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Faculteit Industriële ingenieurswetenschappen

master in de industriële wetenschappen: biochemie

Masterthesis

Uranium Toxicity to the Freshwater Plant *Lemna minor* in an Environmentally Relevant Metal Mixture: Selection and Validation of Potential Uranium-Biomarker Genes

PROMOTOR :

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Scriptie ingediend tot het behalen van de graad van master in de industriële wetenschappen: biochemie

Gezamenlijke opleiding UHasselt en KU Leuven

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UHASSELT

KU LEUVEN

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ABBREVIATIONS

%GC	percentage of guanine and cytosine
$\%I_r$	percentage growth inhibition
μ	average specific growth rate
A. thaliana	*Arabidopsis thaliana*
BIS	Biosphere Impact Studies
Ca	calcium
Cd	cadmium
cDNA	complementary DNA or copy DNA
Cu	copper
DNA	deoxyribonucleic acid
EC_X	effect concentration where the growth is inhibited with X%
FW	fresh weight
L. minor	*Lemna minor*
M0	no addition of metals
M1	metal concentration as it occurs in the Beaverlodge Lake (0.0157 μM Cu, 0.017 μM Ni, 0.0765 μM Zn and 0.0145 μM Pb)
M10	tenfold metal concentration of M1
M100	hundredfold metal concentration of M1
M1000	thousandfold metal concentration of M1
MES	2-(*N*-morpholino)ethanesulfonic acid
Mg	magnesium
MMS	methyl methanesulfonate
mRNA	messenger RNA
Na	sodium
NCBI	National Center for Biotechnology Information
Ni	nickel
NMPA	Normal Melting Point Agarose
OECD	Organisation for Economic Co-operation and Development
Pb	lead
PBS	Phosphate Buffer Solution
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
SE	standard error
Sr	strontium
t_d	doubling time
U	uranium
U0	condition with 0 μM U
U10	condition with 10 μM U
U2	condition with 2 μM U
Zn	zinc
β	beta
γ	gamma

ABSTRACT

Historical uranium (U) mining operations resulted in the contamination of large areas, including surface waters. The effects of U on the freshwater plant *Lemna minor* have been investigated, although they were mainly studied on individual and cellular level. Sequencing of the *L. minor* genome and transcriptome gives possibilities to study the effects on molecular level. The aim of this study is to select and validate potential U-biomarker genes that can possibly predict the effects of U exposure on population level as well as on ecosystem level. Since organisms growing in contaminated waters are not only exposed to U, but also to multiple co-contaminants, it is necessary to find biomarkers that react specifically to U and not to the co-contaminants.

L. minor plants were exposed to 0, 2 or 10 µM U in combination with an environmentally relevant metal mixture consisting of Cu, Ni, Zn and Pb. Plants were exposed during 1, 4 or 7 days. Expression levels of 10 primers for potential U-biomarkers genes were tested using real-time qPCR. A comet assay on *L. minor* was optimized as a second potential biomarker for DNA damage.

The earlier discovered primer 33 could be validated as biomarker for U exposure. In addition, based on gene expression analysis two new U-biomarkers were selected within this project, i.e. primer 48 and primer 52, since their related genes reacted specifically to U already after 4 days of exposure and to the lowest tested U concentration (2 µM). However, further investigations and validation is necessary.

ABSTRACT IN DUTCH

Historische ontginning van uranium (U) heeft geleid tot contaminatie van grote gebieden, zoals oppervlaktewaters. Effecten van U op de zoetwaterplant *Lemna minor* werden voornamelijk op individueel en cellulair niveau bestudeerd. Door de recente sequenering van het *L. minor* genoom en transcriptoom kunnen de effecten nu ook op moleculair niveau bestudeerd worden. Het doel van deze studie is om potentiële U-biomerker genen te selecteren en te valideren, die mogelijk de effecten van U blootstelling op populatie- en ecosysteemniveau kunnen voorspellen. Omdat organismen in verontreinigde wateren niet alleen zijn blootgesteld aan U maar ook aan meerdere co-contaminanten, is het noodzakelijk om biomerkers te vinden die specifiek op U reageren en niet op deze co-contaminanten.

L. minor planten werden blootgesteld aan 0, 2 of 10 µM U in combinatie met een relevant metaalmengsel bestaande uit Cu, Ni, Zn en Pb. Planten werden blootgesteld gedurende 1, 4 of 7 dagen. Expressieniveaus van 10 primers voor potentiële U-biomerker genen werden getest m.b.v. real-time qPCR. Een comet assay op *L. minor* werd geoptimaliseerd als tweede potentiële biomerker.

