MASTER THESIS

Intraspecific variability in aquatic plant response to chemical contaminants

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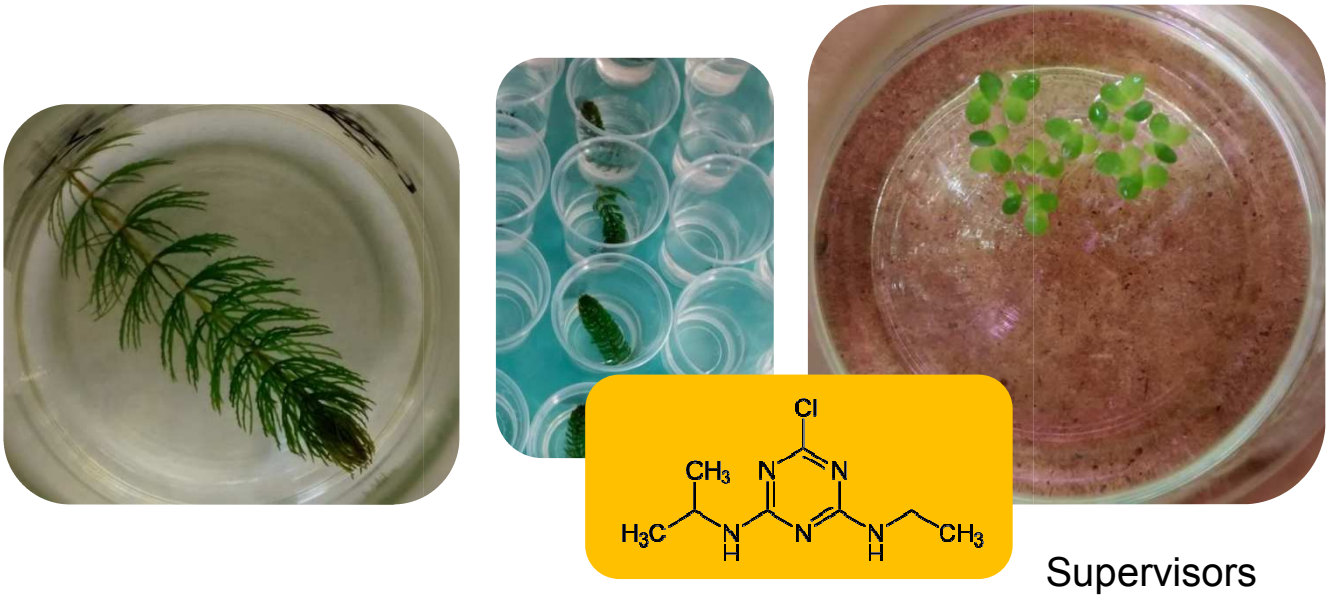

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List of abbreviations

ATR – Atrazine

CU – Copper

D1 – Day one of the PP experiment

D4 – Day four of the PP experiment

D7 – Day seven of the PP experiment

D10 – Day ten of the PP experiment

D14 – Day 14 of the PP experiment

DR_ATR – Dose Response experiment testing the effect of atrazine on *Lemna minor*

DR_CU – Dose Response experiment testing the effect of copper on *Ceratophyllum demersum*

EC_{50} – effective concentration, 50%

EDTA – Ethylene Diamine Tetraacetic Acid

Ecotoxicological endpoint – Specific measurement of a contaminant's effect on organisms *(here: Growth, Photosynthesis Rate, Leaf Area)*

LA – Leaf Area (mm^2)

NOAEL – No Observed Adverse Effect Level

PAR – Photosynthetical Active Radiation

PP – Phenotypic Plasticity experiment

QY – Maximum Quantum Yield of photosystem II (Fv/Fm)

RGR – Relative Growth Rate

SD –Standard Deviation

SL – Stem Length

VG_C – Genetic variability experiment using *Ceratophyllum demersum*

VG_L – Genetic variability experiment using *Lemna minor*

Abstract

Intra-specific variability in the response of *Lemna minor* and *Ceratophyllum demersum,* two aquatic macrophyte species towards chemical contamination was tested in a laboratory environment. In a first **Dose-Response experiment** *L. minor* individuals were exposed to atrazine (0, 3.75, 7.5, 15, 30, 60, 100, 120, 240, 480, 960µgL^{-1}) and copper (0, 0.025, 0.05, 0.1, 0.25, 0.5, 0.75, 1, 2, 5, 10, 15mgL^{-1}) concentrations in order to evaluate the effect of the contaminants under stable environmental conditions and to determine EC_{50} values which were intended to be used for subsequent tests. For atrazine 122.1µg L^{-1} and for copper 0.69mg L^{-1} had been determined as EC_{50} values. In a second **Genetic Variability experiment** *L. minor* and *C. demersum* individuals from geographically distinct sampling sites within the three subcatchments Dordogne, Garonne and Tarn in the south-west of France in order to determine possible differences in the response to the contaminants atrazine (100µg L^{-1}) and copper (1.5mg L^{-1}) due to genetic variabilities. Only *C. demersum* individuals of the Tarn region responded differently, indicating such pattern. Lastly a **Phenotypic plasticity experiment** testing the environmental influence on *L. minor* individuals was conducted. Therefore plants of the Garonne catchment, which were kept under axenic conditions, were exposed to eight different environmental groups in a PRE-CONTAMINATION phase lasting 14 days. They were then in a second step, the CONTAMINTATION-EXPOSURE phase transferred under a fume hood to flasks with the same, and to flasks with different environmental conditions. Additionally a control and an increased exposure group (1.5mg L^{-1} Cu) were established hereby. The result showed that a change in nutrients conditions influences the contamination response. It is therefore concluded that phenotypic plasticity plays an important role in acclimatization to newly occurring environmental conditions. Especially in consideration of climate change and pollution scenarios that will occur in the near future, plastic organisms may benefit from the advantage of being able to live up to changing environments more easily. However further research is required to quantify and elucidate these mechanisms and to rework experimental designs.

Zusammenfassung

Im Zuge dieser Studie wurden Labortests zur Untersuchung des Einflusses von intra-spezifischer Variabilität der beiden Makrophyten *Lemna minor* L. und *Ceratophyllum demersum* L. in Bezug auf Kontaminierung mit den Schadstoffen Atrazin und Kupfer durchgeführt. In einem ersten **Dosis-Wirkungs-Experiment** wurden *L. minor* Individuen verschieden hohe Atrazin (0, 3.75, 7.5, 15, 30, 60, 100, 120, 240, 480, 960µgL^{-1}) und Kupfer (0, 0.025, 0.05, 0.1, 0.25, 0.5, 0.75, 1, 2, 5, 10, 15mgL^{-1}) Konzentrationen ausgesetzt um den negativen Einfluss der Schadstoffe unter stabilen Umweltbedingungen zu bestimmen, und um EC_{50} Werte für die darauffolgenden Tests zur erhalten. Für Atrazin wurden 122,1µg L^{-1} und für Kupfer 0.69mg L^{-1} als EC_{50}-Werte bestimmt. In einem zweiten **Experiment zur Genetischen Variation** wurden *L. minor* und *C. demersum* Individuen von geographisch voneinander getrennten Sub-Einzugsgebieten (Dordogne, Garonne, Tarn) im Süd-Westen von Frankreich auf deren Reaktion auf Atrazin und Kupfer getestet um mögliche Unterschiede auf Grund von genetischer Variation zu finden. Unterschiedliche Genotypen wurden aufgrund von räumlicher Distanz angenommen. Nur *C. demersum* Individuen aus dem Sub-Einzugsgebiet Tarn zeigten unterschiedliche Reaktionen gegenüber den Schadstoffen. Zuletzt wurde ein **Experiment zur Bestimmung phänotypischer Unterschiede** von *L. minor* in Bezug auf Schadstoffe und wechselnden Umweltfaktoren durchgeführt. Individuen aus dem Einzugsgebiet der Garonne, welche unter sterilen Bedingungen kultiviert wurden, wurden acht unterschiedlichen Umweltgruppen in einer 14 Tage andauernden PRE-CONTAMINATION Phase ausgesetzt. In einem zweiten Schritt, der CONTAMINATION-EXPOSURE Phase wurden diese Individuen unter dem Abzug in Gefäße mit gleichen bzw. geänderten Umweltfaktoren übertragen. Das Resultat zeigte, dass unterschiedliche Nährstoff-Konzentrationen unterschiedliche Reaktionen auf Schadstoffe auslösen. Daraus wird gefolgert, dass phänotypische Unterschiede eine wichtige Rolle bei der Anpassung an neu auftretende Umweltbedingungen spielen. In Bezug auf Klimawandel und steigenden Schadstoffbelastungen ist eine erhöhte ökologische Toleranz für Organismen von Vorteil.

1. Introduction

Macrophytes play a key role in aquatic ecosystems where they provide food, shelter and substrate to various aquatic organisms and additionally alter physical and chemical properties (Carpenter and Lodge, 1986; Lodge, 1991). Furthermore they contribute to water clarity since they directly compete with algae for nutrients and often release allelopathic substances which inhibit the propagation of the latter (Scheffer et al., 1993). Due to this importance and a world-wide presence in aquatic ecosystems, macrophytes are often used in ecotoxicological test settings besides algae and other organisms (Coutris et al., 2011; Fairchild et al., 1998; Wang, 1990). Pesticides that are used at agricultural sites can drain into waterbodies and negatively influence the growth of macrophytes. Spatial patterns, such as distance from waterbodies to agricultural sites, soil runoff potential and slope are critical factors that determine the potential accumulation of contaminants (Lerch and Blanchard, 2003). Herbicides particularly pose a risk to macrophytes and are therefore of environmental concern and subject to ecotoxicological testing. Different contaminants influence macrophytes in different ways and some taxa are more susceptible than others. Furthermore environmental factors are determining the toxicity of a contaminant (Brain et al., 2012). Besides that, there are two intrinsic factors in plants regulating the response of a given species towards contaminants:

First, **genetic variability** describes the different gene assemblages that occur within and between populations. They differ because of geographical distinction and evolutionary processes that come along with such separation. The environment the plants are growing in has an influence on these processes and therefore also on the gen setting. Studies have shown that populations of *Lemna minor* L. differed significantly in their sensitivity to the herbicide atrazine (Dalton et al., 2013). Macrophyte species with a wide distribution also exhibit distinct genetic patterns that differ between each other as the work by Mader et al. (1998) on *Potamogeton pectinatus* L. has shown. Also genetic variation of populations of *Ceratophyllum demersum* L. influenced by nitrogen availability were similarly observed (Hyldgaard et al., 2012). Such results suggest that the response of macrophytes towards contaminants may differ between geographically distinct

populations. In this study it is assumed that the subcatchments are separated widely enough to speak of different populations. However this assumption was not tested. In their study Dalton et al. (2013) defined distinct populations by subcatchments being hydrologically not connected. Distribution vectors, such as birds, wind and others might question this assumption. Moreover Vasseur et al. (1993) show in a study on *L. minor* which works with eight populations growing within a radius of about 12km in different habitats (ranging from pond to stream systems) that a high degree of differentiation is present.

