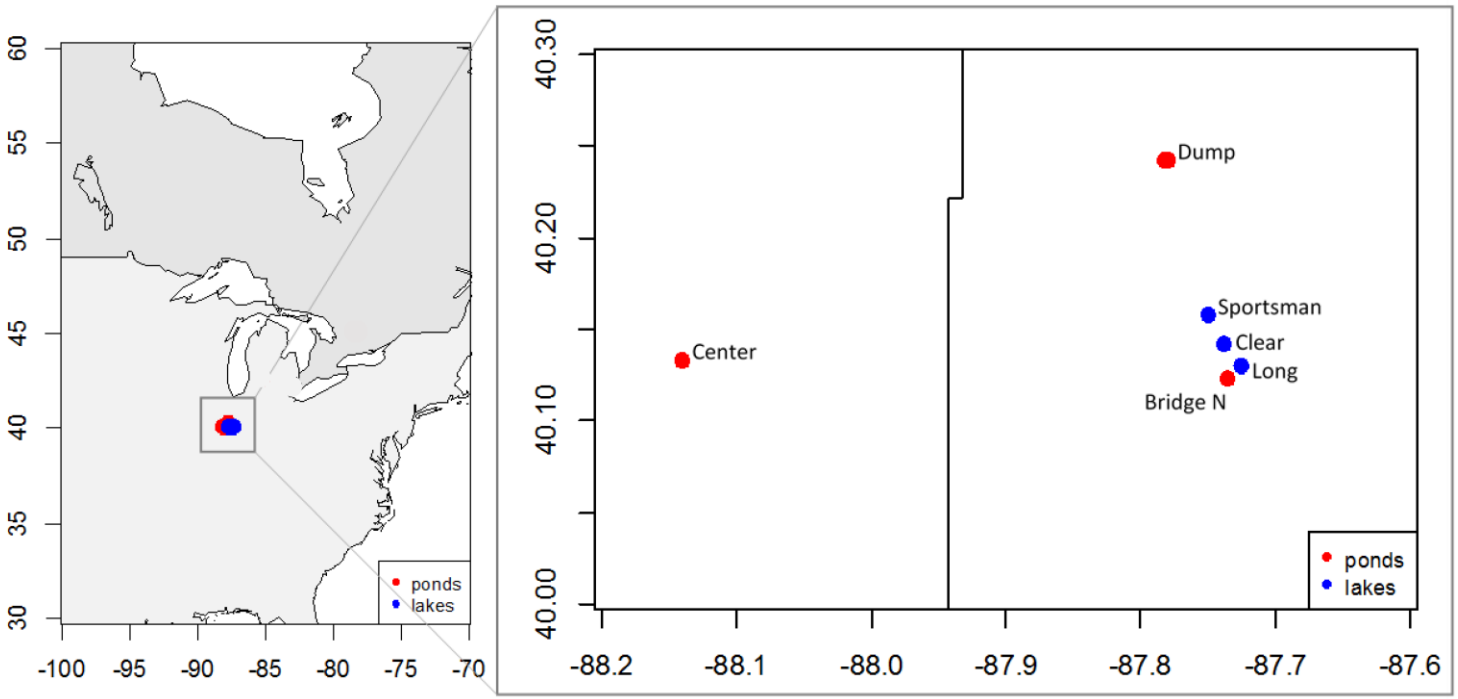


Figure B.2 The sampling protocol included first homogenization through stirring of the tanks and then the collection of ten samples: five at the bottom of the tanks and five in the surface. The samples were collected two by two as shown in the pictures. The numbers in the tank correspond to the samples that were collected in the corresponding picture number.

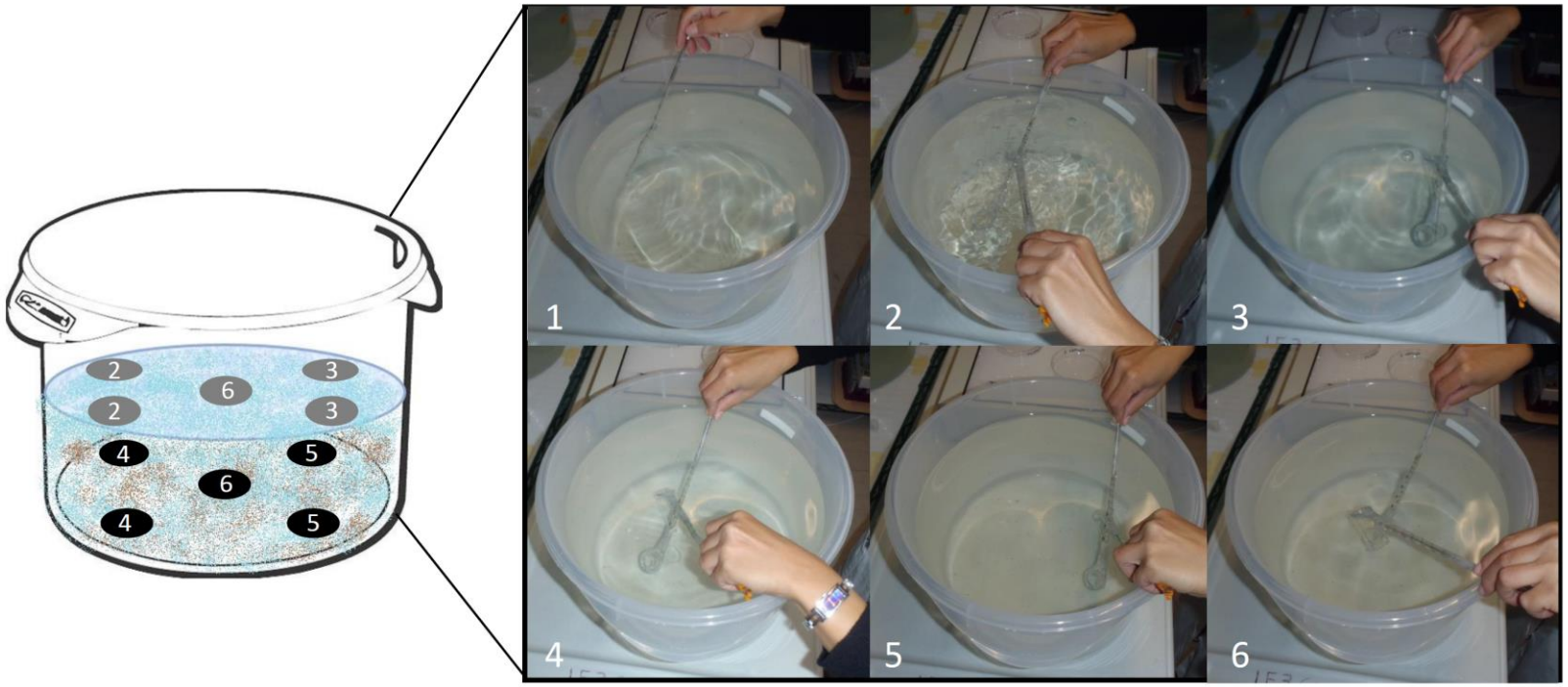


Figure B.3 Validation of the sampling method. The protocol was verified for increments of 100 up to 1,300 individuals, prior to the experiment.