De eerder ontdekte primer 33 werd gevalideerd als biomerker voor U blootstelling. Daarnaast werden ook twee nieuwe U-biomerker genen geselecteerd in dit project op basis van genexpressieanalyse, nl. primer 48 en primer 52. Dit omdat hun verwante genen specifiek reageerden op U al na 4 dagen blootstelling en op de laagste geteste U concentratie (2 µM). Meer onderzoek en validatie is echter nodig.

1 INTRODUCTION

The SCK•CEN is the Belgian Nuclear Research Centre that was established in 1952. One of its goals is to perform research in the nuclear field in order to improve the protection of humans and the environment against ionizing radiation, radionuclides and heavy metals [1].

This thesis is performed in the institute Environment, Health and Safety, and more specifically in the research group Biosphere Impact Studies (BIS). This group has three interlinked research lines. First, there is the understanding of processes and mechanisms for transfer of radionuclides in the biosphere. Process-based models are developed to assess radionuclide dispersion or cycling within different environmental scenarios. Secondly, the group studies the mechanisms behind effects induced in plants following exposure to radiation or radionuclides. Finally, the group develops, improves and applies models for assessing radiological impact on man and environment. This thesis is a part of the second research line. Potential uranium-biomarker genes are selected and validated for the uranium (U) toxicity to *Lemna minor* (*L. minor*), a freshwater plant, in an environmentally relevant metal mixture consisting of copper (Cu), nickel (Ni), zinc (Zn) and lead (Pb).

In the past, several studies have been accomplished with *L. minor* and exposure to radiation/radionuclides at SCK•CEN: 1) uptake and effects of U on *L. minor* in combination with different concentrations calcium (Ca), magnesium (Mg), sodium (Na) and pH [2]; 2) U-cadmium (Cd) mixture experiments on *L. minor*: uptake and growth [3], [4]; 3) recovery of *L. minor* after exposure to gamma (γ) radiation, U or Cd [5]; 4) long-term low dose radiation experiments [6] and 5) effects of γ, strontium (Sr) or U on *L. minor* and ribonucleic acid (RNA)-sequencing [7], [8], [9].

Previously, the effects on *L. minor* plants were only studied on individual and cellular level. The growth inhibition was measured whereby the number and the surface of the leaves, called fronds, were determined. The sequencing and assembly of the *L. minor* genome and the RNA-sequencing of the whole *L. minor* transcriptome by Van Hoeck et al. in 2015 [7] gives new opportunities to study molecular changes of exposed *L. minor* plants. The availability of the transcriptome opens new opportunities for the discovery of biomarkers that can possibly predict the effects of exposure on population level as well as on ecosystem level. In addition, previous experiments mainly studied the effects of single element toxicity on *L. minor*, but it is known that heavy metals can interact with each other. Metals can act independently or have additive, synergistic or antagonistic effects [10]. Therefore, there is an increasing need to study these combined effects. The environmentally relevant metal mixture is based on concentrations that were measured in the Beaverlodge Lake in Canada [11]. As a result of the established U mines local surface waters were contaminated with U and some co-contaminants. The most important co-contaminants (Cu, Ni, Zn and Pb) were selected and used in this research. Prior to this project, in one study the single dose response curves of the different metals of the Beaverlodge Lake were investigated, and a first metal mixture experiment was carried out leading to the discovery of a potential U-biomarker gene (primer 33) [12]. This gene needs to be further investigated and validated by new experiments.

The aim of this research is to validate the potential U-biomarker gene (primer 33) and to select and validate other potential U-biomarker genes for the freshwater plant *L. minor* when they are exposed to U in combination with an environmentally relevant metal mixture. The selection is done based on the available RNA-sequencing data [7]. The first goal is to find genes that specifically react to U and genes that are up- or downregulated,

but only when U is present in the mixture. These genes can then in the future be used as potential U-biomarkers. This will be done by determining the effects on the gene expression levels of selected genes in a 7 days exposure experiment of *L. minor* plants to U in combination with the co-contaminants. The second goal is to find fast-reacting biomarkers in a time kinetics experiment. Hereby the gene expression of the selected genes is followed in function of time by harvesting plants after 1, 4 and 7 days of exposure.

Besides changes at gene expression level, deoxyribonucleic acid (DNA) damage can possibly be used as biomarker for U exposure. Therefore, a comet assay on *L. minor* plants is performed. Since it is the first time that this technique will be performed on *L. minor* within the BIS group, the aim is to test the feasibility of this technique on *L. minor* plants and to optimize some of the parameters. The optimization of the comet assay is performed with plants exposed to methyl methanesulfonate (MMS, i.e. an inducer of DNA damage).