Second, **phenotypic plasticity** is the ability of a single genotype of a plant to react towards changing environmental conditions in different ways and to exhibit a range of different phenotypes, which means morphological and physiological alterations. This trait occurs in any plant but clonal propagating species manifest it more often (Silander, 1985; Vasseur and Aarssen, 1992). Phenotypic plasticity occurs because of different environmental factors whereas genetic differences arise as a result of evolution (Hyldgaard et al., 2012). Vretare et al. (2001) showed that *Phragmites australis* A. exhibited plasticity in some traits when subjected to different water depths. They produced fewer and taller stems and allocated more resources to stem weight. Phenotypic plasticity is of advantage because it might permit the populations survival in a new environment, increase its persistence but also reduce the likelihood of genetic change (Price et al., 2003). Environmental factors, such as light availability are crucial for aquatic primary producers. Some macrophytes are limited to shallow riparian areas where the resource light might be directly influenced by spatial and temporal changes in riparian canopy cover. As a response to low light conditions, aquatic plants can increase light capture or photosynthetic efficiency due to phenotypical plasticity (Going et al., 2008). A study by Going et al. (2008) on watercress (*Nasturtium officinale* R. Br.) tested the plasticity in such traits and showed that plants live up to lowered light conditions by increasing the leaf area representing considerable morphological plasticity. Ganie et al. (2015) make phenotypic plasticity responsible for the successful spread of the genus *Potamogeton* in the Kashmir Himalaya. They compared individuals of the same species sampled from different lentic and lotic habitats and cultivated them under similar conditions which didn't let them exhibit their beforehand observed morphological

alterations. The genus *Potamogeton* generally shows great morphological variations. In a comparative study of 184 different populations of 41 taxa of *Potamogeton* Kaplan (2002) cultivated clones under uniform and different environmental conditions in order to assess the proportion of phenotypic plasticity. According to the study by Ganie et al. (2015), the clones didn't form different morphological traits when cultivated under identical environmental conditions whereas, in different constraining environments, some clones were able to exhibit almost all morphological traits that had been observed for the species in nature. Finally he states that phenotypic plasticity in *Potamogeton* is much more important than genetic variability, which is contrary to Mader et al. (1998) and he is also challenging taxonomical differentiation of varieties and even species within the taxon *Potamogeton*. These examples highlight the importance of Phenotypic Plasticity to cope with a changing environment. Highly plastic traits enable plants to survive in habitats with diverse environmental influences and additionally promote colonization of new habitats. Vasseur and Aarssen (1992) tested in an important piece of work eight genotypes of *L. minor* and their phenotypic plasticity exhibited by differences in temperature, light intensity and nutrient concentrations. They used Rate of Frond Production, Frond Biomass and Root Length as endpoints and found differences due to genetic variability within the populations as well as differences in phenotypic traits caused by different environmental influences. Bradshaw (1965), Levins (1968) and Silander (1985) were the first scientists to see phenotypic plasticity as an alternative to genetic adaption when there is selection pressure and genetic variation within a population is rather low (Vasseur and Aarssen, 1992). How genetic variability influences phenotypic plasticity and vice versa is still partly an open question although Vasseur and Aarssen (1992) found no relationship between genetic and phenotypic variability what they relate to the fact that phenotypic plasticity is not adaptive (Vasseur and Aarssen, 1992). Phenotypic plasticity does not include genetic variability; it is rather the mechanism which allows organisms to live with changing environmental conditions within their lifetime. Considering climate change (Hughes, 2000) and other human induced alterations of the environment that lie ahead, the ability of quickly living up to changing conditions will be of value to organisms (Bradshaw, 1965; Williams et al., 2008).

Environmental conditions influence considerably a plant's growth and living. Factors like nutrient availability, temperature and sunlight are amongst others crucial parameters that shape the physical basis for the plant. Chemical agents and respectively their effects on biota in the environment are also influenced by these parameters. Environmental factors are either influencing the plants physiological capability to absorb the contaminant or are changing the intrinsic properties of the contaminant itself. For instance, Brain et al. (2012) found in a study on *Elodea canadensis* that the toxicity of atrazine is declining with decreasing photosynthetical active radiation (PAR). Since the experiments conducted in this research took place in the laboratory under stable conditions, environmental factors can be controlled and variabilities in the influence on the plants and contaminants excluded. Nevertheless certain predispositions of the plant material have to be taken into account. Plant material collected from different sites may have been in contact with pesticides or other disturbing factors which might already have led to the development of different plastic traits. Vasseur and Aarsson (1992) and Landolt (1957) state that different phenotypical traits are not adaptive and as mentioned before not anymore exhibited when environmental conditions change. Since several months old laboratory cultures were used, predispositions should be negligible.

Although there are several studies that discuss the effect of phenotypic plasticity and to a greater extent the topic of genetic variability there is still a lack of scientific work that examines the genetic and phenotypical differences of plants in **response to contaminants**, except for one article by Dalton et al. (2013) who address the question of the influence of genetic variability on the effect of atrazine on *L. minor* and one by Mazzeo et al. (1997) who test effects of simazine on *Lemna gibba*.

The main focus of the study lay on the **influence of contaminants on plants**. Additionally the two environmental parameters, **nutrient content** and **light availability** were manipulated to see if such influence of contaminants and respectively the plant's sensitivity varies in response to different environments. The idea was to determine the type of alteration which helps the plant to live up to changed conditions. Is either genetic variability of a population or a plant's inherent phenotypic plasticity responsible for a change of morphological or physiological characteristics? The rate of growth inhibition in response to contaminants was determined according to different environmental

conditions and for each genotype. These percentage results are compared among each other and considered as a mark for plasticity and genetic variability, respectively. Following hypotheses were derived from these assumptions:

Negative influence of copper and atrazine:

1) The growth of the aquatic plants *Lemna minor* and *Ceratophyllum demersum is inhibited* by the addition of copper and atrazine.

Genetic variability and phenotypic plasticity:

2) The response of a plant to a contaminant varies in dependence of his genotype.
3) The response of an individual of a certain genotype is variable and depending on environmental conditions.

In order to answer these questions two macrophyte species were used as models in the present project: the common duckweed (*Lemna minor* L.) and the coontail or hornwort (*Ceratophyllum demersum* L.). *L. minor* is a commonly used plant for ecotoxicological testing because it is a fast growing easily cultivable and widely distributed plant (Wang, 1990; Cowgill et al., 1991; Fairchild et al., 1997). Furthermore it is a recommended OECD test species for ecotoxicological test settings (OECD, 2006). Since *L. minor* is a versatile plant it can also be used in various other fields like sanitation and animal feed production (Iqbal, 1999). *L. minor* propagates mostly fissiparously and very rarely produces flowers for sexual reproduction (Vasseur et al., 1993). *C. demersum* is not an OECD test species but since it is also widely distributed there are several publications that perform toxicological tests with it (Coutris et al., 2011; Hyldgaard et al., 2012). *C. demersum* is also recommended as biosorbent for heavy metals such as zinc, lead and not least copper in order to remove these substances from waterbodies.

Atrazine and **copper** were used as **contaminants** in this study. Atrazine is a photosynthesis inhibitor (Tomlin, 2003) and although banned in the EU in 2004, atrazine was still present in river sediments several years thereafter (Devault et al., 2007). In

addition, a study by Barrek et al. (2009) showed that atrazine was present in all of the surface water samples of the Rhône-Alpes region in France at concentrations up to 1.2μgL^{-1} and another study by Barth et al. (2007) could provide evidence for the presence of atrazine and its metabolite, deethylatrazine (up to 1.05 μg L^{-1}) in a spring of the Brévilles catchment. More recent studies by Dalton et al. (2013) find concentrations up to 0.9μg L^{-1} in the South Nation River catchment in Canada. Atrazine is still used as a crop herbicide in North America. It can also be considered as a model compound for the s-triazine herbicide family, based on the huge amount of literature available for it. Copper is on the one hand an essential plant nutrient which is normally present in plants at concentrations around 10μg g^{-1} dry tissue (Baker and Brooks, 1989; Kanoun-Boulé et al., 2009). On the other hand this metal becomes toxic beyond a certain concentration because of its two oxidation states (in the form of Cu2+) (Kanoun-Boulé et al., 2009). Copper is taken up by the plant and accumulates in the plant tissue where elevated concentrations can lead to oxidative stress (Razinger et al., 2007). It is furthermore used as a fungicide and together with copper-based fertilizers it is widely used in agriculture (de Oliveira-Filho et al., 2004). Also other human activities like mining, combustion of fossil fuels, metal-working industries lead to the emissions of copper (Schützendübel and Polle, 2002).

In a first experiment the inhibition effect of atrazine and copper on *L. minor* and *C. demersum* was tested and in two further experiments the question of genetic variability and phenotypic plasticity were addressed. For these two experiments nutrient and light conditions were increased in order to gain higher growth rates.

2. Material and methods

2.1 Macrophytes

Individuals of *L. minor* and *C. demersum* were sampled in August and September 2014 from the Dordogne, Garonne and Tarn catchment in the south-west of France. They were kept in the laboratory at 20°C and were transferred to fresh medium every 14 days. Only the Toulouse pool population was sterilized in a 1% calcium hypochlorite solution for at least one minute and subsequently rinsed with sterile media to eliminate algae associated with the plants. All sampled populations were collected on the different rivers upstream the confluence sectors, reducing the risk to have identical genotypes.

Table 1–Population names and relating river catchments

	Water bodies and location
L. minor	
Tarn (T)	Tarn river at Sainte-Livrade
Toulouse pool (P)	pond in Paul Sabatier campus, Toulouse
Garonne (G)	Garonne river at Bourret
C. demersum	
Tarn (T)	Tarn river at Sainte-Livrade
Dordogne (D)	Dordogne river at Lalinde
Channel Ramonville (C)	Canal du Midi at Ramonville

2.2 Experiments

Five experiments were conducted during the internship period whereupon two dose-response-tests, one using atrazine (DR_ATR) and the other copper (DR_CU) were established in order to get an idea of the adverse influence of the contaminants on *L. minor* individuals. Furthermore an experiment in order to address the question of genetic variability was conducted using *L. minor* (GV_L) and *C. demersum* (GV_C) individuals of three different populations. Lastly an experiment testing the possibility for phenotypic plasticity (PP) in *L. minor* was set up. The two 14 days lasting dose-response experiments (DR_ATR, DR_CU) were established in the “ECLOSERIE” within the facilities of ECOLAB. The GV_L, GV_C and the PP experiment were conducted in a phytotron, equipped with high pressure sodium lamps (Philips 600 W, Netherlands).

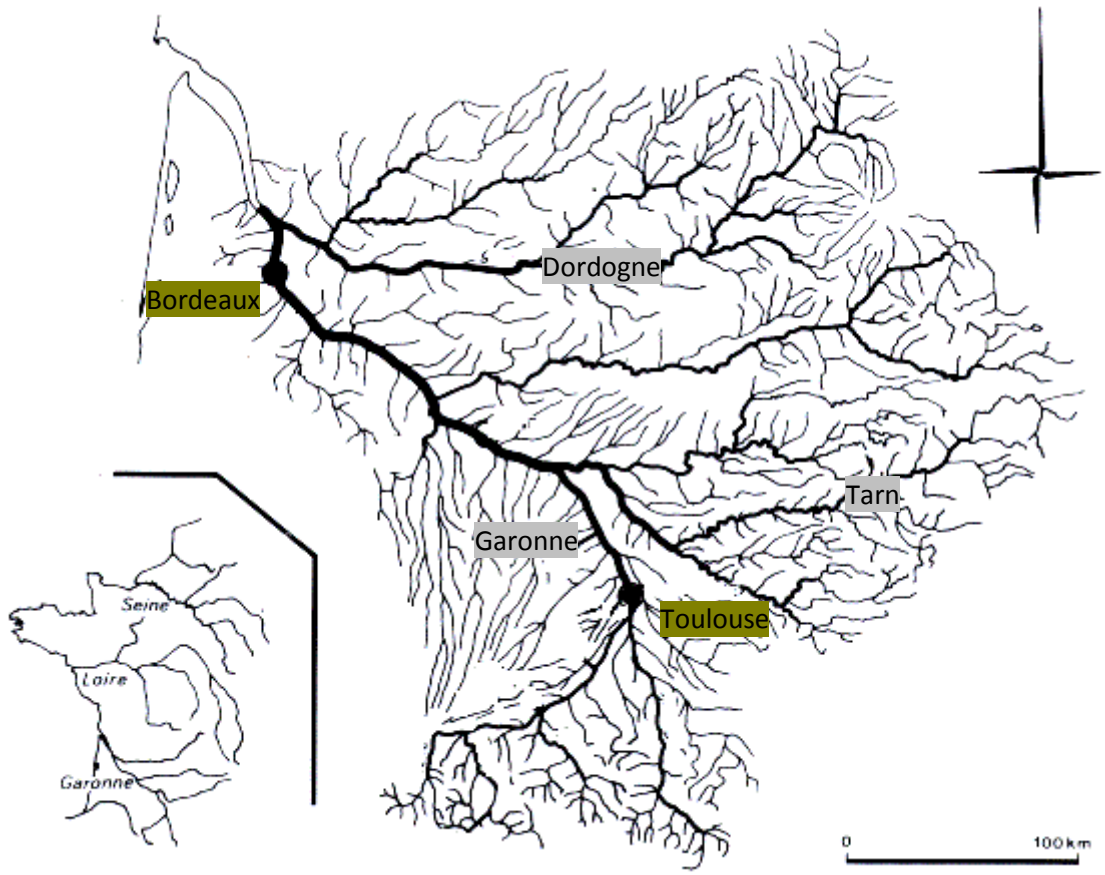

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Figure 1 – Map of the Dordogne, Garonne and Tarn subcatchments and their situation in France

The experiment measuring the influence of atrazine (DR_ATR) lasted from 24 March to 8 April 2015 and the second one using copper (DR_CU) as contaminant started on the 2 April and ended at the 14 April. These experiments were not conducted under axenic conditions and the plants were exposed to atrazine and copper through a single addition on the first day of the experiment. A day-night cycle of 12:12 was set and the temperature regulated to 20°C. Light mean intensity was 42.64 µmol m^{-2} s^{-1} (SD: 4.5, n=16). The experiment was conducted in order to determine the **EC_{50}** value, which presents the concentration of a toxicant which induces a response halfway between the baseline and the maximum effect after a specified exposure time. The experiments discussing the questions of genetic variability (GV_L, GV_C) and phenotypic plasticity (PP) were conducted in the "PHYTOTRON4" within the facilities of ECOLAB. The temperature was set to 20°C and a day-night cycle of 14:10 was established providing mean light intensities around 183 µmolm^{-2} s^{-1} (SD: 36, n=13). They lasted 14 days whereupon the two genetic variability experiments were conducted from 26 June to 9 July 2015 following a one week period (19–25 June) of preculture. The experiment addressing the question of phenotypic plasticity was performed from 7 July to 20 July following a preculturing phase of two weeks (23 June – 6 July).