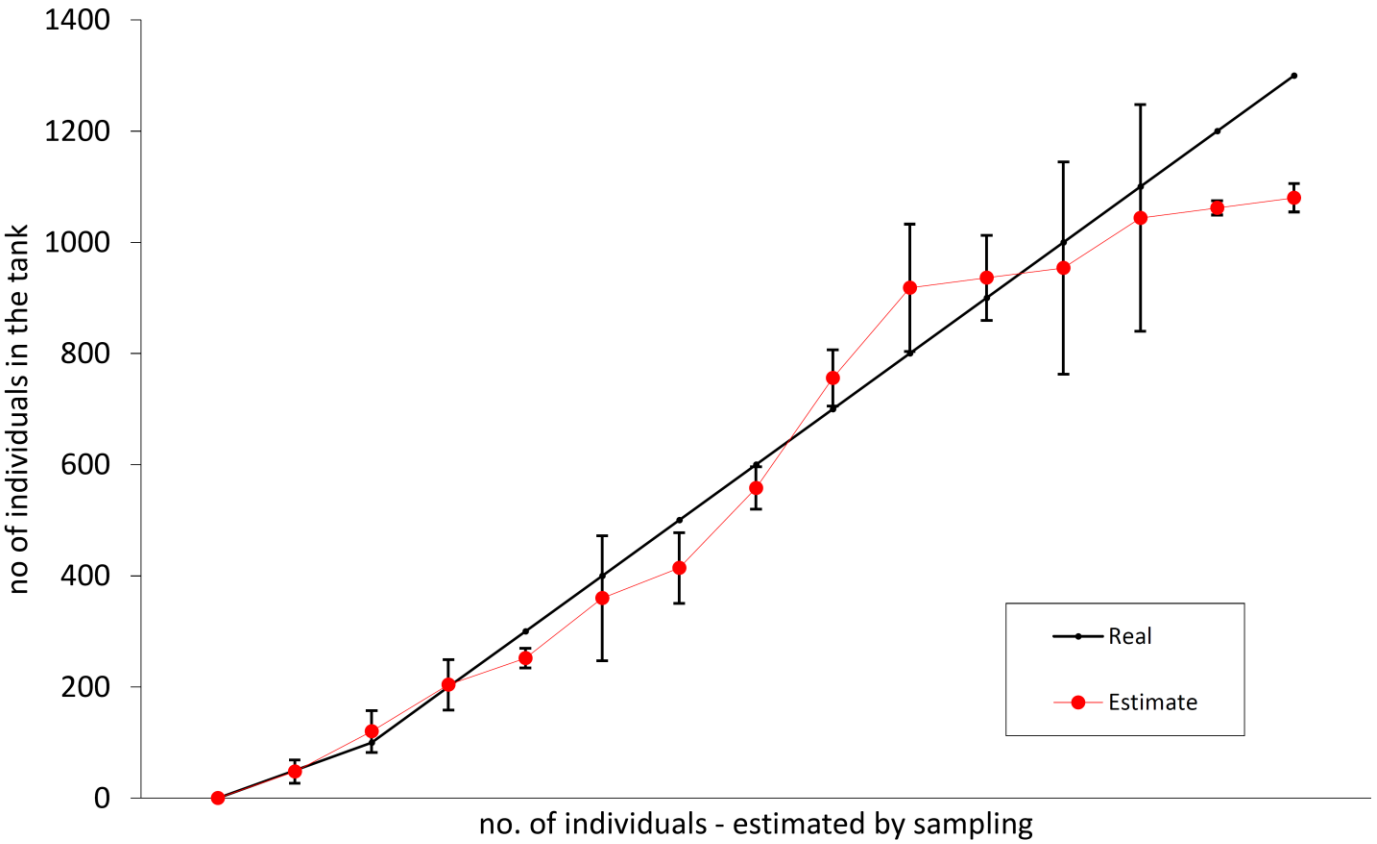
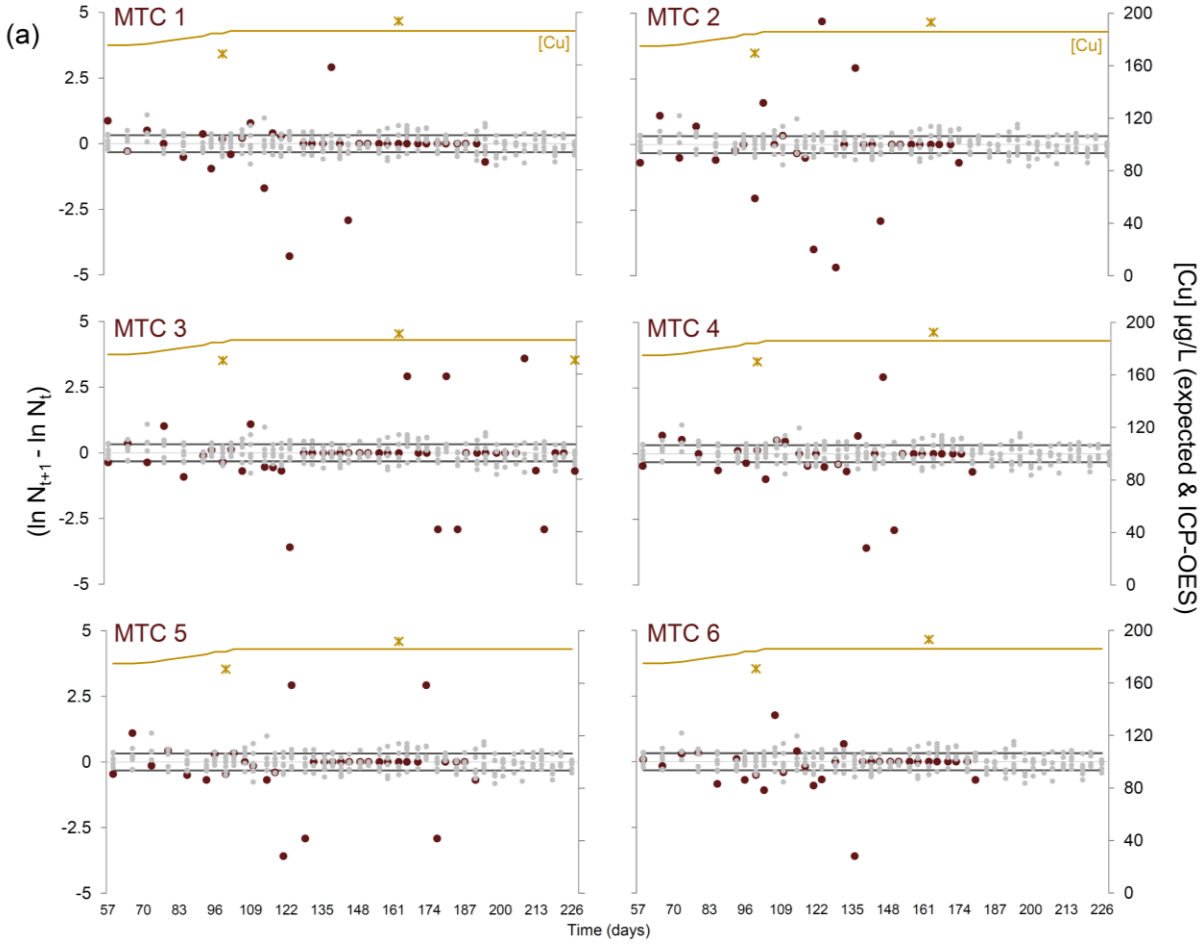


Figure B.4. Population growth rates [ln(N_{t+1}-N_t)] in **(a)** MTC populations and **(b)** MNC populations. Controls 'growth rates are shown in gray including the standard deviation range. Expected copper concentrations and ICP-OES estimates are also shown.