2 ENVIRONMENTAL POLLUTION

All living organisms are exposed to environmental pollution coming from, for example, disposal of industrial effluents, modern agricultural practices, mining and milling… [13]. This can not only affect the human population, but also animals and plants resulting in an increasing worldwide health concern [14]. Heavy metals are important contributors to the pollution. Although they are mostly naturally occurring elements, when they are released in high concentrations changes on cellular and individual level can occur since they are non-biodegradable, potentially toxic to biota and can bio-accumulate [15], [16]. In addition, also radionuclides are an important source of contamination. Radionuclides can be naturally occurring (e.g. U-238, Co-60) or artificially produced (e.g. Cs-137), but both are present in all ecosystems by nature, accident or human activities [17]. In addition historical U mining operations resulted in the contamination of large areas, including surface waters. Therefore there is a need to study the effects of U and heavy metals on plants to preclude major problems to the environment. The selection of potential biomarkers can help us with the prediction of these effects.

2.1 Uranium

Uranium is a naturally occurring radionuclide as well as a heavy metal that is present in aquatic and terrestrial environments. The average concentration of dissolved U in freshwater is 0.03-2.1 $\mu g.L^{-1}$ [18], [19], [20]. Due to anthropogenic activities such as U mining and milling, processing of U-containing ores and processing of U as fuel for nuclear reactors, high concentrations of U can be found that can reach up to 0.45 $mg.L^{-1}$ which lead to increased toxicological and ecotoxicological concerns [11], [20], [21].

Natural U consist of three isotopes: U-238 (99.27%), U-235 (0.72%) and U-234 (0.0055%) [22], [23]. All of these U isotopes have different radioactive properties, but the chemical properties are the same. The radioactivity depends on the half-life, which is given in Table 1 along with the relative mass and specific activity of the three U isotopes [21], [24], [25]. U is characterized with a higher chemical toxicity than radiotoxicity because the decay half-life is very long [21], [23], [24], [26].

Table 1: Characteristics of uranium isotopes in natural uranium [24, p. 95]

Isotope	Half-life (years)	Relative mass (%)	Specific activity ($Bq.g^{-1}$)
^{238}U	$4.47x10^9$	99.3	12455
^{235}U	$7.04x10^8$	0.72	80,011
^{234}U	$2.46x10^5$	0.0055	$231x10^6$

The chemical toxicity depends on the U species which can be divided into three predominant species: uranyl cation (UO_2^{2+}), uranyl carbonates (UO_2CO_3, $UO_2[CO_3]_2^-$), and uranyl hydroxides (UO_2OH^+, $[UO_2]_3[OH]_7^-$) [18], [27]. The U speciation is controlled by the pH value, redox potential, ionic strength, and the presence of complexing agents [18], [20], [21]. The uranyl cation (UO_2^{2+}) is the most common form in the environment and it can form complexes with carbonate, phosphate or sulfate ions [23], [24]. It is suggested that U toxicity is mainly caused by UO_2^{2+} due to a number of factors: 1) UO_2^{2+} can replace Ca and Mg ions, which results in structural changes in cell membranes, enzyme inactivation, and damage to RNA and DNA; 2) it can interact with phosphate groups, DNA and it can also induce membrane damage resulting in more permeable membranes; 3) it

can interact with carboxylic groups; 4) it can bind to DNA causing mutations in cells [18], [27], [28].

U is, along with other heavy metals and radiation, a genotoxic agent that changes the balance in chromatin metabolism which results in loss of genomic stability and lower rate of cell division. Genotoxicity can be described as the possibility to destructively effect the genetic material (DNA or RNA) of a cell which can lead to mutations [29]. U can induce DNA damage directly or indirectly by the production of free radicals or reactive oxygen species or by metabolic activation [29], [30], [31], [32]. DNA damage can occur as single-strand breaks, double-strand breaks, alkali-labile sites, incomplete excision repair sites, DNA crosslinks… depending on the concentration or oxidation state of the metal or the exposure time [29], [33], [34]. Studies with the zebrafish *Danio rerio* proved that U is genotoxic and can induce oxidative stress [35], [36]. U also affects the expression of genes of e.g. the detoxification and DNA repair [28].

Humans are exposed to U through food, water and air and the daily intake is approximately 0.037 $\mu g.kg^{-1}$ of body weight [37], [38]. In general the effects of U in humans are mainly caused by the chemical toxicity (chemotoxic) [38], [39]. U can enter the body in different ways and has also multiple targets. There are targets with a low risk e.g. lymph nodes, digestive tract, muscles, nervous tissue, liver, spleen and blood; medium risk e.g. lungs and gonads and high risk e.g. bones and kidneys. The kidneys have the most chemotoxic risk whereby U binds on biological molecules [40].