Table 2 – Five experiments that were conducted during the internship together with number of initial fronds used, location, duration, date of the experiments, their light situation and number of replicates.

Experiment	**plants**	**fronds**	**location**	**dur.**	**date**	**mean light intensity± SD**	**rep.**
				days		*µmol (m^{-2} s^{-1})*	
DR_ATR	L. minor	6	ECLOSERIE	14	24.3 – 8.4.2015	42.6 ± 4.4	5
DR_CU	L. minor	6	ECLOSERIE	14	2.4 – 14.4.2015	39.5± 4.7	5
GV_L	L. minor	6	PHYTOTRON	14	26.6 – 9.7.2015	183± 36	5
GV_C	C. demersum	shoot	PHYTOTRON	14	26.6 – 9.7.2015	183± 36	5
PP	L. minor	6	PHYTOTRON	14	7.7 – 20.7.2015	L+ 183± 36 L- 14.6 ± 3.8)	6

2.3 Growth Medium

The growth medium used in these experiments differs between the first two Dose-response experiments conducted with atrazine and copper (DR_ATR, DR_CU), where the medium *NORMAL* was used and the experiments examining the question of genetic variability (GV) and phenotypic plasticity (PP). For the GV setting only the medium *RICH* was used whereas for the plasticity experiment (PP) the media *RICH* and *POOR* were used. The nutrient medium *RICH* resembles the STEINBERG medium (After ISO 20079) which is recommended by the OECD (2006) for ecotoxicological test settings including the *Lemna* genus. *NORMAL* and *POOR* were nutrient media already used at ECOLAB. The pH was regulated at 5.8 ± 0.2 for all the experiments. The medium RICH was added for the GV and PP experiment in order to increase growth rates of *Lemna minor* and *Ceratophyllum demersum* individuals in comparison to plants in the medium NORMAL. This step was taken because the results of the DR experiments indicated rather low growth rates.

Table 3 - nutrient media (POOR, NORMAL and RICH) used for the experiments. (Ammonium (NH4+) was avoided)

nutrients	POOR	NORMAL	RICH
Macronutrients	µM	µM	µM
KNO3	86	819	4095
NH4NO3	0	0	0
CaCl2, 2H2O	250	250	250
K2HPO4	4	42	210
KH2PO4	12	119	595
MgSO4, 7H2O	150	150	150
NaHCO3	750	750	750
Ca(NO3)2, 4H2O	51	635	1270
Fe EDTA	10	10	50
Oligonutrients			
MnSO4, 2H2O	0.414	0.414	0.414
CuSO4, 5H2O	0.004	0.004	0.004
ZnSO4, 7H2O	0.077	0.077	0.077
H2SeO3	0.012	0.012	0.012
Na2MoO4, 2H2O	0.1	0.1	0.1
H3BO3	31.54	31.54	31.54

2.4 Dose-response-tests (DR_ATR, DR_CU)

The stock solution for atrazine was prepared by dissolving herbicide crystals (purity >99%, Pestanal, Aldrich) in acetone (pesticide analysis quality, Pestipur, Carlo Erba-SDS) and subsequently in deionized water (5mg atrazine, 1mL acetone, 500mL H_2O). A second stock solution with copper in the form of $CuSO_4$ was prepared by diluting the crystals in deionized water (500mg $CuSO_4$, 500mL H_2O) as well. They were then serially diluted to obtain the herbicide concentrations used in the different experimental treatments. The experiment investigating the effect of atrazine included 11 groups, two of them being controls (with or without acetone but without atrazine) and 9 different contaminant concentrations (3.75, 7.5, 15, 30, 60, 100, 120, 240, 480, 960μgL^{-1}) (Dalton et al., 2013). An extra stock solution containing acetone (0.5mL mL^{-1}) was prepared in order to adjust the different acetone levels to an identical value of 19.2 µL acetone per Erlenmeyer flask. The copper experiment included one control, and 11 contamination groups were set up (0 (control), 0.025, 0.05, 0.1, 0.25, 0.5, 0.75, 1, 2, 5, 10, 15mgL^{-1}). Although EC_{50} values for atrazine, but less for copper are known from other examples in the literature Dose Response tests were repeated in this case because EC_{50} calculations usually vary between experiments due to different nutrient media used, light conditions established and general stochastic inter-laboratory differences (de Oliveira-Filho et al., 2004).

2.5 Genetic variability experiments (GV_L, GV_C)

L. minor (VG_L) and *C. demersum* (VG_C) individuals (Figure 2) of different locations within different subcatchments of the Garonne river (Figure 1) were used in the experiment in order to determine the inhibition effect caused by contamination for each group of individuals by comparing the control and the corresponding contaminated treatment. The concentration of atrazine (100µg L^{-1}) and copper (1.5mg L^{-1}) were used for *L. minor*. The value of 20µg L^{-1} for *C. demersum* concerning atrazine was looked up in an article by Fairchild (1998) and for copper 4mg L^{-1} were used (Sharma et al., 2009). The populations used in this experiment are listed in Table 1. No allozymic study in order to assess the genotypic differentiation was performed and differing genetic patterns were assumed due to the spatial distribution of the sampling sites. For the experiment two three-frond individuals of *L. minor* were put into Erlenmeyer flasks containing 100mL of nutrient solution (RICH). The experiment was not conducted under sterile conditions. The individuals used for the preculture were put into a beaker glass. For each population three groups were established ([0] - control; ATR contaminated; CU contaminated), each having five replicates.

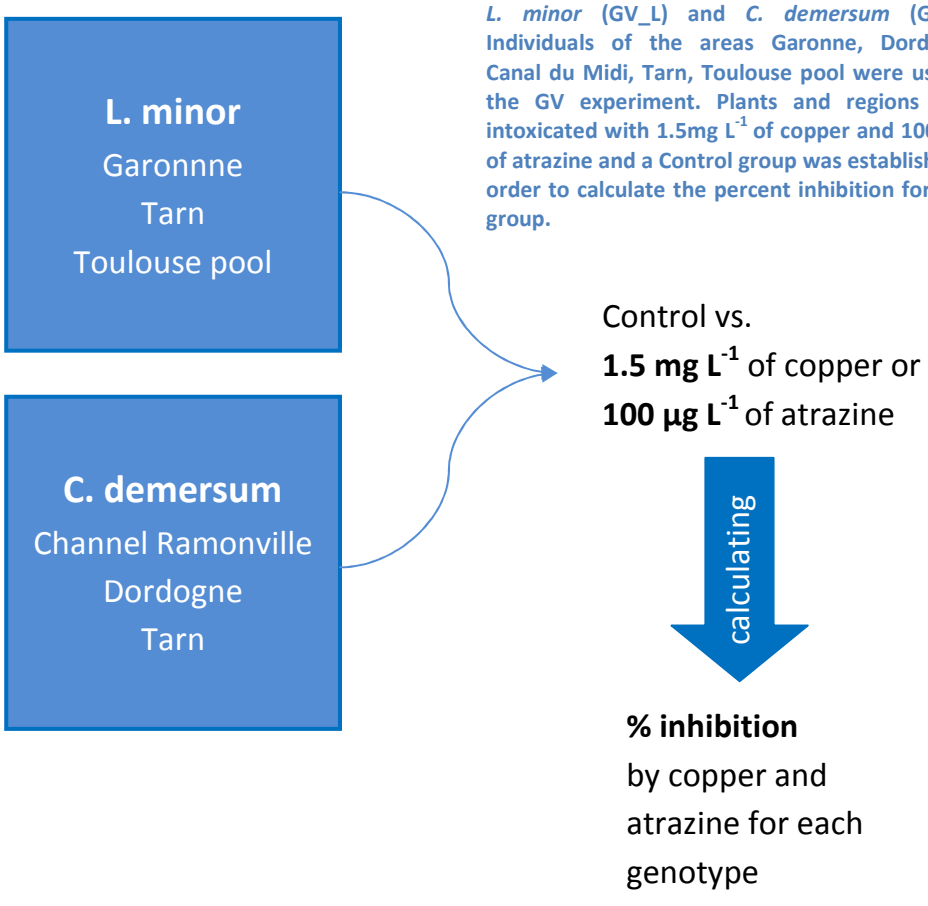

Figure 2 – schema explaining the experimental design of the genetic variability experiment with *L. minor* (GV_L) and *C. demersum* (GV_C): Individuals of the areas Garonne, Dordogne, Canal du Midi, Tarn, Toulouse pool were used in the GV experiment. Plants and regions were intoxicated with 1.5mg L^{-1} of copper and 100µgL^{-1} of atrazine and a Control group was established in order to calculate the percent inhibition for each group.

2.6 Phenotypic plasticity experiment (PP)

For the experiment addressing the question of phenotypic plasticity only the *Lemna minor* population collected at a pool in Toulouse (T) and kept under sterile conditions for several months was used. The experiment was being operated in two steps: First a PRE-CONTAMINATION phase (23 June – 6 July) was adapting the plants towards the two different environmental condition groups (each having two subdivisions), namely either low or high nutrients, or low or high light availability. All the environmental factors (and levels within factors) are listed in Table 4. In order to start the pre-contamination phase, three-leaf *L. minor* individuals were transplanted under a fume hood into autoclaved Erlenmeyer flasks each containing 100mL of nutrient solution (*POOR* for N- or *RICH* for N+, L+ and L-). Secondly the contamination exposure experiment (7 June – 20 July) was started by transplanting again two three-frond *L. minor* individuals under the fume hood into new Erlenmeyer flasks having the appropriate new environmental conditions.

Table 4 - Groups that were established for the phenotypic plasticity experiment (PP) – Nutrient content and light availability for both the pre-contamination phase and the contaminant exposure experiment are listed here. Empty cells in the table refer always to "rich (N+)" and respectively "high (L+)" conditions. Contaminants added during the exposure experiment are listed in a separate column. An extra group having "high (L+)" conditions during both pre-contamination phase and exposure experiment was not established because group 1 and 2 are exhibiting such conditions.