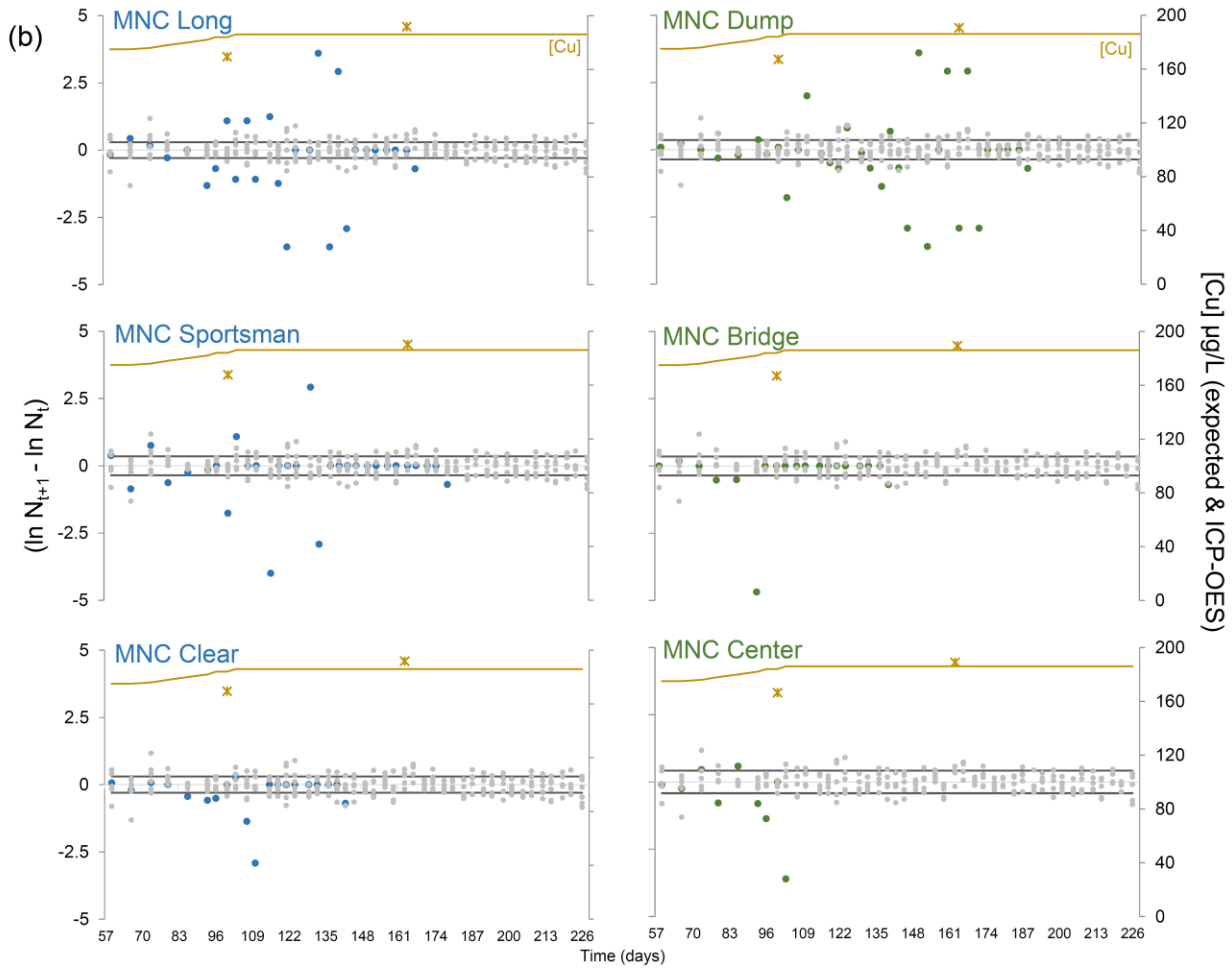
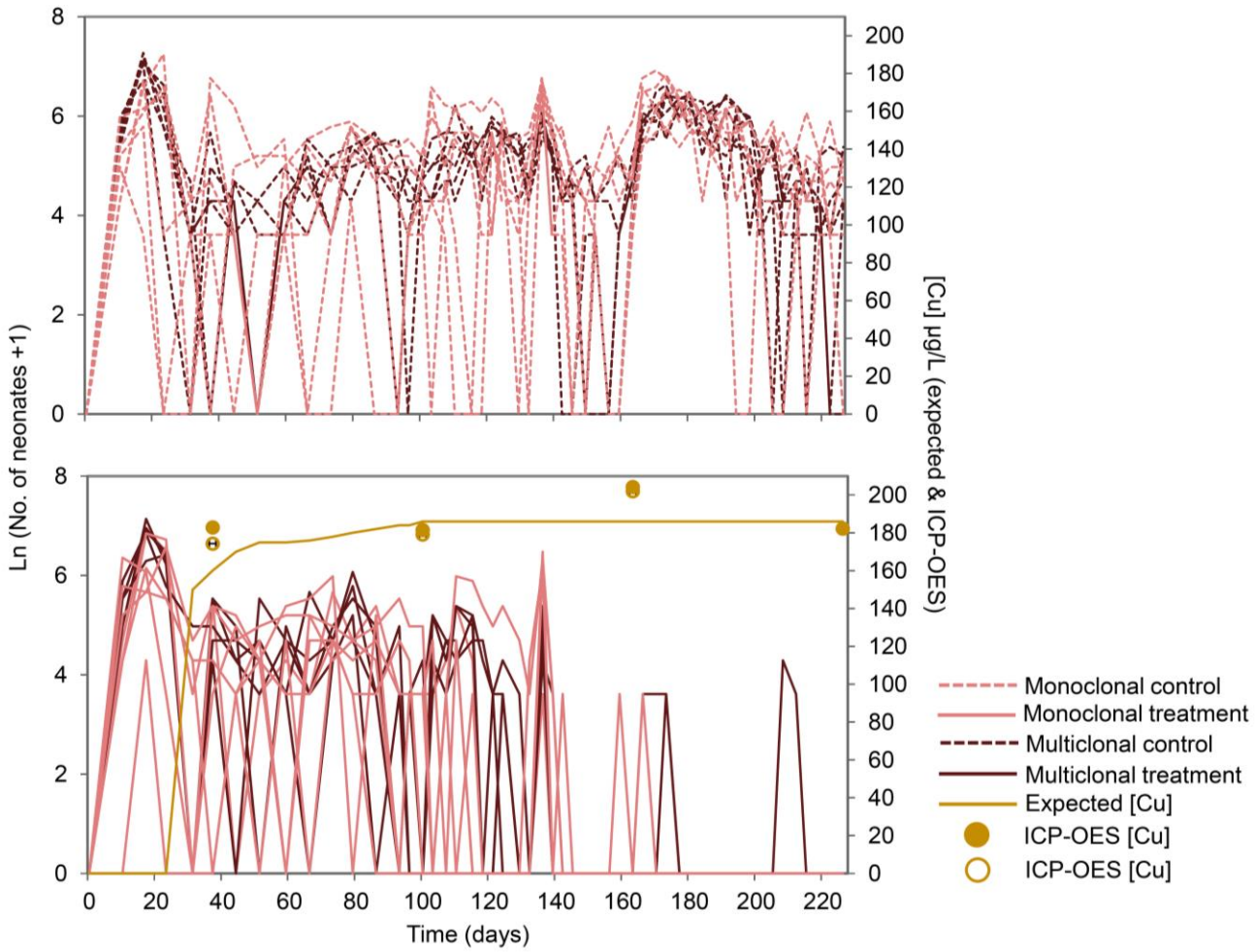

(b)
MNC Long
MNC Dump
MNC Sportsman
MNC Bridge
MNC Clear
MNC Center
[Cu]
(ln N$_{t+1}$ - ln N$_t$)
[Cu] µg/L (expected & ICP-OES)
Time (days)
5
2.5
0
-2.5
-5
200
160
120
80
40
0
57 70 83 96 109 122 135 148 161 174 187 200 213 226

Figure B.5. Number of neonates (ln) produced by control and treatment populations throughout the experiment.



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Appendix C

Supplementary material for Chapter 3

Mock communities

Methods

Two mock communities were included in the molecular analysis to assess the quality of sequencing and to calibrate the bioinformatic pipeline. The mock communities consisted of single individuals of several zooplankton species, covering broad taxonomic groups (Mollusca, Rotifera, Tunicata, Crustacean). The first mock community (mock community *a*) included 10 species of zooplankton and the second one 27 species (mock community *b*; Appendix C, Table C.1). The species were identified morphologically by experienced taxonomists and DNA was extracted using Qiagen DNeasy Blood & Tissue kits and stored in ultra pure water at -20 °C, as described in Brown *et al.* (2015).

Results

The detection of mock community species ranged from 30% to 50% for replicates of the mock community *a* and was 63% for mock community *b*. Replicate 1 of the mock community *a* showed an increase of one species when quality filtering parameter increased from 20 to 23 while the other mock community samples showed the same level of detection for all quality parameters.

Discussion

The missing species from the mock community assemblages were not retrieved by allowing lower quality of sequences during filtering and merging or lower percentages of identity during taxonomic sequence assignment. The mock community samples used in this study have been previously used in other studies (Brown *et al.* 2015; Zhang *et al.* 2018). Zhang *et al.* (2018) used the same mock community samples to test the effectiveness of multiplexing molecular markers to maximize species detection. They used two barcode markers (COI and 18S) and four primer pairs including Leray COI (Leray *et al.* 2013). They found that detection increased with the number of primer and marker used and it ranged from an average of 77% with the use of a single marker and single primer pair to 89-93% with the combination of COI and 18S. Similar results were found by Alberdi *et al.* (2018) using COI and 16S. The overall detection success of the Leray COI was 38.5%. COI primers were designed to target a wide range of phyla (as

observed in this study with fungi, protists, and the detection of other taxa) but they found that amplification success was dependent on the species group. This, perhaps, explains the lack of full amplification success of the mock communities' species.

Table C.1. Mock communities species lists and information about the source of each specimen. All species were represented by one single individual.

Mock community	Order	Family	Species	Source
a	Cyclopoida	Cyclopidae	*Acanthocyclops vernalis*	See Brown *et al.* 2015
a	Diplostraca	Bosminidae	*Bosmina longirostris*	See Brown *et al.* 2015
a	Diplostraca	Daphniidae	*Ceriodaphnia lacustris*	See Brown *et al.* 2015
a	Diplostraca	Daphniidae	*Daphnia parvula*	See Brown *et al.* 2015
a	Diplostraca	Daphniidae	*Daphnia pulex*	See Brown *et al.* 2015
a	Diplostraca	Daphniidae	*Daphnia pulicaria*	See Brown *et al.* 2015
a	Diplostraca	Holopediidae	*Holopedium gibberum*	See Brown *et al.* 2015
a	Diplostraca	Leptodoridae	*Leptodora kindtii*	See Brown *et al.* 2015
a	Diplostraca	Polyphemidae	*Polyphemus pediculus*	See Brown *et al.* 2015
a	Calanoida	Clausocalanidae	*Pseudocalanus mimus*	See Brown *et al.* 2015
b	Calanoida	Acartiidae	*Acartia hudsonica*	See Brown *et al.* 2015
b	Trachymedusae	Rhopalonematidae	*Aglantha digitale*	See Brown *et al.* 2015
b	Anostraca	Artemiidae	*Artemia spp*	See Brown *et al.* 2015
b	Isopoda	Asellidae	*Asellus*	See Brown *et al.* 2015
b	Sessilia	Balanidae	*Balanus crenatus*	See Brown *et al.* 2015
b	Stolidobranchia	Styelidae	*Botrylloides violaceus*	PBS-DFO
b	Stolidobranchia	Styelidae	*Botryllus schlosseri*	PBS-DFO
b	Ploima	Brachionidae	*Brachionus calyciflorus*	See Brown *et al.* 2015
b	Diplostraca	Cercopagididae	*Bythotrephes longimanus*	See Brown *et al.* 2015
b	Amphipoda	Caprellidae	*Caprella mutica*	PBS-DFO
b	Diplostraca	Cercopagididae	*Cercopagis pengoi*	See Brown *et al.* 2015
b	Venerida	Cyrenidae	*Corbicula fluminea*	See Brown *et al.* 2015
b	Ostreida	Ostreidae	*Crassostrea gigas*	PBS-DFO
b	Diplostraca	Daphniidae	*Daphnia magna*	See Brown *et al.* 2015
b	Decapoda	Varunidae	*Hemigrapsus oregonensis*	PBS-DFO
b	Mysida	Mysidae	*Hemimysis anomala*	See Brown *et al.* 2015
b	Amphipoda	Hyalellidae	*Hyalella azteca*	See Brown *et al.* 2015
b	Ploima	Branchionidae	*Keratella quadrata*	See Brown *et al.* 2015
b	Thecosomata	Limacinidae	*Limacina helicina*	See Brown *et al.* 2015
b	Mytilida	Mytilidae	*Limnoperna fortunei*	See Brown *et al.* 2015
b	Decapoda	Panopeidae	*Lophopanopeus bellus*	PBS-DFO
b	Cardiida	Tellinidae	*Macoma secta*	PBS-DFO
b	Cyclopoida	Cyclopidae	*Mesocyclops edax*	See Brown *et al.* 2015
b	Decapoda	Cancridae	*Metacarcinus magister*	PBS-DFO
b	Harpacticoida	Ectinosomatidae	*Microsetella norvegica*	See Brown *et al.* 2015
b	Stolidobranchia	Styelidae	*Styela clava*	PBS-DFO
b	Calanoida	Tortanidae	*Tortanus discaudatus*	See Brown *et al.* 2015