Plants are easily exposed to high concentrations of U [41], [42]. Previous experiments with plants showed that U has important effects on growth, nutrient uptake and oxidative stress related responses in plants [43], [44], [45], [46]. But the mechanisms for the uptake and tolerance of U have not yet been fully understood [47]. However experiments with *Arabidopsis thaliana* (*A. thaliana*) have shown that the concentration of U is higher in roots than in shoots due to the adsorption on the cell wall reducing the root-to-shoot translocation [18], [44], [47], [48]. It is not clear yet if experiments with aquatic plants such as *L. minor* give the same results, as they are exposed differently to U (see paragraph 3.1) [49].

2.2 Environmentally relevant metal mixture

In Uranium City, located in northern Saskatchewan Canada, historical U mining operations produced a total of 25939 tonnes of U_3O_8 between 1953 and 1982. This resulted in the contamination of local surface waters like lakes and streams, including the Beaverlodge Lake. Organisms in this area are not only exposed to U, but also multiple co-contaminants (Table 2). This results in a need to determine effects from contaminants in real environmental conditions, including metal mixtures [11], [50], [51]. The co-contaminants of interest from the Beaverlodge Lake are Cu, Ni, Zn and Pb. They are released by natural or anthropogenic activities and can be assimilated by aquatic organisms via different routes possibly leading to toxic effects [52], [53], [54], [55].

Table 2: Trace metal, metalloid, uranium and thorium concentrations in the Beaverlodge Lake [11, p. 102]

Contaminants	Concentration ($\mu g.dm^{-3}$)
As	1.8
Cu	1
Mn	nm
Fe	65
Ni	1
Zn	5
Se	4.8
Mo	nm
Ba	0.56
Pb	3
U	483.6
Th	79000

*nm = not measured

Cu is an essential micronutrient for all organisms, including plants, and is involved in various physiological processes such as photosynthesis, respiration, cell wall metabolism and hormone signaling [54], [56], [57]. In addition, it is a cofactor in various enzymes [57], [58]. However, an excess of Cu can lead to toxic effects e.g. by forming free radicals which can cause oxidative stress [52]. Cu in freshwater is normally found in concentrations of 0.44 $\mu g.L^{-1}$, but due to anthropogenic activities high concentrations of Cu were found, which are not only toxic to plants but also to human and animals. The concentration in the plants depends not only on the plant species, but also on the growth stage [52], [56], [57], [59].

Ni is found at concentrations from 1-3 $\mu g.L^{-1}$ in freshwaters and can reach up to 10-15 $\mu g.L^{-1}$ in polluted areas [54], [60], [61]. It is an essential micronutrient for plants, e.g. it is an important element of the enzyme urease and thus essential for plants which produce this enzyme [58]. Since Ni is a micronutrient, a Ni deficiency is rare but an excess of Ni is more common leading to toxic effects. Depending on the plant species, growth stage, cultivation conditions, Ni concentration and exposure time, different effects are ascertained [60]. For example, Ni can cause inhibition of growth, chlorosis and necrosis in plants [62].

Zn is a non-ferrous metal that in freshwaters is found in concentrations of 2.8 $\mu g.L^{-1}$ [63]. It is an essential micronutrient for all biota [53], [64]. It has an important role in the physiology of the cell and for the structure of proteins [54]. It is also necessary for enzymes that have a role in carbohydrate and protein synthesis, gene regulation, structure and integrity of biomembranes and protection of cells from damage due to free radicals [58]. It is also known that Zn reduces metal toxicity (e.g. Cu and Cd) which improves the plant growth [65]. But toxic Zn concentrations no longer have the protective function and can cause ecotoxicological effects on plants such as reduced biomass, leaf chlorosis and root growth inhibition [64], [65], [66].

Pb is a toxic element that is naturally present in small quantities in the earth's crust. Anthropogenic activities increase the concentration of Pb in the environment, which results in elevated Pb concentrations in surface and groundwater's [67], [68], [69]. In freshwater the concentration of Pb can reach up to 0.18-1 $\mu g.L^{-1}$ [70]. Pb occurs as inorganic Pb(II)

and is one of the few metals that has no biological function [69], [71], [72]. It is known that Pb(II) can disrupt biological systems by modifying molecular interactions, cell signaling and cellular function [68], [71]. Plants mainly absorb Pb(II) in their roots, translocation to the shoots is not very common. Pb(II) can accumulate and critical levels will affect the plant itself by a decrease of biomass or will eventually kill it [62], [73], [74].