Group	Pre-contamination phase		Contaminant exposure experiment		
	Nutrients	*Light*	*Nutrients*	*Light*	*Contaminants*
1	rich (N+)	high (L+)	rich (N+)	high (L+)	[0]
2	rich (N+)	high (L+)	rich (N+)	high (L+)	[1.5 mg L^{-1}]
3	rich (N+)		poor (N-)		[0]
4	rich (N+)		poor (N-)		[1.5 mg L^{-1}]
5	poor (N-)		rich (N+)		[0]
6	poor (N-)		rich (N+)		[1.5 mg L^{-1}]
7	poor (N-)		poor (N-)		[0]
8	poor (N-)		poor (N-)		[1.5 mg L^{-1}]
9		high (L+)		low (L-)	[0]
10		high (L+)		low (L-)	[1.5 mg L^{-1}]
11		low (L-)		high (L+)	[0]
12		low (L-)		high (L+)	[1.5 mg L^{-1}]
13		low (L-)		low (L-)	[0]
14		low (L-)		low (L-)	[1.5 mg L^{-1}]

Beginning with this transplantation, the Erlenmeyer flasks where not anymore completely in axenic condition but were closed with a plastic cap in order to minimize the

entering possibility of unwanted organisms. The reduced light availability (**L-** 14.6 ± 3.8µmol (m^{-2} s^{-1})) was established by using black textiles imposed onto the Erlenmeyer flasks. In the whole experiment just one contaminant concentration was used, namely 1.5mg L^{-1} of copper. This value was based on the experiment DR_CU which found an EC_{50} of 0.69mg L^{-1}. The differences between the nutrient contents of *RICH* and *POOR* media are listed in Table 3.

An experiment testing a possible habituation effect of *L. minor* individuals towards increased copper exposure during the pre-contamination phase was additionally set up in the course of the phenotypic plasticity experiment (Table 5). 12 additional Erlenmeyer flasks were contaminated with 0.15mg L^{-1} of copper during the pre-contamination phase and hereupon separated during the contaminant exposure experiment into two groups ([0] – control, [1.5mg L^{-1}] – Cu contaminated)

Table 5 – Groups in the experiment testing a possible habituation effect towards small copper concentrations. These groups were compared to groups 1 and 2 – both with 0.25µg L^{-1} (as a part of the nutritive medium) during the pre-contamination phase (Table 4), to examine the influence of increased copper exposure during the pre-contamination phase.

Group	Pre-contamination phase	Contaminant exposure experiment
15	0.15mg L^{-1}	[0]
16	0.15mgL^{-1}	[1.5 mg L^{-1}]

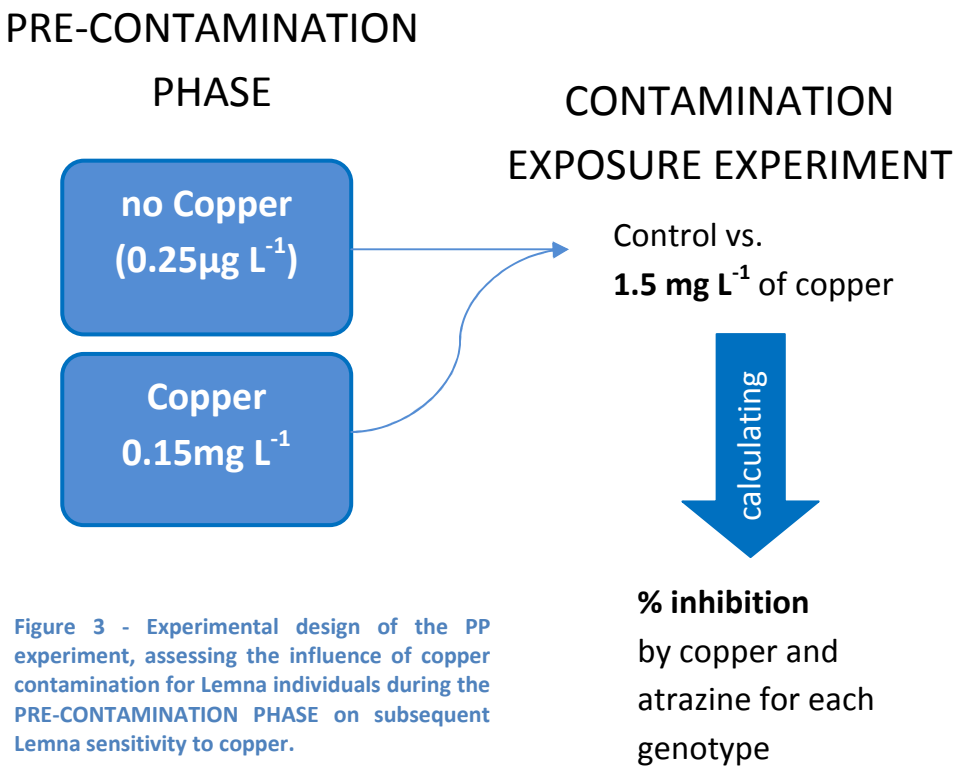

Figure 3 - Experimental design of the PP experiment, assessing the influence of copper contamination for Lemna individuals during the PRE-CONTAMINATION PHASE on subsequent Lemna sensitivity to copper.

2.7 Endpoints

The two endpoints **Relative Growth Rate (RGR)** and **Quantum Yield (QY)** were determined at the end of a 14 days period of growth and a third one, **Leaf Area (LA)** was measured regularly every three days for the Phenotypic Plasticity experiment only. The **RGR** was calculated by the formula: $\ln(m_2 - m_1)d^{-1}$, where m_1 and m_2 are initial and final fresh mass (or initial and final stem length for the *C. demersum* experiment respectively) of a plant sample and *d* is the duration of the contamination period in days (Cedergreen et al., 2004). For the initial fresh mass only six separate measurements were made at the start of the experiment and their mean result was used for all other RGR calculations. This was done once for the Dose-Response (DR) experiment and a second time for the Genetic Variability (GV) and the Phenotypic Plasticity (PP) experiment because they were conducted apart in different rooms under different pre-conditions. In order to measure the **QY** the plants were subsequently dark-adapted for a minimum period of 15 minutes and afterwards an UNDERWATER FLUOROMETER DIVING-PAM (Heinz Walz GmbH, Germany) was used to determine the Quantum Yield (Fv/Fm ; Fv=maximal variable fluorescence, Fm=maximal fluorescence yield) of photosystem II. The basic settings of the DIVING-PAM, namely measuring intensity (50: MEAS-INT) and GAIN were set to 3. The **LA** was recorded on Day 1, 4, 7, 10 and 14 of the experiment by taking a photo at the top of the Erlenmeyer flask focusing the whole water surface inside. The camera of a Moto G (first generation) telephone was used. Since the leaves of *L. minor* are floating on the surface, their one-sided area can be determined in this way. The photos were analyzed with the ImageJ (Fiji) software (version: 1.50e) using a macro-script which can be found in the appendix 9.2. The Macro which colour thresholds images and calculates areas can be executed with the help of the *Multiple Image Processor* tool (The threshold values have to be adapted to given light conditions and importantly to given background colours).

2.8 Data analyses Statistics

Dose-Response experiment

In the dose-response experiment a percentage of growth in comparison to the control (growth (vs. control)) was calculated from the fresh weight for each treatment trial (herbicide concentration *j*, replicate *k*) relative to the average dry mass ($\bar{m}$) measured in the control trials *(j=0)* by the formula:

$$Growth(\%)_{j,k} = \frac{m_{j,k}}{\bar{m}_{j,k=0}}$$

ANOVA analyses were performed on the effect of concentrations of atrazine and copper on **Growth (%)** and **RGR.** If a significant dose effect was found, data was fitted to a log-logistic dose-response model with 4 parameters using the *drc*-package in R:

$$U = c + \frac{d - c}{1 + \exp\left[b(\log(dose) - \log(EC_{50}))\right]}$$

U denotes the plant response, *d* and *c* denote the upper and lower limit and *b* is proportional to the slope of the curve around EC_{50} (Cedergreen et al., 2004; Knezevic et al., 2007).

Genetic Variability and Phenotypic Plasticity experiment

For the endpoints RGR, QY and LA mean growth inhibitions were calculated out of the six replicates. Furthermore boxplot diagrams, ANOVA and respectively non-parametric KRUSKALL-WALLIS (when data couldn't reach homoscedasticity assumption) tests were conducted. Statistical analyses were performed using the R software (version 3.0.3) implemented in R Studio (version 0.98) (The R Foundation for Statistical Computing, 2014).

2.9 Copper effective Concentration

In order to determine the effective concentration, samples were taken from each experiment and the copper content was analysed using Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES, IRIS INTREPID II XSP9597 – Thermo Elemental, USA). Before the measurement the samples were stored in a cooling chamber (5°C) for some time with added drops of nitric acid HNO_3.

3. Results

3.1 Dose-Response-Tests

The mean result of the six initial fresh masses was 7.3mg (SD: 0.3, n=6) and the Dose-Response tests of one population of *L. minor* determined an EC_{50} of 124.4µg L^{-1} for the **RGR** and an EC_{50} of 34.7 mg $L^{-1}$for the **Growth (%)** for individuals contaminated with atrazine. The maximum mean RGR was 0.198day^{-1} (SD: 0.011, n=8), and was observed for the control group with acetone. A significant reduction of plant growth can be observed for atrazine concentrations equal or higher than 7.5µg L^{-1} (ANOVA, Tukey's HSD test, F=84.15, P < 0.001) (Figure 5). The two control groups, one with acetone and the other without, which were established in this experiment, could show that acetone has an influence on the plant's growth: the control group with acetone showed an increased growth by 60%, on average, compared to the control without acetone (Figure 4).

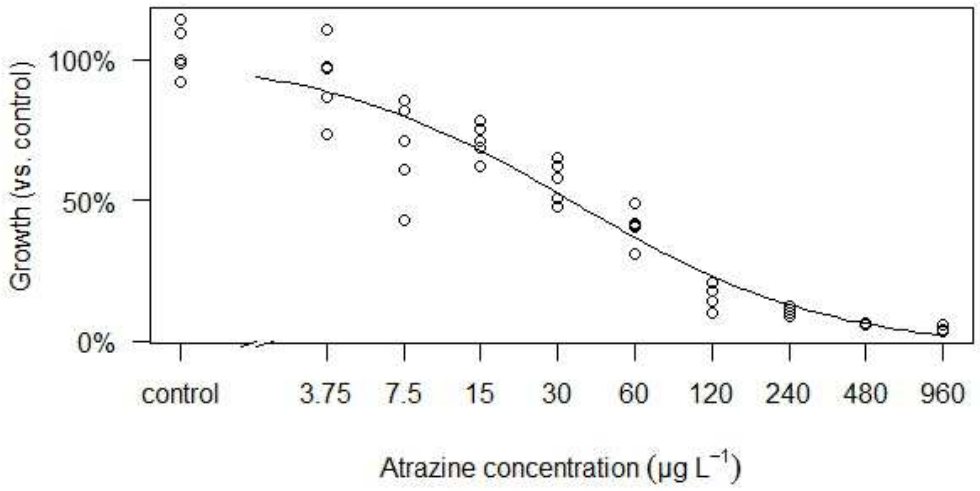

Figure 5 - Dose-response-test conducted for the effect of atrazine on *L. minor* (logarithmic scale on the X axis).

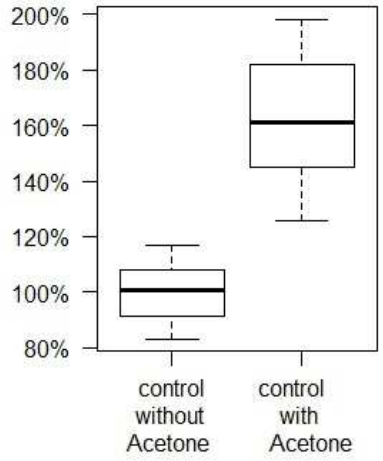

Figure 4 - influence of acetone on growth (n=8)

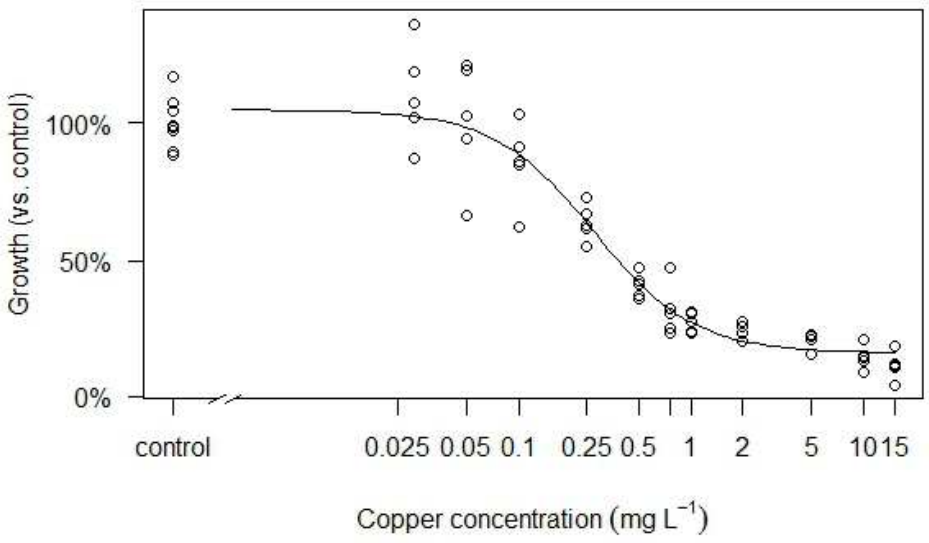

Figure 6 - Dose-response-test conducted for copper on *L. minor* (logarithmic scale on the X axis).