Table C.2. Output of the DADA2 analyses based on different combinations of parameters: "Quality"= minimum quality average allowed before trimming; "Primer errors"= maximum number of "expected errors" allowed in a forward and reverse read; "ASV size"= minimum size of an ASV. Data include the total number of reads recovered, number of reads and species relative to mock communities, and the percentage of recovered species based on the original sample (10 species for a, and 27 for b), and number of reads and species detected in negative controls. Taxonomic information of mock community samples was assigned using a database including only sequences of the mock community species while for negative controls I used a database including sequences of all Eukaryota (NCBI database). All species assignments were done with a percentage identity of 98%. Numbers in bold represent data for the selected pipeline: quality of 23, 4 and 5 maximum errors in forward and reverse read respectively, and 4 as minimum size of ASV.

Quality	Primer errors	ASV size	Total # reads	Mock communities									Negative controls					
				a replicate 1			*a* replicate 2			*b*			blank 1		blank 2		blank 3	
				# reads	# species	%	# reads	# species	%	# reads	# species	%	# reads	# species	# reads	# species	# reads	# species
18	ef4er5	min 4	9,607,402	65,048	3	30	88,579	5	50	141,008	17	63	3,709	3	2,009	3	331	2
		min 8	9,604,338	65,048	3	30	88,572	5	50	140,911	17	63	3,709	3	2,009	3	331	2
	ef5er6	min 4	10,013,469	67,957	3	30	97,140	5	50	146,946	17	63	3,939	3	2,214	3	348	2
		min 8	10,011,754	67,956	3	30	97,110	5	50	146,862	17	63	3,939	3	2,214	3	348	2
20	ef4er5	min 4	9,980,947	76,283	3	30	119,584	5	50	147,899	17	63	3,861	3	2,148	4	350	4
		min 8	9,977,256	76,283	3	30	119,573	5	50	147,777	17	63	3,861	3	2,148	4	350	4
	ef5er6	min 4	10,391,546	79,347	3	30	127,896	5	50	153,855	17	63	4,046	3	2,296	4	364	4
		min 8	10,388,724	79,346	3	30	127,885	5	50	153,745	17	63	4,046	3	2,296	4	364	4
23	**ef4er5**	**min 4**	**11,546,487**	**81,324**	**4**	**40**	**134,642**	**5**	**50**	**157,444**	**17**	**63**	**4,166**	**4**	**2,377**	**6**	**461**	**4**
		min 8	**11,544,189**	**81,324**	**4**	**40**	**134,629**	**5**	**50**	**157,258**	**17**	**63**	**4,166**	**4**	**2,377**	**6**	**461**	**4**
	ef5er6	min 4	11,858,704	83,435	4	40	138,443	5	50	161,649	17	63	4,309	4	2,489	6	481	4
		min 8	11,856,512	83,435	4	40	138,430	5	50	161,478	17	63	4,,309	4	2,489	6	481	4
25	ef4er5	min 4	11,567,277	81,436	4	40	134,794	5	50	157,696	17	63	4,177	4	2,385	6	461	4
		min 8	11,565,227	81,436	4	40	134,789	5	50	157,519	17	63	4,177	4	2,385	6	461	4
	ef5er6	min 4	11,872,797	83,535	4	40	138,652	5	50	161,837	17	63	4,315	4	2,493	6	481	4
		min 8	11,871,192	83,535	4	40	138,647	5	50	161,658	17	63	4,315	4	2,493	6	481	4
28	ef4er5	min 4	11,705,950	83,402	4	40	138,804	5	50	161,745	17	63	4,289	4	2,469	6	481	4
		min 8	11,703,758	83,402	4	40	138,771	5	50	161,549	17	63	4,289	4	2,469	6	481	4
	ef5er6	min 4	11,923,205	84,807	4	40	141,414	5	50	164,654	17	63	4,402	4	2,542	6	494	4
		min 8	11,920,944	84,806	4	40	141,384	5	50	164,461	17	63	4,398	4	2,542	6	494	4

Table C.3. List of families that were detected but not considered for the analyses: microalgae, protists, fungi, fish, mammals, terrestrial invertebrates and insects without an aquatic life stage.

Taxonomic group	Phylum	Class	Family
Microalgae	Chlorophyta	Chlorophyceae	Scenedesmaceae
	Haptophyta	Prymnesiophyceae	Prymnesiaceae
	Miozoa	Dynophyceae	Amphidomataceae
	Miozoa	Dynophyceae	Pfiesteriaceae
	Miozoa	Dynophyceae	Suessiaceae
	Miozoa	Dynophyceae	Thoracosphaeraceae
	Ochrophyta	Chrysophyceae	Ochromonadaceae
	Ochrophyta	Synurophyceae	Paraphysomonadaceae
Protists	Amoebozoa	Discosea	Cochliopodiidae
	Amoebozoa	Discosea	Vannellidae
	Amoebozoa	Discosea	Vexilliferidae
	Amoebozoa	Tubulinea	Hartmannellidae
	Ciliophora	Oligohymenophorea	Tetrahymenidae
Fungi	Ascomycota	Ascomycetes	Helotiaceae
	Ascomycota	Dothideomycetes	Astrosphaeriellaceae
	Ascomycota	Eurotiomycetes	Aspergillaceae
	Ascomycota	Eurotiomycetes	Herpotrichiellaceae
	Ascomycota	Lecanoromycetes	Parmeliaceae
	Ascomycota	Sordariomycetes	Bionectriaceae
	Ascomycota	Sordariomycetes	Glomerellaceae
	Ascomycota	Sordariomycetes	Nectriaceae
	Basidiomycota	Agaricomycetes	Bolbitiaceae
	Basidiomycota	Agaricomycetes	Entolomataceae
	Basidiomycota	Agaricomycetes	Hericiaceae
	Basidiomycota	Agaricomycetes	Pluteaceae
	Basidiomycota	Agaricomycetes	Russulaceae
	Basidiomycota	Agaricomycetes	Schizophyllaceae
	Basidiomycota	Basidiomycetes	Cortinariaceae
	Basidiomycota	Pucciniomycetes	Melampsoraceae
	Heterokonta	Oomycetes	Peronosporaceae
	Heterokonta	Oomycetes	Pythiaceae
	Heterokonta	Oomycetes	Saprolegniaceae

Taxonomic group	Phylum	Class	Family
Invertebrates	Annelida	Polychaeta	Nereididae
	Annelida	Polychaeta	Sabellidae
	Arthropoda	Arachnida	Acaridae
	Arthropoda	Arachnida	Dermationidae
	Arthropoda	Arachnida	Philodromidae
	Arthropoda	Crustacea	Varunidae
	Arthropoda	Entognatha	Fujientomidae
	Arthropoda	Insecta	Mycetophilidae
	Arthropoda	Insecta	Phlaeothripidae
	Arthropoda	Insecta	Phoridae
	Arthropoda	Insecta	Simuliidae
	Arthropoda	Insecta	Tipulidae
	Arthropoda	Malacostraca	Caprellidae
	Arthropoda	Malacostraca	Lithodidae
	Arthropoda	Malacostraca	Panopeidae
	Arthropoda	Malacostraca	Varunidae
	Cnidaria	Hydrozoa	Campanulariidae
	Cnidaria	Hydrozoa	Corynidae
	Cnidaria	Hydrozoa	Mitrocomidae
	Cnidaria	Hydrozoa	Tiaropsidae
	Echinodermata	Asteroidea	Asteriidae
	Mollusca	Bivalvia	Corbiculidae
	Mollusca	Bivalvia	Hiatellidae
	Mollusca	Bivalvia	Limacinidae
	Mollusca	Bivalvia	Mytilidae
	Mollusca	Gastropoda	Ostreidae
	Platyhelminthes	Catenulida	Stenostomidae
Fish	Chordata	Actinopterygii	Cluepidae
Mammals	Chordata	Mammalia	Homo sapiens
	Chordata	Mammalia	Muridae

Table C.4. List of sequences and number of libraries (for each mesocosm) that were not considered due to suspected contamination from families of mock community libraries (same sequence ID). Mesocosm ponds are coded as: MC (mesotrophic control), Mc (mesotrophic control with final severe pulse), Mg (mesotrophic moderate-glyphosate), MG (mesotrophic high-glyphosate), EC (eutrophic control), Ec (eutrophic control with final severe pulse), Eg (eutrophic moderate-glyphosate), EG (eutrophic high-glyphosate).

Subphylum	Family	Family	Sequence ID	MC	Mc	Mg	MG	EC	Ec	Eg	EG
Rotifera	Monogononta	Brachionidae (*Keratella cochlearis*)	3	2	10	1	3	11	5	8	5
			5	1	8	2	2	11	6	9	4
			16	1	7	1	2	11	6	7	1
Crustacea	Cladocera	Daphniidae	61	0	0	0	0	0	2	0	0
			65	0	0	0	4	1	0	0	0
			84	0	0	3	0	0	0	0	0
		Leptodoridae	57	0	0	1	0	0	2	0	0

Table C.5. Model selection table for the presence/absent of the taxonomic families Chydorydae and Cyclopidae. Abundance estimates were represented by the number of individuals. Models were sorted by increasing values of second-order Akaike information criterion (AICc). Models in bold represent the best-fit models.

Model ID	Fixed terms	Random terms	ΔAICc	df
Chydorydae				
Null model	**na**	**na**	**0**	**1**
Linear model 1	mesocosm	na	8.5	8
Linear model 2	abundance	na	3.1	1
M1	abundance	mesocosm ID	5.3	2
M2	abundance + [glyphosate]	mesocosm ID	6.2	4
M3	[glyphosate]	mesocosm ID	3.9	3
M4	[glyphosate] + time	mesocosm ID	6.2	4
M5	time	mesocosm ID	4.4	3
M6	abundance + [glyphosate] + time	mesocosm ID	8.7	5
M7	[chlorophyll *a*]	mesocosm ID	3.3	3
M8	nutrient level	mesocosm ID	4.1	3
Cyclopidae				
Null model	na	na		
Linear model 1	mesocosm	na	5.7	8
Linear model 2	abundance	na	19.2	1
M1	abundance	mesocosm ID	10.9	2
M2	abundance + [glyphosate]	mesocosm ID	9.1	4
M3	[glyphosate]	mesocosm ID	7.1	3
M4	**[glyphosate] + time**	**mesocosm ID**	**0**	**3**
M5	time	mesocosm ID	4.6	2
M6	abundance + [glyphosate] + time	mesocosm ID	4	5
M7	[chlorophyll *a*]	mesocosm ID	9.9	3
M8	nutrient level	mesocosm ID	9.7	3

Table C.6. Comparison between metabarcoding data and morphological assessments on family composition of zooplankton across experimental mesocosms. Percentages represent the average number of families that were detected with morphological assessment that were also detected by metabarcoding in each mesocosm. Standard deviations are also shown along with the average percentage of the total families detected by metabarcoding across all ponds. The number of species refers to the species that were detected in each pond through morphological assessments and its total refers to the species that were detected in the whole experiment. Estimates of undetermined nauplii obtained with the morphological approach were not considered.

Mesocosm	%	SD	No. of species
Mesotrophic control (MC)	35.6	20.9	17
Mesotrophic control lethal pulse (Mc)	46.3	41.8	16
Mesotrophic moderate-glyphosate (Mg)	27.7	23.0	17
Mesotrophic high-glyphosate (MG)	8.3	20.4	9
Eutrophic control (EC)	63.4	12.8	16
Eutrophic control lethal pulse (Ec)	65.1	36.7	17
Eutrophic moderate-glyphosate (Eg)	45.2	28.5	15
Eutrophic high-glyphosate (EG)	15.0	23.4	10
TOTAL	38.3	32.0	23

Table C.7. List of models tested with AICc model selection with their respective AICc values. In bold the AICc values of the AIC-best models. The factor time is categorical. Morpho = morphological assessments; meta = metabarcoding.

Models	**No. of taxonomic families**		***Effective numbers of species***		**Rotifers**		**Crustaceans**		**Insects**	***Keratella cochlearis***	***Polyarthra sp.***
	AIC										
	morpho	meta	morpho	meta	morpho (abundance)	meta (no. of sequences)	morpho (abundance)	meta (no. of sequences)	meta	meta	meta
[glyphosate]	**124**	**106**	**165**	**193**	**141**	**124**	141	**120**	120	**138**	**138**
[chlorophyll *a*]	134	126	**164**	195	**141**	**124**	**132**	130	122	**138**	140
[glyphosate]*time	135	118	172	198	151	133	146	132	121	143	144
[chlorophyll *a*]*time	165	128	165	185	152	136	**131**	136	**115**	147	149

Table C.8. AICc-best model coefficients for total number of taxonomic families, the *effective numbers of species*, number of sequences and abundance (number of individuals) within rotifers and crustaceans estimated with morphological assessments and metabarcoding for day 0, 6, 14, 29, 34, and 42 (phase I). Diversity within insects was only assessed for estimates obtained with metabarcoding. Significance codes: 0 '***', 0.001 '**', 0.01 '*', 0.05 '.'.

Response variable	**Morphological assessments**			**Metabarcoding**		
No. of families	**Model**			**Model**		
	no. of families ~ [glyphosate] + (1|mesocosm)			no. of families ~ [glyphosate] + (1|mesocosm)		
	Fixed terms	**Estimate**	**SE**	**Fixed terms**	**Estimate**	**SE**
	[glyphosate]***	-0.55	0.12	[glyphosate]***	-0.68	0.12
	Random term	**Variance**	**SD**	**Random term**	**Variance**	**SD**
	mesocosm ID	0	0	mesocosm ID	0.09	0.3
	Residual	0.69	0.83	Residual	0.41	0.64
***Effective numbers of species* (Hill numbers)**	**Model**			**Model**		
	Hill numbers ~ [glyphosate] + (1|mesocosm)			Hill numbers ~ [glyphosate] + (1|mesocosm)		
	Fixed terms	**Estimate**	**SE**	**Fixed terms**	**Estimate**	**SE**
	[glyphosate]	-0.25	0.21	[glyphosate]	-0.48	0.29
	Random term	**Variance**	**SD**	**Random term**	**Variance**	**SD**
	mesocosm ID	6.31	2.51	mesocosm ID	9.85	3.14
	Residual	0.85	0.92	Residual	1.58	1.26
Rotifers	**Model**			**Model**		
	rotifers abundance ~ [glyphosate] + (1|mesocosm)			no. of sequences ~ [glyphosate] + (1|mesocosm)		
	Fixed terms	**Estimate**	**SE**	**Fixed terms**	**Estimate**	**SE**
	[glyphosate]	-0.16	0.14	[glyphosate].	-0.26	0.15
	Random term	**Variance**	**SD**	**Random term**	**Variance**	**SD**
	mesocosm ID	0	0	mesocosm ID	0.36	0.6
	Residual	0.98	0.99	Residual	0.53	0.72

Response variable	Morphological assessments (day 0-6-14-29-34-42)			Metabarcoding (day 0-6-14-29-34-42)		
Crustaceans	**Model** crustaceans abundance ~ [chlorophyll *a*] + (1|mesocosm)			**Model** no. of sequences ~ [glyphosate] + (1|mesocosm)		
	Fixed terms	**Estimate**	**SE**	**Fixed terms**	**Estimate**	**SE**
	[chlorophyll *a*]**	0.44	0.13	[glyphosate]***	-0.5	0.13
	Random term	**Variance**	**SD**	**Random term**	**Variance**	**SD**
	mesocosm ID	0.03	0.18	mesocosm ID	0.14	0.37
	Residual	0.79	0.89	Residual	0.53	0.73
Insects				**Model** no. of sequences ~ [chlorophyll *a*]*time+ (1|mesocosm)		
				Fixed terms	**Estimate**	**SE**
				[chlorophyll *a*]* day 0	-0.18	0.45
				[chlorophyll *a*]* day 6*	0.91	0.44
				[chlorophyll *a*]* day 14	0.83	0.54
				[chlorophyll a]* day 29*	0.84	0.41
				[chlorophyll *a*]* day 34	0.14	0.17
				[chlorophyll *a*]* day 42***	1.08	0.25
				Random term	**Variance**	**SD**
				mesocosm ID	0.14	0.17
				Residual	0.59	0.77

Table C.9. Distribution of zooplankton haplotypes (rotifers and insects) across mesocosms. Numbers in brackets represent the number of haplotypes for the subphylum, family and species. Mesocosm ponds are coded as: MC (mesotrophic control), Mc (mesotrophic control with final severe pulse), Mg (mesotrophic "low" glyphosate), MG (mesotrophic "high" glyphosate), EC (eutrophic control), Ec (eutrophic control with final severe pulse), Eg (eutrophic "low" glyphosate), EG (eutrophic "high" glyphosate).