The second Dose-Response-test found an EC_{50} of 0.69mg L^{-1} of the **RGR** and an EC_{50} of 0.27mg L^{-1} for the **Growth (%)** for the experiment with copper and *L. minor* lasting 14 days. The development of the contaminant's influence can be observed in Figure 6. At a concentration of 0.25mg L^{-1} a statistically significant decrease (ANOVA, Tukey's HSD test, F=66, P<0.001) in growth compared with the control group can be observed. Effective concentrations which were obtained from an ICP-OES verified the nominal concentrations used in this experiment (Figure 4).

Table 6 – ICP-OES result (effective concentration) of the DR_CU experiment (3 samples for each concentration were taken)

Nominal concentration (mg L^{-1})	Mean + SD Effective Concentration (mg L^{-1})
0.025	0.0372±0.012
0.05	0.0523±0.002
0.1	0.0946±0.019
0.25	0.2326±0.001
0.5	0.3684±0.013
0.75	0.6875±0.007
1	0.9254±0.004
2	1.8307±0.010
5	4.6500±0.061
10	9.5187±0.124
15	14.2433±0.145

3.2 Genetic Variability Tests (GV)

The average initial fresh mass that was used for calculating the RGR for all the individuals from the Genetic Variability and the Phenotypic Plasticity experiment was 8.7mg (SD=1.3: n=6). Effective concentrations were measured in the GV and PP experiment and could verify the nominal concentrations that were used in the experiment (Table 7).

Ceratophyllum demersum

The Stem Length measurements of the plants of the Tarn (T) river populations showed both statistically significant differences concerning the control groups between Channel Ramonville (C) individuals (ANOVA, HSD, p=0.00017) and individuals from the Dordogne (D) (p=0.0001) (data not shown). All in all the Tarn individuals manifest a decrease in length in presence of atrazine whereas the other two groups gained length. Concerning the length gain there was not a notable difference between the Channel Ramonville and the Dordogne population. The QY measurement of *C. demersum* shoots of the populations Dordogne (D) and the channel Ramonville (C) showed a negative influence of atrazine on photosynthetical activity. A mean decrease of **15%** (n=5) of individuals from Dordogne and a decrease of **9%** (n=6) for individuals of the Channel Ramonville was observed.

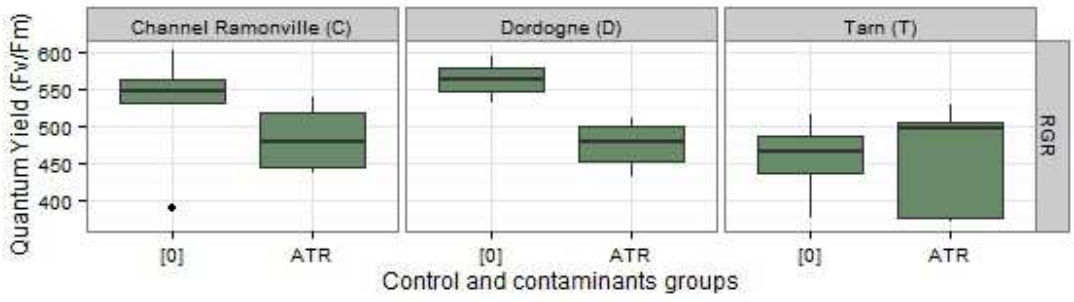

Figure 7–QuantumYield measurement on *C. demersum* for three different populations (from Channel Ramonville (C), Dordogne (D) and Tarn (T)); [0] – control group, ATR – treatment with 100µg L^{-1}atrazine.

This difference was not found to be statistically significant. Generally no significant differences (KRUSKAL-Wallis) concerning the Quantum Yield measurements between the control and the contaminated treatment and across groups could be detected.

Lemna minor

The experiment addressing genetic variability of *L. minor* showed that the control groups across the populations of the Garonne (G), the pool at Toulouse (P) and the Tarn(T) grew similarly (Figure 8). No inhibition by atrazine or copper could be observed for any

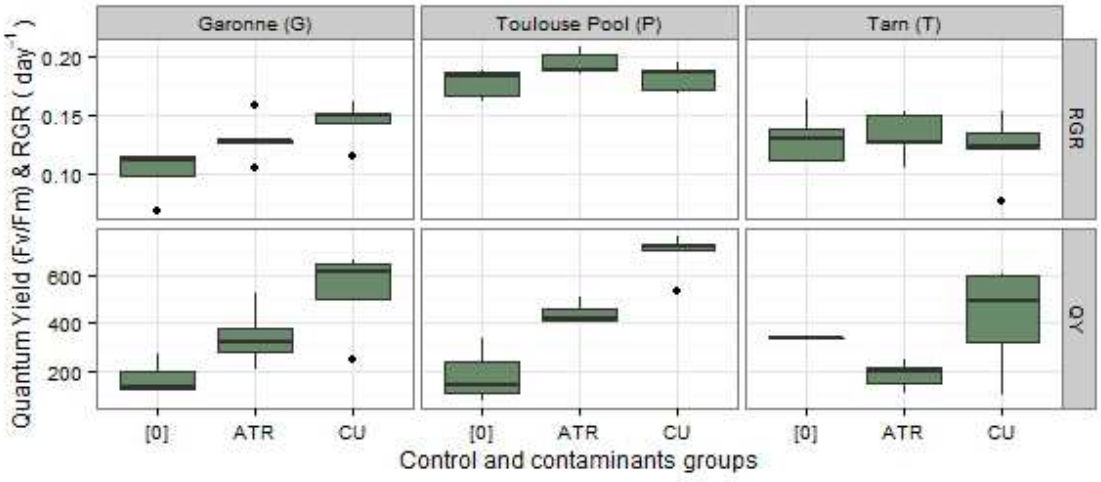

Figure 8 – RGR and quantum yield measurement on *L. minor* for three different populations (from Garonne (G), pool at Toulouse (P) and Tarn (T)); [0] – control group, ATR – group contaminated with atrazine, CU – group contaminated with copper

populations except for the Quantum Yield results of the Tarn population for atrazine (**46%**). In contrast to the expectations the growth of the Garonne population seems to increase when adding copper (ANOVA, Tukey's HSD test, p=0.033).The quantum yield measurement draws a similar picture and indicates an enhanced photosynthetic activity for the Toulouse Pool (p=0.001) and the Garonne (p=0.04) populations in presence of copper as well. In general the values of the quantum yield were very low which indicates that the plants (including controls) are stressed. Further ANOVA and HSD analyses showed that there's a significant difference between the control groups of the Toulouse pool (P) and the Garonne (G) population (p=0.0001) regarding the RGR.

Table 7 - ICP-OES result (effective concentration) of the VG and PP experiments (3 samples per experiment)

experiment	**Nominal concentration (mg L^{-1})**	**Effective concentration 1 (mg L^{-1})**	**Effective concentration 2 (mg L^{-1})**	**Effective concentration 3 (mg L^{-1})**
VG_C	**4**	4.016	3.862	3.917
VG_L	**1.5**	1.479	1.503	1.451
PP	**1.5**	1.523	1.498	1.462

3.3 Phenotypic plasticity Tests (PP)

Influence of nutrients on plant response to copper contamination.

The **RGR** of the plants **not treated with copper** is significantly different between the groups [N-/N+] and [N-/N-] (ANOVA, HSD, p=0.003)as well as **[N+/N+]**and [N-/N+] (p=0.003).The highest RGR values were observed for the groups **[N+/N+]** and [N-/N-] which are the treatments having stable environmental conditions (during pre-contamination and exposition phase).However **LA** measurements indicated a better growth performance for [N+/N+] in comparison to the other groups.

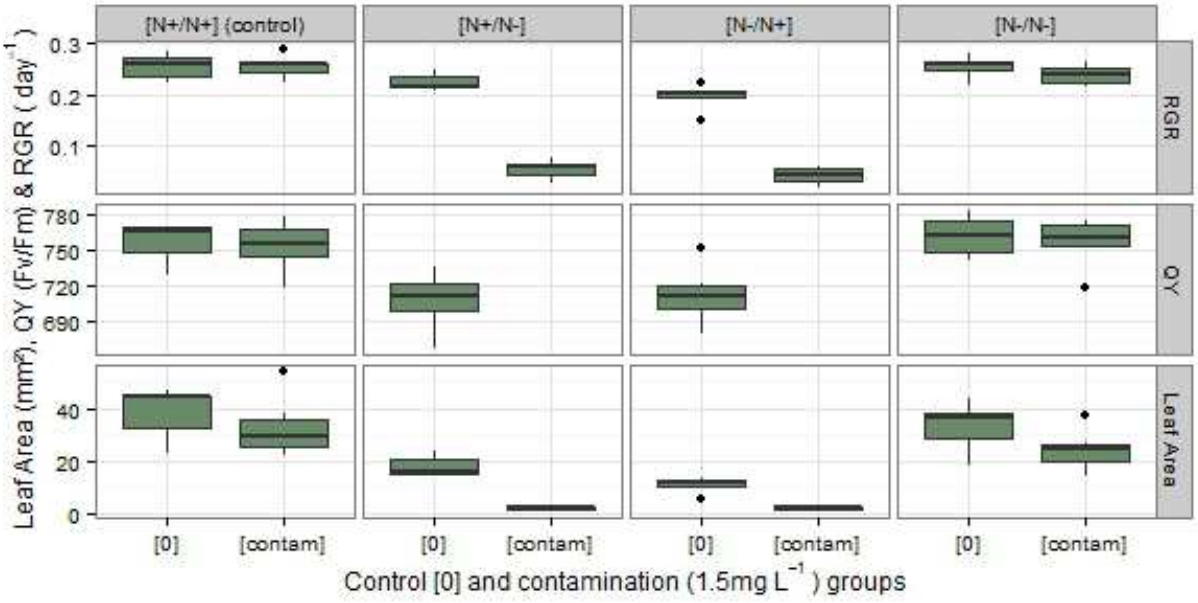

Figure 9 - Results of the phenotypic plasticity experiment showing the influence of nutrient water content on plant response to copper.: The box-plots indicate growth RGR (day^{-1}), Quantum Yield (Fv/Fm) & Leaf Area (mm²) measurements for four different environmental conditions concerning nutrients ([N+/N+], [N+/N-], [N-/N+], [N-/N-] – ([pre-contamination phase/contamination exposure experiment]), in absence [0] or presence [contam] of copper (n=6 for each combination: Nutrient x contamination treatments)

QY measurements showed similar results for the combinations of the groups controls of [N-/N+] – [N-/N-] (p=0.003) and **[N+/N+]** – [N-/N+] (p=0.012). Furthermore they showed that there is also a significant difference between the groups [N+/N-] and [N-/N-] (p=0.0014)as well as for the groups**[N+/N+]**and [N+/N-] (p=0.005).

ANOVA analyses of the **differences between control and treatment group** could show that a copper contamination had a significant negative influence concerning

growth on the [N+/N-] (RGR decrease of **76.6%**) and [N-/N+] (RGR decrease of **79.6%**) group (p-values<0.0001).A negative influence of contamination on the photosynthetical activity for the same two groups were not measured but could be assumed because of leaf chlorosis.

There is no decrease of the RGR of the group **[N+/N+]** between the control and the copper contaminated group. Similarly a RGR reduction of just **6.4%** for the group [N-/N-] can be observed when control and contamination treatment are compared.