Subphylum	Family	Species	Sequence ID	MC	Mc	Mg	MG	EC	Ec	Eg	EG
Rotifera (53)	Asplanchnidae (7)	***Asplanchna sieboldi*** (7)	139		x						
			189	x	x			x	x		
			240	x		x		x	x	x	
			272	x		x		x	x		x
			336	x	x	x			x	x	
			502	x	x			x	x		
			829	x				x			
			Total	6	4	3	0	5	5	2	1
			%	86	57	43	0	71	71	29	14
	Brachionidae (22)	***Keratella cochlearis*** (22)	369		x			x	x	x	
			385								x
			404		x			x	x	x	
			538		x			x	x		
			556		x			x	x	x	
			751					x			
			755					x			
			786	x							
			791						x		
			838		x			x	x		
			862						x		
			880	x							
			938							x	
			980		x				x		
			1411					x			
			1477		x						
			1701						x		
			1800						x		
			1871		x						
			2288								x
			2327				x				
			2394							x	
			Total	2	8	0	1	8	10	5	2
			%	9	28	0	3	28	34	17	7

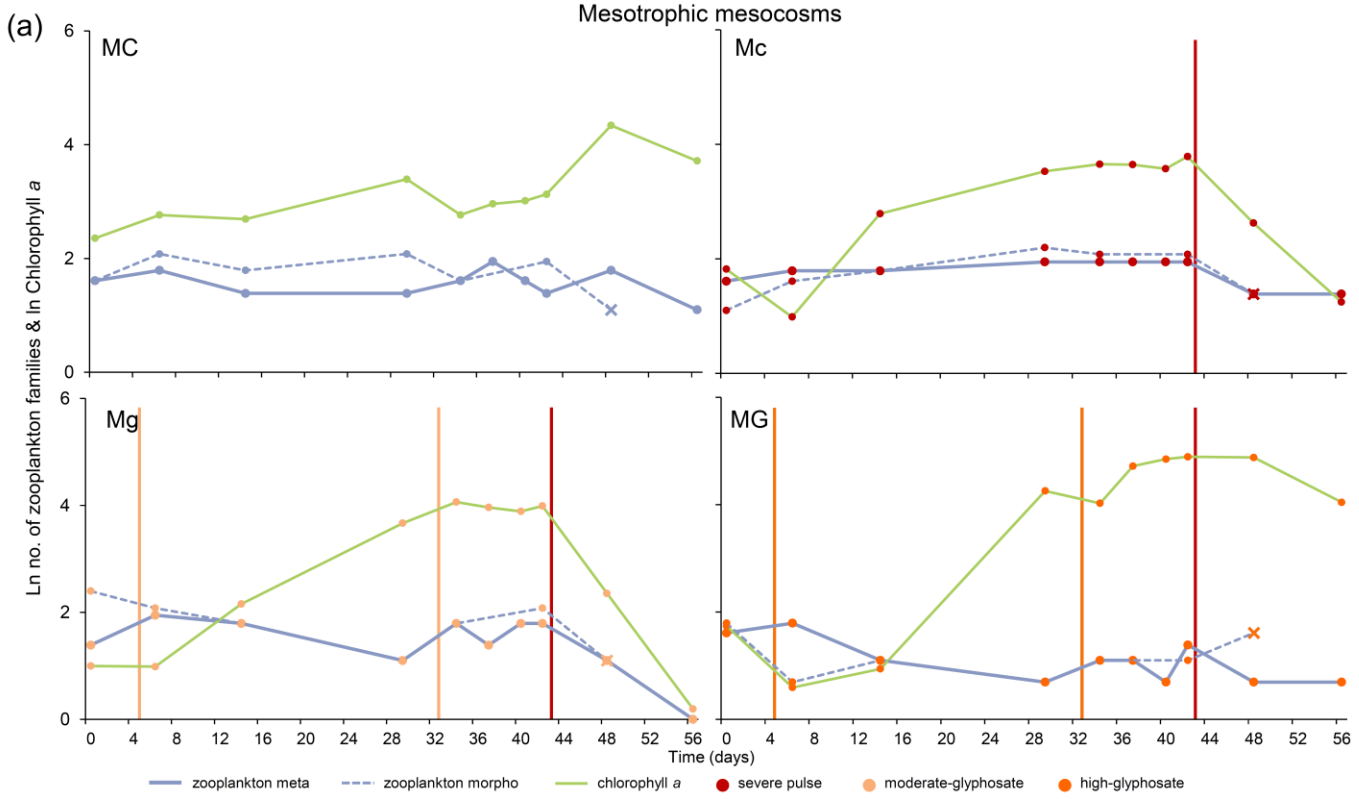
Subphylum	Family	Species	Sequence ID	MC	Mc	Mg	MG	EC	Ec	Eg	EG
Rotifera (53)	Synchaetidae (24)	***Polyarthra sp.*** (24)	34	x	x	x	x	x	x	x	
			257	x				x	x		
			389	x	x		x		x		
			426	x	x	x		x	x		x
			428	x	x						
			430	x	x				x		
			526		x				x		
			533	x	x			x			
			544		x						
			568		x	x				x	
			614					x			
			623	x				x	x	x	
			738	x	x				x		
			809					x			
			889	x							
			894						x		
			900	x							
			949	x							
			969	x							
			995	x							
			1206	x							
			2093		x						
			2283		x						
			2368		x						
			Total	15	13	3	2	7	9	3	1
			%	63	54	13	8	29	38	13	4
			Total Rotifera	23	25	6	3	20	24	10	4
			% Rotifera	43	47	11	6	38	45	19	8
Insecta (16)	Baetidae (16)	***Callibaetis fluctuans*** (10)	131	x	x			x	x	x	
			217			x					
			319	x							x
			522			x					
			794	x							
			965					x			
			1082			x					
			1121					x			
			1497					x			
			1655	x							
			Total	4	1	3	0	4	1	1	1
			%	40	10	30	0	40	10	10	10

Subphylum	Family	Species	Sequence ID	MC	Mc	Mg	MG	EC	Ec	Eg	EG
Insecta (16)	Baetidae (16)	***Cloeon dipterum*** (6)	103				x				
			414	x	x	x	x	x			x
			517	x	x		x	x		x	
			797								x
			982				x				
			1509	x							
			Total	3	2	1	4	2	0	1	2
			%	50	33	17	67	33	0	17	33
			Total Insecta	7	3	4	4	6	1	2	3
			% Insecta	44	19	25	25	23	6	13	19
			Total	30	28	10	7	26	25	12	7

Table C.10. Best-fit model coefficients for haplotype diversity in the rotifer species *Keratella cochlearis* and the genus *Polyarthra* sp. for day 0, 6, 14, 29, 34 and 42 (phase I). Glyphosate concentration is expressed in ppb. Significance codes: 0 '***', 0.001 '**', 0.01 '*', 0.05 '.'.

Species	**Metabarcoding** (day 0-6-14-29-34-42)		
Keratella cochlearis	**Model**		
	no. of sequences ~ [glyphosate]		
	Fixed effects	**Estimate**	**SE**
	[glyphosate]	-0.17	0.16
	Random term	**Variance**	**SD**
	mesocosm ID	0.14	0.38
	Residual	0.81	0.9
Polyarthra sp.	**Model**		
	no. of sequences ~ [glyphosate]		
	Fixed effects	**Estimate**	**SE**
	[glyphosate].	-0.27	0.14
	Random term	**Variance**	**SD**
	mesocosm ID	0	0
	Residual	0.93	0.96

Figure C.1. Dynamics of the estimated number of taxonomic families of zooplankton (ln-transformed) obtained from metabarcoding ("zooplankton meta") and morphological identification ("zooplankton morpho") in **(a)** mesotrophic and **(b)** eutrophic mesocosms. Insect families detected with metabarcoding are not shown. Sampling point at day 44 is not shown given that samples were collected only a few hours after glyphosate application. In phase II, zooplankton was collected only on day 48 for morphological identification and abundance estimates (cross symbol). Chlorophyll *a* concentration (ln) trends are also shown. Mesocosm ponds are coded as: MC (mesotrophic control), Mc (mesotrophic control with final severe pulse), Mg (mesotrophic moderate-glyphosate), MG (mesotrophic high-glyphosate), EC (eutrophic control), Ec (eutrophic control with final severe pulse), Eg (eutrophic low-glyphosate), EG (eutrophic high-glyphosate).



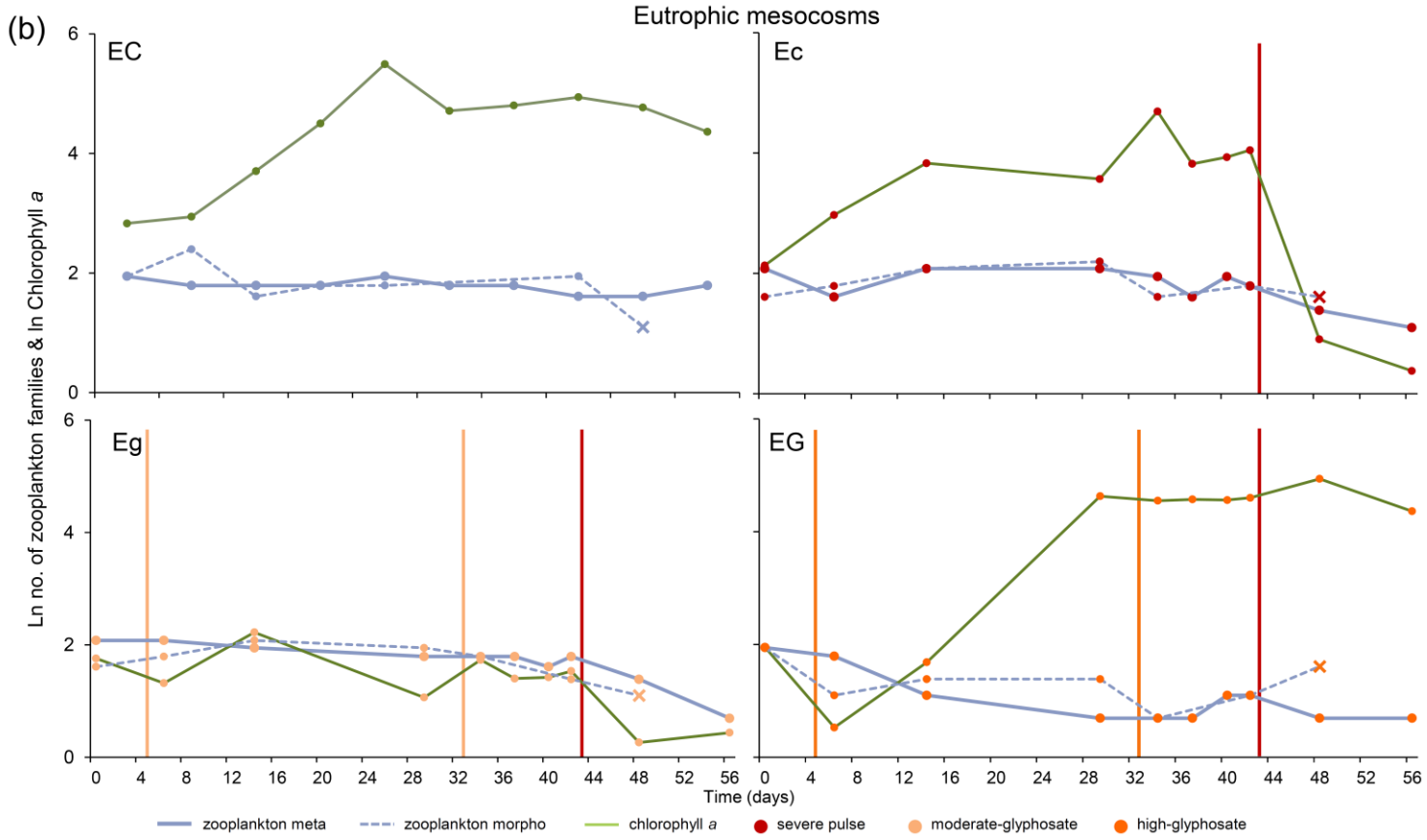
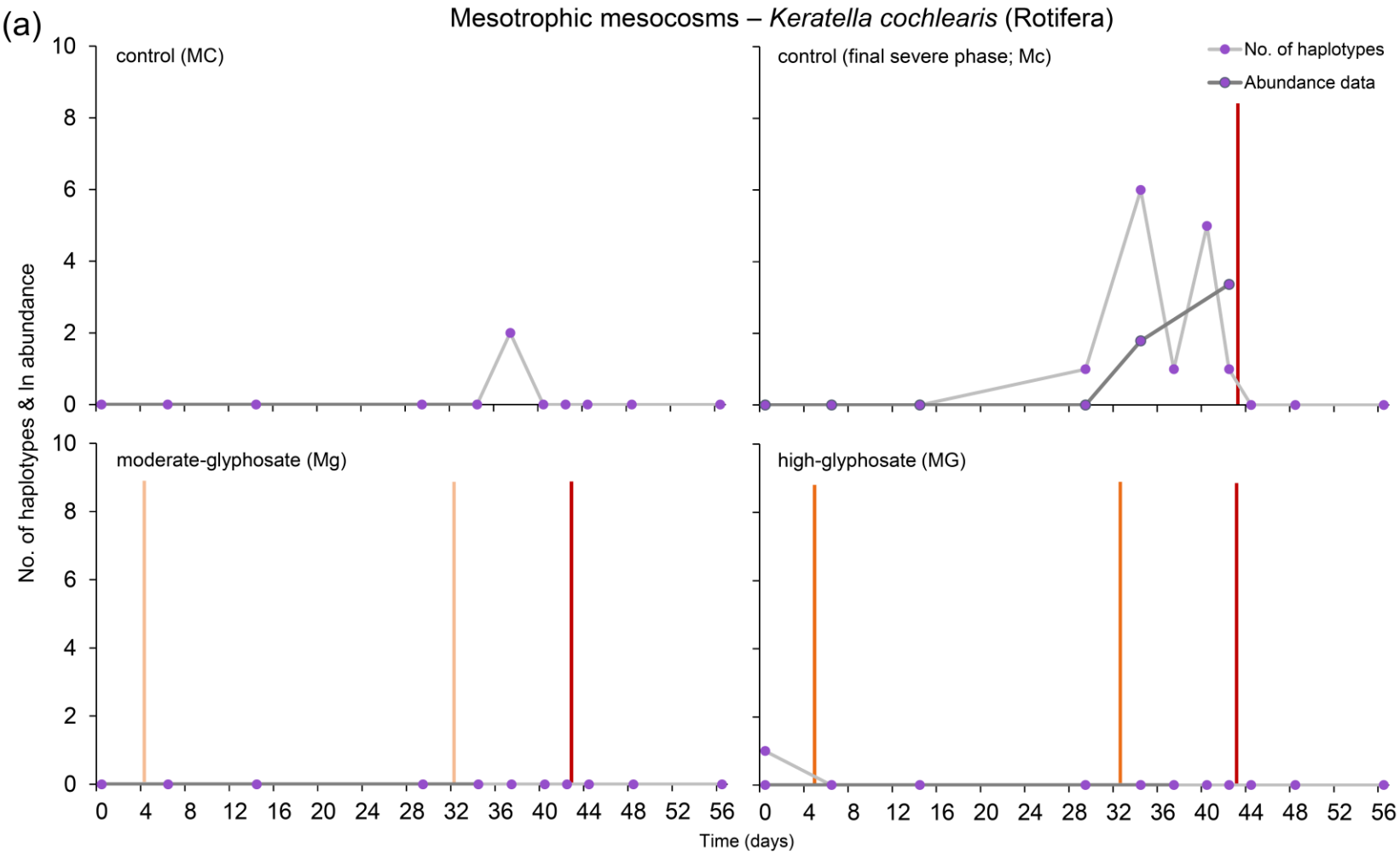
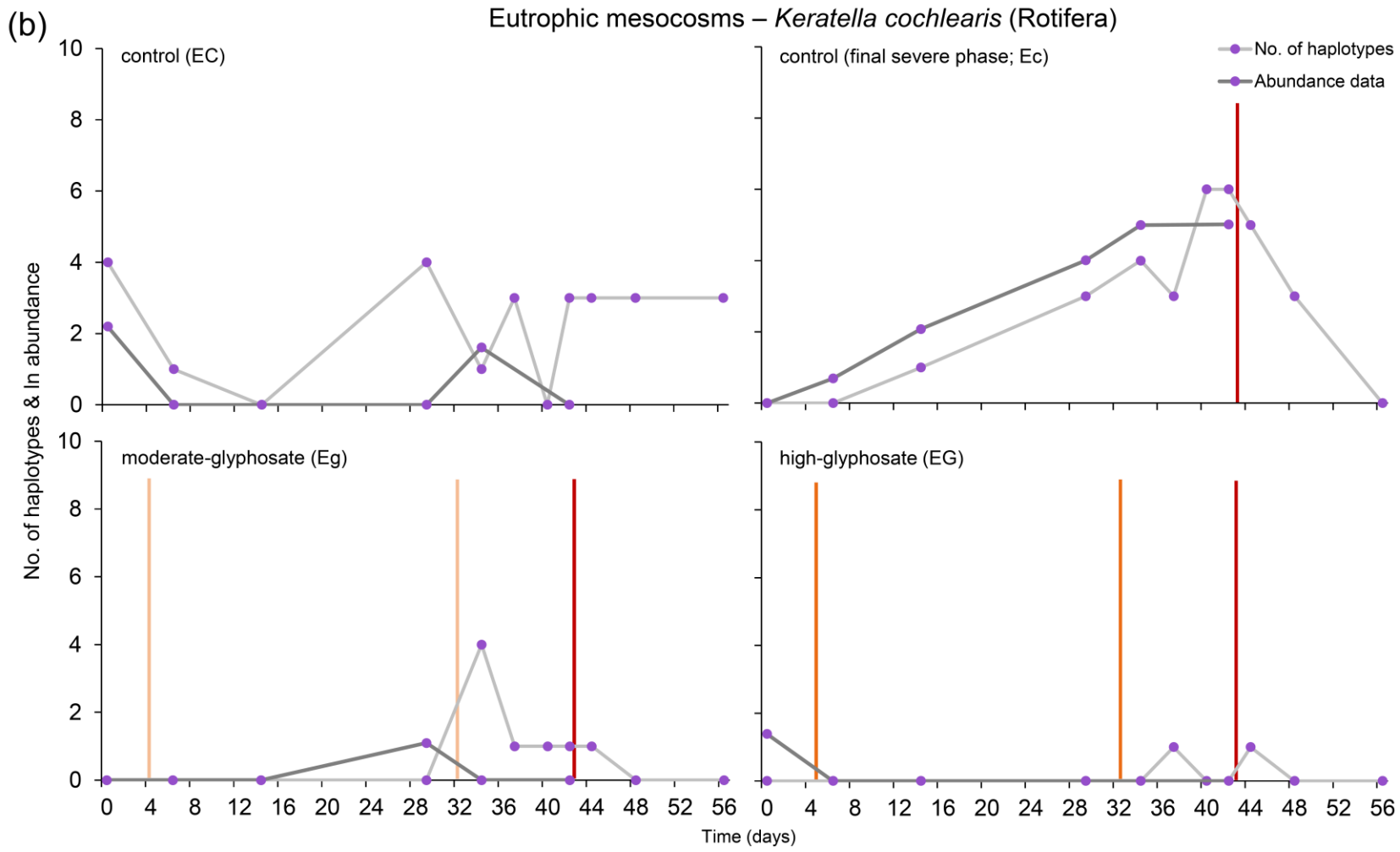
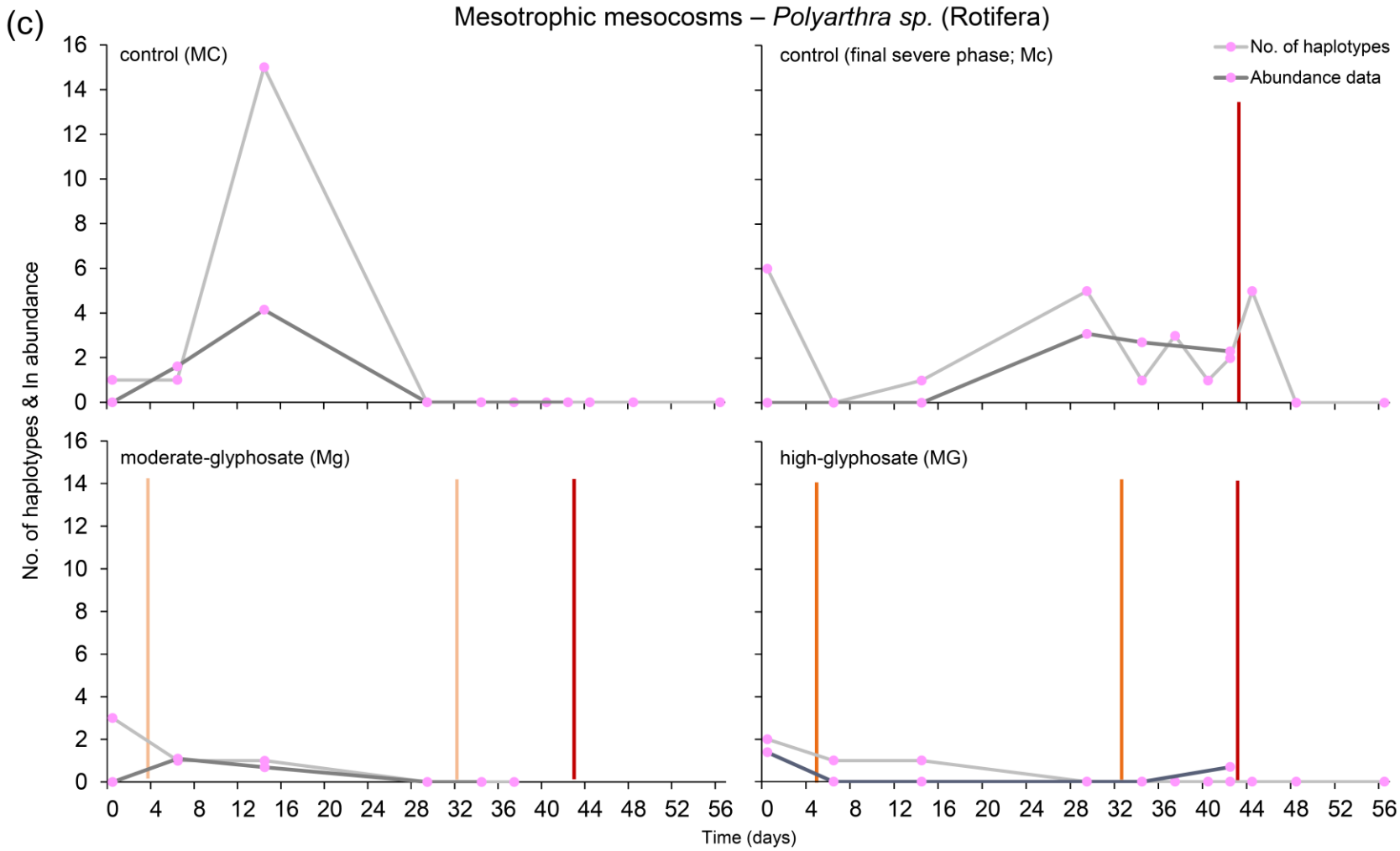
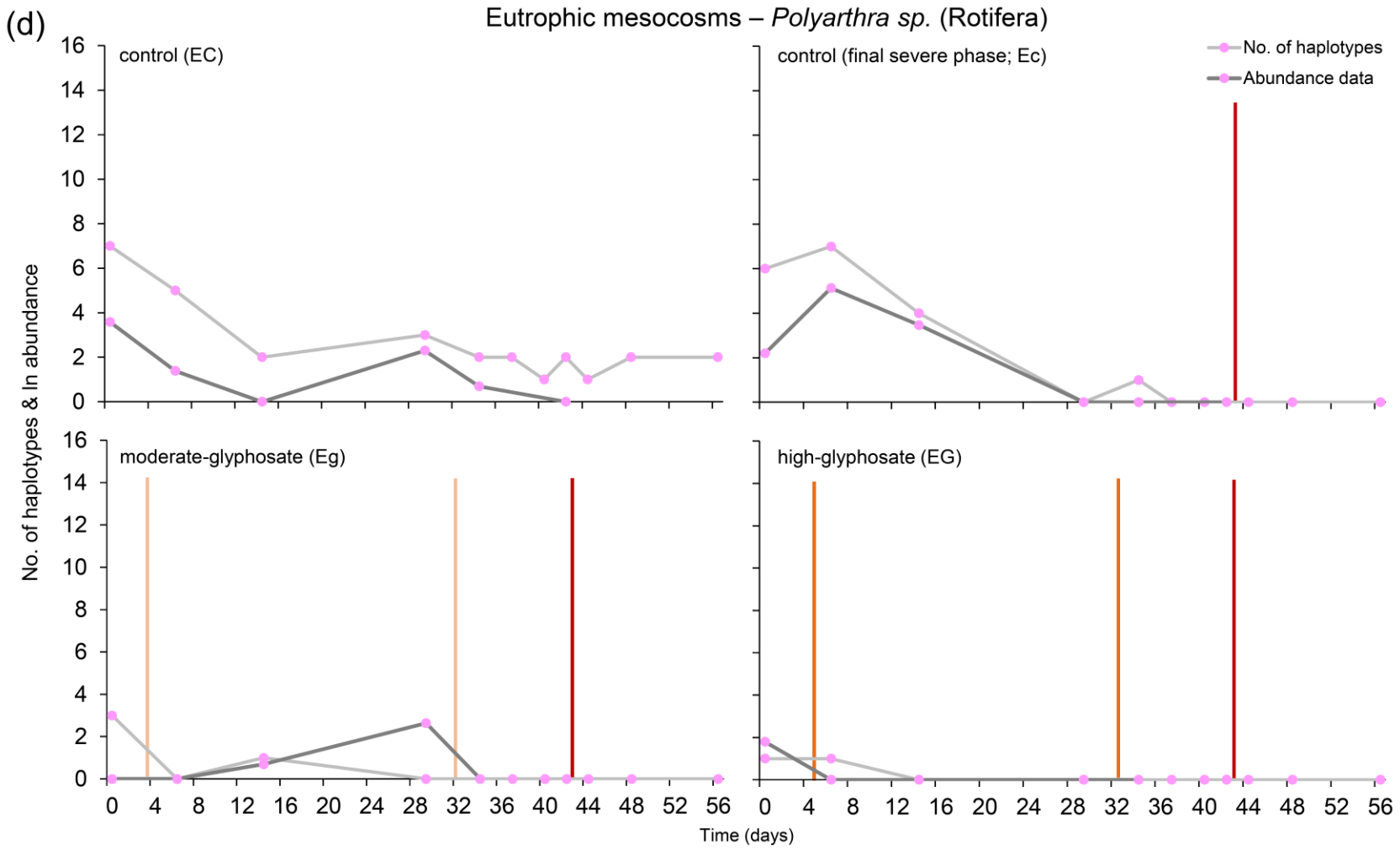