The **LA** measurement on **D10** showed a decrease of leaf area in the groups **[N+/N+]**, [N+/N-] (p<0.0001), [N-/N+] (p<0.0001) and [N-/N-] of 11.1%, 80.9%, 72.4% and 23.1% due to copper contamination. This development is perpetuated for the leaf areas measured four days later at **D14**: **[N+/N+]**, [N+/N-] (p<0.0001), [N-/N+] (p<0.0001) and [N-/N-] show a copper inhibition of 14.6%, 87.6%, 81.1% and 26.3% respectively (Table 11) (The inhibitions where statistically significant when p-values are shown).

Influence of light on plant response to copper contamination

In the absence of contamination, the light group **[L+/L+]**exhibited significantly higher **RGR** in comparison to the groups [L+/L-] (p=0.001) and [L-/L-] (p=0.001). No difference in the **QY** measurement could be observed for the treatments without contamination. ANOVA analyses of the contamination response, the difference between the control [0] and the contamination [contam] group could not find any significant reduction of RGR and QY measurements throughout the treatment groups in dependence of light availability (Table 4).

The **LA** measurement showed mean percentage inhibitions for the groups [L+/L-], [L-/L+] and [L-/L-] at **D10** (data not shown) of 10.7%, 5.8% and 6.1% and at **D14** 0%, 6.9% and 16.6% respectively (Table 11). However none of them were statistically significant.

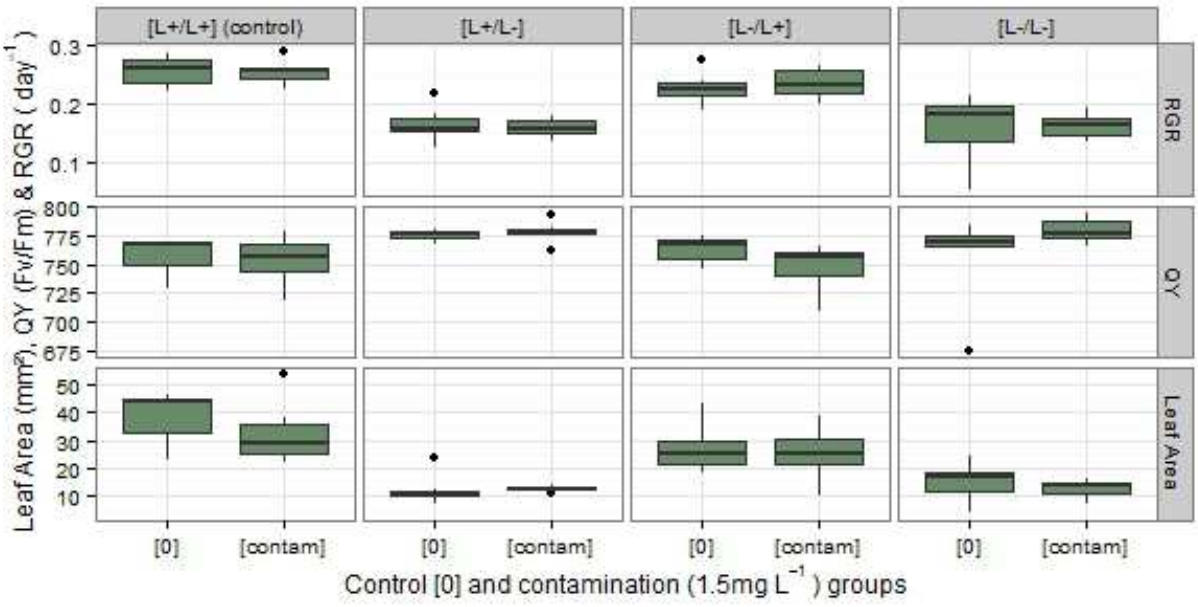

Figure 10 - Results of the phenotypic plasticity experiment showing the influence of light availability on plant response to copper.: The box-plots indicate RGR (day^{-1}), Quantum Yield (Fv/Fm) & Leaf Area (mm²) measurements for four different environmental conditions concerning light availability ([L+/L+], [L+/L-], [L-/L+], [L-/L-] – ([pre-contamination phase/contamination exposure experiment]), in absence [0] or presence [contam] of copper (n=6 for each combination: Light x contamination treatments)

Influence of copper pre-treatment

The group [CU+] which 0.15mg L^{-1} of copper was added during the pre-contamination phase (Figure 5) in order to detect possible habituation effects showed a decrease in **RGR** of 8% (not sign.), no decrease of **QY** and again a decrease of 16.1% on **D10** (Figure 12) and a significant decrease of 22.9% on **D14** concerning the **LA** (Figure 11, Table 11).

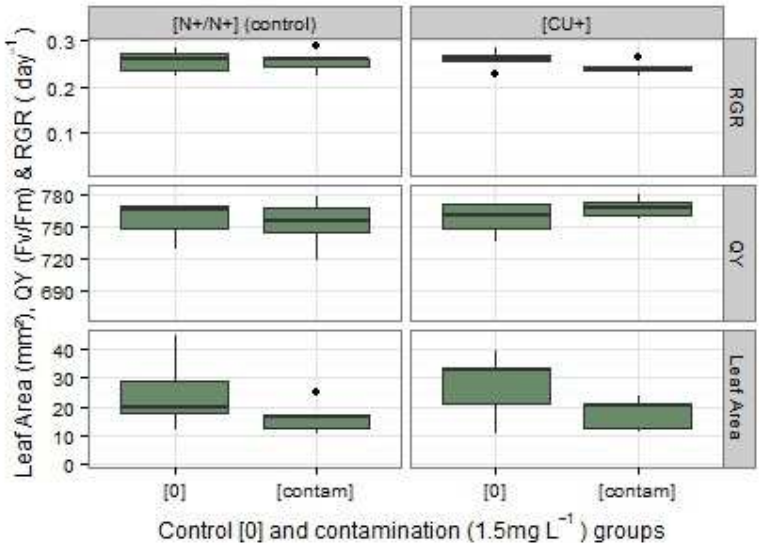

Figure 11 – Results of the phenotypic plasticity experiment using copper pre-treated individuals: The box-plots indicate growth RGR (day^{-1}), QY quantum yield (Fv/Fm) and Leaf Area (mm²) for copper non-treated and copper-treated individuals, in absence [0] or presence [contam] of copper (n=6 for each combination)

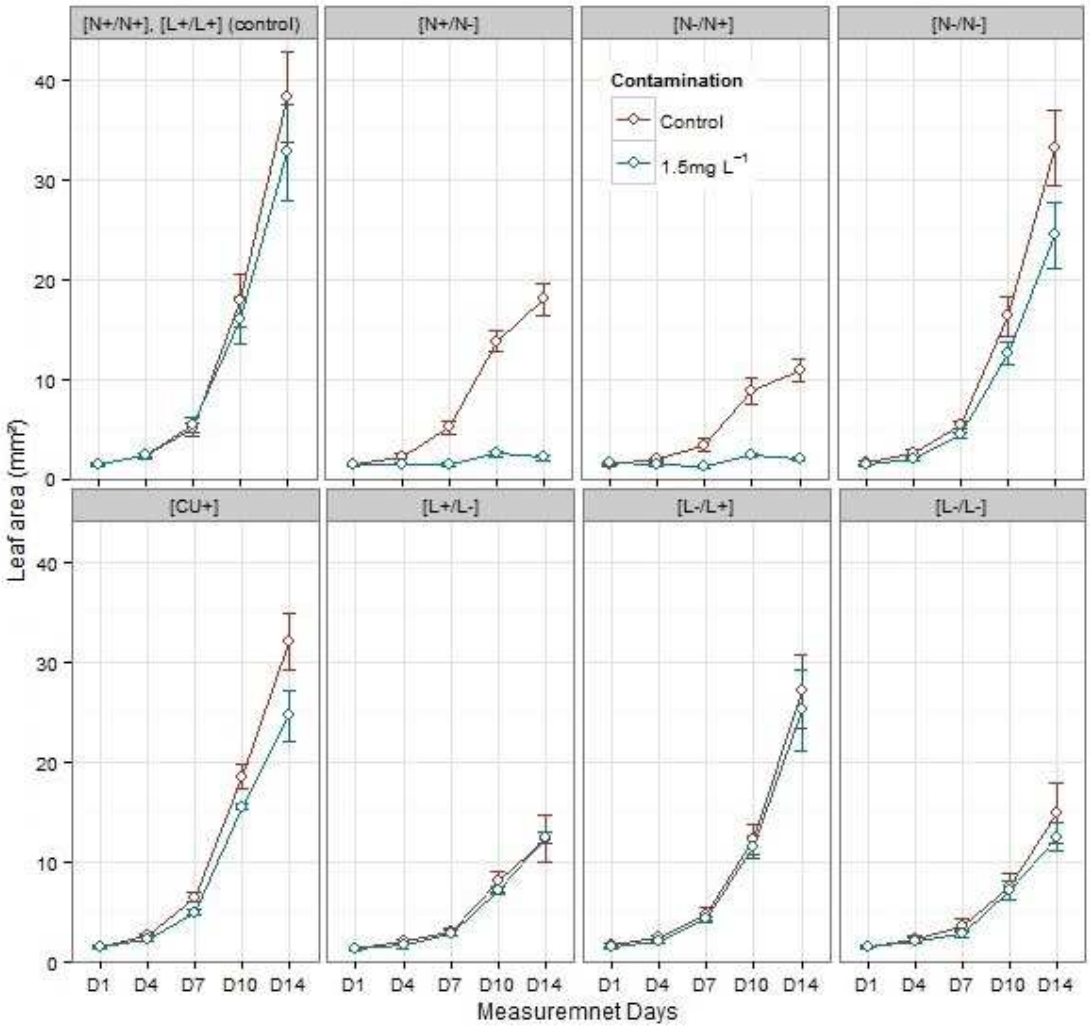

Figure 12 - Results of the Leaf Area (mm²) measurements of the phenotypic plasticity experiment of all the test groups: [N+/N+], [N+/N-], [N-/N+], [N-/N-], [L+/L+], [L+/L-], [L-/L+], [L-/L-] and [CU+] over a time period of 14 days. D1 – D14 indicate the measurement day and red data points show the Control group whereas the green ones represent the copper contaminated groups (1.5mg L^{-1}).

4. Discussion

Dose-response-test (DR_ATR, DR_CU)

The results from the **Dose-Response-test** on *L. minor* showed an EC50 of 122.1 µg L^{-1} for atrazine and an EC50 of 0.69 mg L^{-1} for copper. A statistically negative influence of contamination could be observed from a concentration of 7.5 µg $L^{-1}$for atrazine and 0.25mg L^{-1} for copper. These results are slightly different compared to the results by Fairchild et al. (1998) who found an EC50 of 92 ± 6 µg L^{-1} for atrazine and *L. minor* (test-duration: 96h).Another 10 day lasting experiment by Kirby and Sheahan(1994)found an EC50 of 60µg L^{-1} for fresh weight as an endpoint, but generally the results obtained lie within the range of already conducted similar experiments. Solomon et al. (1996) defined the ecological importance in natural systems of atrazine beginning with >50 µg L^{-1} and they stated that atrazine concentrations of 5 µg L^{-1} or less, typically encountered in the environment pose no significant ecological risk. The significant influence of 7.5 µg L^{-1} found in this study reflects laboratory conditions and might therefore accord with their results. Khellaf and Zerdaoui (2009) found in four days lasting experiment (pH: 6.1 ± 0.1; 16:8 – day:night – cycle) an EC_{50} of 0.47 mg L^{-1} for copper.

Genetic variability experiment (GV_L GV_C)

As a basis for the experiment Genetic Variability between the sampled populations was assumed to exist due to the wide spatial distribution of the sampling sites (Figure 1, Table 1). However, this assumption was not tested.

Genetic Variability experiment with *L. minor*

The results of the **RGR** and **QY** measurement of the GV experiment on *L. minor* suggest an influence of genetic variability in the plant response to contaminants. However due to methodological problems it is difficult to assign these differences in RGR and QY only to genetic variability (Figure 8). The **RGRs** differ between the control groups which would indicate genetic variability. Also the control group of the Tarn population showed increased values in the **QY** measurement in comparison to the two other populations, indicating likewise different genetic patterns. Furthermore differences between the

populations **in response to the contaminants** atrazine and copper can be observed. However the inhibition effect developed differently than expected: Except for the atrazine contaminated group of the Tarn population all other treatment groups showed compared to the control group and in contrast to our expectations **increased** or **equal** RGRs and QYs. This outcome questions if the variability in the results can only be assigned to genetics. Other factors, such as algae occurring in the samples were additional influencing parameters. Atrazine and copper might have inhibited initially algae growth and led therefore to an increase in RGRs and QYs of *L. minor* (Table 10). A similar study by Mazzeo et al. (1997) testing the reaction of fourteen different clones of *Lemna gibba* L. from locations all over the world in response to the triazine simazine found no significant difference in the influence of the contaminant between the populations. Also Dalton et al. (2013) found only a small difference in atrazine sensitivity between distinct populations of *L. minor* and they concluded that laboratory cultures provide a good estimate for natural populations all over the world. Furthermore in comparison to this study they did not just use one contaminant concentration but conducted dose-response-tests for each population.

Genetic Variability experiment with *C. demersum*

The experiment on *C. demersum* (Figure 7, Table 9) showed more comprehensible results indicating no variability between the inhibition effect in the Channel Ramonville (9%) and the Dordogne (15%) populations in response to contaminants. Only the control group of the Tarn (T) population showed decreased QYs and therefore also a different inhibition pattern (0.2%) in comparison to the two other populations. This difference is significant and can be ascribed to genetic variability.

Generally the genetic variability experiments showed **rather low RGRs** (Table 8). Although the experiment was conducted under the same environmental conditions as the experiment for phenotypic plasticity (with just a small delay of one week) it showed compared to the PP experiment (0.255 day^{-1}) rather small mean RGRs of around 0.177, 0.130 and 0.101 $day^{-1}$of the three populations Toulouse pool (P), Tarn (T) and Garonne (G) respectively. The Toulouse pool population was kept under axenic conditions until ten days before the experiment. The plants were then put under the same non-axenic

conditions as the individuals of the Tarn and the Garonne population in order to provide similar initial growth conditions. Nevertheless a high difference between the Toulouse pool population (P) and the two others (T, G) concerning RGRs could be observed (which can be related to the axenic storage). Coming back to unexpected development in the contaminant treatment groups in the *L. minor experiment*, the toxicity of atrazine should rather be increased with light availability (Brain et al., 2012) and not diminished. Furthermore algae testing showed that light availability is positively correlated with the toxicity of metals (Munovar et al., 1988). Compared to these findings, the individuals of the Toulouse pool (P) and the Tarn (T) population neither showed signs of an inhibition effect in response to the two contaminants. The toxicity of copper for *L. minor* individuals might be reduced due to the high nutrient content in the media RICH or more probably due to the higher amount of EDTA (50µM) (Table 3) used in the media RICH in comparison to the media NORMAL (10µM), which was used during the DR experiment.

To give a résumé, patterns of variability in RGRs and QYs cannot clearly be assigned to **Genetic Variability** in the experiment with *L. minor*. Testing different populations of *C. demersum* showed the Tarn population to react differently in comparison to the two others verifying the hypothesis for **Genetic variability** in this case.

In order to avoid influencing vectors such as algae or other competitive organisms and to guarantee equal growth conditions it is recommended to perform similar studies under axenic conditions in future.

Table 8 – Mean fresh mass at the end of experiments (Erlenmeyer flask, 250mL)and relative growth rates (mean ± SD) of *Lemna minor* in the different test settings in the absence of herbicides (controls).

Experiment	Fresh mass ± SD (contr.)	RGR (control)
	mg	day^{-1}
DR_ATR (without acetone)	59.92 ± 6.89	0.161 ± 0.008
DR_ATR (with acetone)	97.35 ± 14.4	0.198 ± 0.011
DR_CU	53.33 ± 4.99	0.152 ± 0.007
GV_L *(all 3 populations)*		
Toulouse pool (P)	88.78 ± 13.9	0.177 ± 0.012
Tarn (T)	49.16 ± 14.6	0.130 ± 0.021
Garonne (G)	33.62 ± 8.51	0.101 ± 0.022
PP	253.02 ± 82.30	0.255 ± 0.026

Phenotypic plasticity experiment (PP)

The **groups with changing nutrient conditions** showed that a change in nutrient content between pre-contamination and exposition phase had a considerable influence on the contamination response of the plants. Individuals that were not contaminated with copper already showed a significant decrease of RGR of 12.8% [N+/N-] and 23.4% [N-/N+] in comparison to the control group **[N+/N+]**.Inhibition effects differed throughout the groups. A statistically significant decrease in growth caused by copper within the nutrient groups (compared to uncontaminated controls with similar nutrient conditions) could be asserted for the [N+/N-] (RGR: 76.6%, LA: 87.6%) and the [N-/N+] (RGR: 79.6%, LA: 81.1%) group. For the endpoint LA other inhibitions were found as well but they were not statistically significant (Table 11), nevertheless suggesting different responses in dependence of nutrient conditions: The LA of the group **[N+/N+]** was inhibited by 11.1% on D10 and by 14.6% on D14 whereas the group containing only low amount of nutrients [N-/N-] was inhibited by 23.1% on D10 and 26.3% on D14 due to copper contamination. The **groups with changing light conditions** [L+/L-], [L-/L+] and [L-/L-] showed only small effects of copper contamination and no significant differences among their response to contaminants. The **copper pre-treated group** [CU+] (0.15mg L^{-1} Cu) also showed a significant decrease for the endpoint LA on D14 by 22.9%. However compared to other groups it didn't differ significantly.

Phenotypic plasticity in *L. minor* could therefore only be shown for groups [N+/N-] and [N-/N+] with changing nutrient conditions and the hypothesis attesting environmental influence for the plant response to contaminants can be confirmed.

Light groups responded too weakly to contamination in order to get meaningful results. Also the plants of the group [CU+] exhibited only slight non-significant differences in inhibition compared to other groups. Generally phenotypic plasticity could be asserted best for the LA measurement because all groups (except for [L+/L-] on D14) showed inhibition effects on D10 and D14, whereas RGR and QY measurements were less susceptible to contamination (Figure 12). In addition to that the endpoint LA had the advantage that measurement processes were not interfering with the plant and that it could be recorded at any time during the experiment, providing information on the

development of plants over time. Vasseur and Aarssen (1992) found in their study on phenotypic plasticity in *Lemna minor* that the rate of frond production was the best estimate for fitness and root length was the best measurement for them to observe plasticity. It was not measured in this study though some observations were made that support these findings and suggest putting this aspect into consideration for future work. The results of this study are in favour that LA is a good estimate for fitness and plasticity in *Lemna minor*. When nutrient conditions were stable in the groups [N+/N+] and [N-/N-], RGR and QY measurements did not show inhibition effects, whereas LA measurements found reduced leaf areas (Figure 12, Table 11). At the same time groups with changing nutrient conditions [N+/N-] and [N-/N+] showed very high copper inhibitions for all the three endpoints RGR, QY and LA. The QY measurement was not recorded because all the leaves suffered from chlorosis, a loss of photosynthetical pigments. It was assumed that QY measurements would have been low.

Influence of environmental factors on the toxicity of copper

Variability in the influence of environmental factors on the toxicity of copper was not part of the research objectives and since the experiment was conducted under laboratory conditions, factors like nutrients, light, sterility could be controlled. However some influences of these factors might have influenced copper toxicity considerably.

Although having used very high amounts of copper (1.5mg L^{-1}), toxic effects were not present in some groups. As already discussed before copper toxicity could have been mitigated by the increased concentration of **EDTA** in the medium RICH (50µM EDTA) which was used for the nutrient groups [N+/N+], partly for the groups [N+/N-] and [N-/N+] and for all three light groups [L+/L-], [L-/L+] and [L-/L-] and the [CU+] group. The only group which was solely using the medium POOR with 10µM EDTA was [N-/N-]. As during the DR tests the medium NORMAL, which contained also only 10µM EDTA showed no decreased copper toxicity, we would have expected the group [N-/N-] to show considerable toxic effects. However it did not occur and the plants of the group [N-/N-] developed similarly to the **[N+/N+]** control group, regarding RGR and QY measurements. The endpoint LA showed some copper toxicity effects but similar low ones as the **[N+/N+]** group (Figure 12). Still EDTA might be the cause for reduced toxicity

for groups with the medium RICH and also for those with the medium POOR. EDTA is a chelating agent and reduces the bioavailability and toxicity of Cu ions (Nasu et al., 1983; Teisseire et al., 1999). Nasu et al. (1983) found 30µM of EDTA to be sufficient to suppress the absorption of copper at a concentration of 5-10µM. In this study copper concentrations of 1.5mg L^{-1}(23µM) were put into media containing 50µM EDTA. Possible different EDTA influences between the two media NORAML and POOR might have occurred due to big differences in nutrient contents. To avoid such uncertainties in future experiments, media with no EDTA are recommended to be used. Besides EDTA, **pH** and **nutrient content** are important factors that influence the toxicity of copper. A publication by Sharma et al. (2009) stated that copper toxicity for *Lemna aequinoctialis* as well as for *C. demersum* is higher in a nutrient-poor alkaline medium at a pH of 8.3 – 8.7 (EC50s of 100-170µg L^{-1} for *L. aequinoctialis* and 104-200µg L^{-1} for *C. demersum*) in comparison to a nutrient rich acidic medium (pH 5.4 – 5.7; *C. demersum*: EC50 of 2600-3175µg L^{-1};*L. aequinoctialis*: EC50 4350-4715µg L^{-1}. They used a HOAGLAND medium and also discussed the possibility that FeEDTA (as used in this study) might bind Cu ions. Furthermore they make cations (K and Ca) in nutrient rich media responsible for reduced Cu toxicity (Sharma et al., 2009). Nasu et al. (1983) found that copper inhibits plant growth more strongly with increased pH (Growth inhibition significantly higher at pH 5.1 in comparison to pH 4.1). Leblebici et al. (2010) proved in their study on *Lemna gibba* L. that nutrient addition is slightly reducing the toxic effect of copper. Sharma et al. (2009) support these findings and showed additionally that a alkaline pH (8.3-8.7) is enhancing copper toxicity. In contrast to that Lepp (1981) finds that a pH between 3.7 and 4.5 favour the dissolution of carbonate and hydroxide minerals, thus freeing associated metals. Therefore most trace metals are more toxic at lower pH levels (Guilizzoni, 1991; Lepp, 1981). Since the pH was adjusted to a constant level of 5.8 ± 0.2, and light should increase the toxicity of copper (Munovar et al., 1988), increased nutrient content (in comparison to the DR_CU experiment) might be a reason for reduced copper toxicity. However different nutrient concentrations might have also changed the pH during the experiment in different ways. Unfortunately the pH was measured only once at the beginning of the experiment, thus changes in pH throughout the experiment could not be excluded. In the Genetic Variability experiment *C. demersum*(GV_C) individuals were intoxicated with 4mg L^{-1} of copper sulphate ($CuSO_4$)

which was higher than the EC50 results for an acidic nutrient rich medium published by Sharma et al. (2009). It led to an immediate loss of leaves of *C. demersum* sprouts within just a few days should therefore be lowered in further studies. **Temperature** is not influencing the copper toxicity substantially and during all experiments stable temperatures of 20°C.were provided. A combination effect of atrazine and copper was not tested because such circumstance is not of ecological importance (Roberts et al., 1990).