(b)
Eutrophic mesocosms
EC
Ec
Eg
EG
Ln no. of zooplankton families & ln Chlorophyll a
Time (days)
zooplankton meta
zooplankton morpho
chlorophyll a
severe pulse
moderate-glyphosate
high-glyphosate

Figure C.2. Comparison between dynamics of species abundance and number of haplotypes during phase I of the experiment (only phase I pulses) for rotifer species **(a, b)** *Keratella cochlearis*, and **(c, d)** *Polyarthra* sp.




(b)
Eutrophic mesocosms – *Keratella cochlearis* (Rotifera)
control (EC)
control (final severe phase; Ec)
moderate-glyphosate (Eg)
high-glyphosate (EG)
No. of haplotypes
Abundance data
No. of haplotypes & ln abundance
Time (days)
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56
0 2 4 6 8 10


(c)
Mesotrophic mesocosms – *Polyarthra sp.* (Rotifera)
control (MC)
control (final severe phase; Mc)
moderate-glyphosate (Mg)
high-glyphosate (MG)
No. of haplotypes
Abundance data
No. of haplotypes & ln abundance
Time (days)


(d)
Eutrophic mesocosms – *Polyarthra sp.* (Rotifera)
control (EC)
control (final severe phase; Ec)
moderate-glyphosate (Eg)
high-glyphosate (EG)
No. of haplotypes
Abundance data
No. of haplotypes & ln abundance
Time (days)
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56
0 2 4 6 8 10 12 14 16

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