5. Conclusion

The results of the Dose Response (DR) tests have proven toxicity effects of atrazine and copper on *L. minor* and *C. demersum,* with EC_{50} values (ATR: 122.1µg L^{-1}; CU: 0.69mg L^{-1}) that are in line with those from the literature (Dalton et al., 2013; Fairchild et al., 1997; Kirby and Sheahan, 1994; Sharma et al., 2009). The genetic variability experiment (GV_C) on *C. demersum* showed no difference between the populations concerning the inhibition effect due to copper except for the Tarn population. But this might have been related to a decreased photosynthetic activity in the control group and not to a different response of the population to the contaminant. The genetic variability experiment (GV_L) on *L. minor* showed less comprehensive results. The reduced RGR of the control individuals is already a sign for disturbing influences during growth. Generally tests with plants under axenic conditions should be preferred. The phenotypic plasticity experiment showed that, depending on the environmental conditions, *L. minor* individuals react differently; therefore a considerable amount of phenotypic plasticity must be present. EDTA has to be used with caution since it acts as a chelating agent, and its role in the present results is not clear. Although OECD (2006) test guidelines recommend growth media containing EDTA several researchers omit this acid. Generally intra-specific variability can be regarded as an asset for changing environments. Especially for plant individuals, as they are normally attached to solid grounds or spatially limited to their habitats such inherent mechanisms can be of advantage. In a world impacted by human development, genetic variability enables evolutionary changes that have the potential to adapt to newly emerging problems. Likewise phenotypic plasticity, the ability of an individual to change its physiology or morphology within a generation to acclimatize to changing systems is a beneficial feat for survival. Global warming will increase temperatures and pollution will probably, due to population growth not be reduced. Organisms will have to cope with these turnovers and flexibility is certainly of importance (Hughes, 2000; Williams et al., 2008). The ability for phenotypic plasticity to cope with contamination is in contrast to genetic variability not yet studied in depth and needs more clarification. Besides human induced changes, phenotypic plasticity is also regarded as a crucial parameter for invasion (Hyldgaard and

Brix, 2012). Although there are already a lot of studies on the toxicity of chemicals in nature, further research into this direction is required, since a high number of new chemical substances are introduced, usages of, for example pesticides are increasing, newly mode of actions and risks discovered and topics such as combined effects of contaminants are only poorly studied (Cedergreen, 2014).

6. List of figures

7. List of tables

8. Literature

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9. Appendix

9.1 Additional tables

Table 9 – Mean results of length and quantum yield measurements of Ceratophyllum demersum shoots of three different populations (Channel Ramonville (C), Dordogne (D) and Tarn (T) in comparison with contamination groups. Growth inhibition caused by contamination and number of observations.

C. demersum population	cont.	length gain (cm)	quantum yield (Fv/Fm)	length (control) %	QY(control) %	length inhibition %	QY inhibition %	n (len.)	n (QY)
Channel Ram. (C)	[0]	0.9	530	*100.0*	*100.0*			*6*	*6*
Channel Ram. (C)	ATR	0.5	496	59.6	91.0	*40.4*	*9*	*6*	*6*
Dordogne (D)	[0]	1.3	555	*100.0*	*100.0*			*3*	*3*
Dordogne (D)	ATR	1.0	472	75.4	85.0	*24.6*	*15.0*	*5*	*5*
Tarn (T)	[0]	-0.9	456	*100.0*	*100.0*			*5*	*4*
Tarn (T)	ATR	-1.6	455	186.0	99.8	*-86.0*	*0.2*	*6*	*6*

Table 10 - Mean results of growth and quantum yield measurements of Ceratophyllum demersum shoots of three different populations (Channel Ramonville (C), Dordogne (D) and Tarn (T) in comparison with contamination groups. Growth inhibition caused by contamination and number of observations.

Lemna minor populations	cont.	RGR (day^{-1})	quantum yield (Fv/Fm)	RGR (control) %	QY (control) %	length inhibition %	QY inhibition %	n (len.)	n (QY)
PAU-SAB	*[0]*	*0.178*	*183*	*100.0*	*100.0*			5	3
PAU-SAB	CU	0.183	692	102.7	378.4	*-2.7*	*-278.4*	5	5
PAU-SAB	ATR	0.195	400	109.6	218.5	*-9.6*	*-118.5*	5	3
TAR-SLI	*[0]*	*0.131*	*338*	*100.0*	*100.0*			5	1
TAR-SLI	CU	0.122	423	93.2	125.3	*6.8*	*-25.3*	5	4
TAR-SLI	ATR	0.132	182	101.1	53.8	*-1.1*	*46.2*	5	4
GAR-BOU	*[0]*	*0.102*	*172*	*100.0*	*100.0*			4	4
GAR-BOU	CU	0.145	537	142.1	312.1	*-42.1*	*-212.1*	5	4
GAR-BOU	ATR	0.129	334	127.1	193.8	*-27.1*	*-93.8*	5	5

Table 11 – Mean results of the phenotypic plasticity experiment showing the influence of nutrient and light availability on plant response to copper.: Mean RGR (day^{-1}) & mean QY - quantum yield (Fv/Fm) for different environmental conditions concerning nutrient content ([N+/N+], [N+/N-], [N-/N+], [N-/N-]) and light availability ([L+/L+], [L+/L-], [L-/L+], [L-/L-] – ([pre-contamination phase/contamination exposure experiment]), in absence([0]) or presence (EC50) of copper. Furthermore the inhibition (%) of RGR and QY in dependence of their of their group controls and the inhibition (%) of RGR and QY compared to the group [N+/N+] are indicated along with number of observations (n1: growth measurement, n2: PAM measurement).

nutrient, light& pre-cont. groups	contam.	RGR (mean)	RGR (SD)	QY (mean)	QY (SD)	LA: D10 (mean)	LA: D10 (SD)	LA: D14 (mean)	LA: D14 (SD)	inhib. RGR (%)	inhib. QY (%)	inhib. LA: D10 (%)	inhib. LA: D14 (%)	n RGR	n QY	n LA
[N+/N+]	[0]	0.26	0.03	757	18	19.01	5.79	24.53	12.63					5	5	5
[N+/N+]	contam	0.26	0.02	754	22	16.63	5.85	16.28	5.19	0.1	0.5	11.1	14.6	5	6	5
[N+/N-]	[0]	0.22	0.02	708	24	13.84	2.68	11.15	6.94					6	6	6
[N+/N-]	contam	0.05	0.02	NA	NA	2.64	0.72	2.21	0.54	76.6	NA	80.9	87.6	6	NA	6
[N-/N+]	[0]	0.20	0.03	713	24	9.34	3.61	4.08	1.22					6	6	6
[N-/N+]	contam	0.04	0.02	NA	NA	2.44	0.37	2.07	0.44	79.6	NA	72.4	81.1	6	NA	6
[N-/N-]	[0]	0.25	0.02	763	17	17.80	4.25	18.75	8.62					6	6	6
[N-/N-]	contam	0.24	0.02	757	20	13.27	3.13	13.21	3.64	6.4	0.7	23.1	26.3	6	6	6
[L+/L-]	[0]	0.17	0.03	776	5	8.09	2.61	12.33	5.80					6	6	6
[L+/L-]	contam	0.16	0.02	779	10	7.23	0.91	12.42	1.18	4.3	-0.4	10.7	-0.7	6	6	6
[L-/L+]	[0]	0.23	0.03	763	13	13.35	3.93	13.45	4.07					6	5	6
[L-/L+]	contam	0.23	0.03	747	21	12.86	2.76	12.85	4.89	-3.2	2.0	5.8	6.9	6	6	6
[L-/L-]	[0]	0.16	0.06	756	40	7.61	3.31	14.96	7.36					6	6	6
[L-/L-]	contam	0.16	0.02	780	12	7.15	2.18	12.47	3.45	-1.5	-3.1	6.1	16.6	6	6	6
[CU+]	[0]	0.26	0.02	758	15	19.79	3.54	27.85	10.99					6	6	6
[CU+]	contam	0.24	0.01	768	9	16.00	2.27	17.80	5.53	8.0	-1.3	16.1	22.9	5	5	5

9.2 Image-J macro

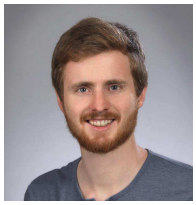
```
setBatchMode(true);
makeRectangle(399, 3, 1761, 1453);
run("Crop");
imgName = getTitle();
run("Duplicate...", imgName);

run("Color Threshold...");
// Threshold Colour v1.13------
// Autogenerated macro, single images only!
// G. Landini 30/Aug/2011.
//

min=newArray(3);
max=newArray(3);
filter=newArray(3);
a=getTitle();
run("HSB Stack");
run("Convert Stack to Images");
selectWindow("Hue");
rename("0");
selectWindow("Saturation");
rename("1");
selectWindow("Brightness");
rename("2");
// setting of the colour threshold values [0,1,2]
min[0]=40;
max[0]=125;
filter[0]="pass";
min[1]=78;
max[1]=255;
filter[1]="pass";
min[2]=0;
max[2]=255;
filter[2]="pass";
for (i=0;i<3;i++){
selectWindow(""+i);
setThreshold(min[i], max[i]);
run("Convert to Mask");
if (filter[i]=="stop") run("Invert");
}
imageCalculator("AND create", "0","1");
imageCalculator("AND create", "Result of 0","2");
for (i=0;i<3;i++){
selectWindow(""+i);
close();
```

```
}
selectWindow("Result of 0");
close();
selectWindow("Result of Result of 0");
rename(a);
// Threshold Colour ----------

run("8-bit");
run("Create Selection");
run("Set Measurements...", "area limit display redirect=None decimal=3");
// setting the distance (ratio between bottom diameter [pixel]and [cm]of Erlenmeyer flask)
run("Set Scale...", "distance=872known=9 pixel=1 unit=cm");
run("Measure");
// directory to save the resulting images and the measurement results
saveAs("Results", "...\\Result.xls");

selectWindow(imgName);

run("Restore Selection");
setBackgroundColor(255, 255, 255);
run("Make Inverse");
run("Clear", "slice");
```

Curriculum Vitae

PERSONAL INFORMATION

Andreas SCHARMÜLLER BSc.



Mühlwang 14, 4690 Schwanenstadt, Austria

0043 660 7319935

andschar@gmail.com

Sex Masculin | Date of birth 16 Feb 88 | Nationality Austria

EDUCATION

Sep 2014 – Sep 2015
Master: Ecologie et Biosciences de l'Environnement (EBEN)
École Nationale Supérieur Agronomique de Toulouse (ENSAT), France

Nov 2012 – Mar 2016
Master: Applied Limnology
University of Natural Resources and Life Science (BOKU), Vienna

Sep 2008 – Nov 2012
Bachelor: Landscape planning and -architecture
University of Natural Resources and Life Science (BOKU), Vienna

2007
A-levels

WORK EXPERIENCE

1 Feb 16 – 20 Feb 16
Research Assistant
University of Natural Resources and Life Sciences (BOKU), Vienna (Austria)

Carbonate analysis of sediment samples

2 Feb 15 – 31 Jul 15
Internship: Ecotoxicology
Laboratoire écologie fonctionnelle et environnement (ECOLAB), Toulouse (France)

“Intraspecific variability in aquatic plant response to chemical contaminants” Laboratory experiments in order to study the effects of atrazine and copper on two aquatic macrophytes.

30 Apr 14 – 8 Mai 14
Student tutor: Hydrobiology
University of Natural Resources and Life Sciences (BOKU), Vienna (Austria)

Teaching and guiding students through a 9 days field course

1 Oct 13 – 31 Jan 14
1 Oct 12 – 31 Jan 13
Student tutor: GIS
University of Natural Resources and Life Sciences (BOKU), Vienna (Austria)

Assisting a GIS-course, hold weekly tutorials

1 Oct 12 – 31 Jan 13
1 Oct 11 – 31 Jan 12

Student tutor: Plant Anatomy

University of Natural Resources and Life Sciences (BOKU), Vienna (Austria)

Assisting in a plant anatomy course

1 Mar 12 – 30 Jun 12

Student tutor: Dendrology

University of Natural Resources and Life Sciences (BOKU), Vienna (Austria)

4 Jul 11 – 23 Sep 11

Internship: Landscape Ecology

National park: Hohe Tauern, Matrei i. O. (Austria)

2 Nov 07 – 31 Jul 08

Civilian service

Alternative civilian service at Lebenshilfe Voecklabruck in lieu of conscription (Austria)
Working with people with disabilities, children and the elderly

PERSONAL SKILLS

Mother tongue: German

Other languages: English - fluent
French - advanced

Computer and technical skills:

GIS

Statistics: R, SPSS

Databases: Ms Access, SQL, WRDB (Water Resource Database)

Image analysis: ImageJ

Notions in Programming: Python, R, Macro scripting

CAD

Analytical techniques: ICP-OES

Photosynthesis measurements

Other skills:

Diving-Licence

Mountaineering, Ski-Instructor

Guitar

Driving Licence: A, B