Although the toxic effects of the single metals have been investigated before, the environment is contaminated with more than one heavy metal. In addition, the concentration of the metals is not constant. When the concentration rises above the tolerated concentration, toxicity effects appears and changes can be induced [54], [56], [75]. This is not caused by the individual metals itself, but by those mixtures of metals that can have joint effects. The observed effects with co-contaminants can diverge from the results of single-stressor experiments. Metals can have additive, synergistic or antagonistic effects [76] or act independently, depending on the concentration, temperature, pH and light intensity [10]. For example, Vellinger et al. (2012) [77] found an antagonistic metal interaction of arsenate and Cd on *Gammarus pulex*. Also Charles et al. (2006) [78] found an antagonistic effect, but between U and Cu in *Lemna aequinoctialis*. The metal effects are also dependent on the relationship of the metals to other mineral elements. Therefore more research is needed to study the joint effects of the metal mixtures [75], [79], [80].

2.3 *Lemna minor*

The freshwater plant used in this study is *L. minor* (Figure 1). It is an aquatic plant that has an important role in aquatic ecosystems. It is a free-floating freshwater macrophyte that belongs to the subfamily of duckweeds (*Lemnaceae*). It is a relatively small vascular plant from a few mm up to 1 cm [21], [81]. These plants consists of one, two or three leaves, called fronds, which have a single root. The roots are approximately 1-2 cm long, whereas the fronds are oval shaped with a length of 0.6-5 mm thick, light green in color with three nerves and a small air pocket to advance floating. For their growth the conditions should be as ideal as possible: high level of nutrients, pH between 5 and 9 (optimum 6.5-7.5), and temperature between 6 and 33°C [81]. They have a high growth rate via asexual reproduction. On average, one adult plant splits into two new plants every 2.5 days. This results in identical clones [7], [81], [82]. As a result of the genetically identical clones potential effects due to genetic variability can be eliminated [7].

Plants are essential to a healthy ecosystem. The duckweed species have an ecological significance as the primary producers of food for waterfowl, fish and small organisms, and oxygen [7], [82]. *L. minor* plants are sensitive towards different kinds of stress (e.g. heavy metals) and are able to absorb and accumulate high amounts of it in their biomass [52], [56], [82], [83]. Due to their easy growth in controlled and sterile laboratory conditions *L. minor* is often used as a model test organism for environmental studies and fundamental plant research [7], [82]. The Organisation for Economic Co-operation and Development (OECD) describes a growth inhibition test for *L. minor* (guideline 221) to test the toxicity of pollutants in existing freshwaters [21], [52], [56], [84]. Toxicity of compounds is expressed in EC_X, the effect concentration where the growth is inhibited with X% [81].

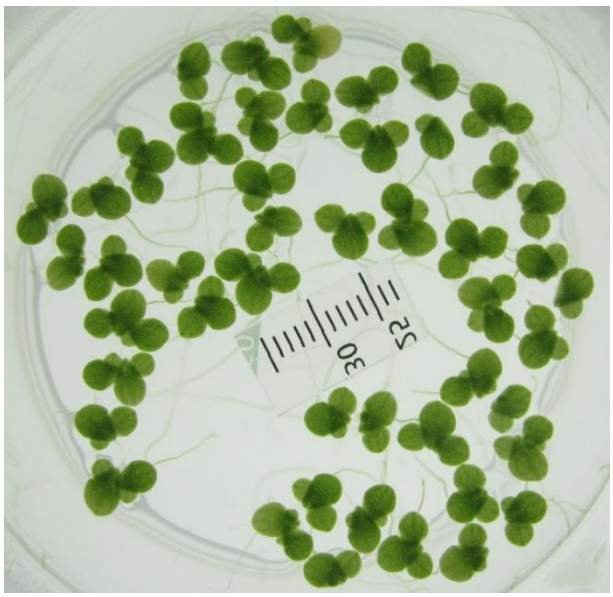


Figure 1: L. minor plants with floating ruler of 10 mm

2.4 Biomarkers

The interest for biomarkers is still growing to determine the toxic effects on the environment. They can be used as early warning systems for organisms that are exposed to single or complex mixtures of chemicals resulting in a changed biological response [81], [85], [86]. There are two different types: biomarkers of exposure and biomarkers of effect. Biomarkers of exposure are used as functional measure of exposure to toxicants, whereby the expression is on a suborganism level. Biomarkers of effect, also called bioindicators, describes changes in biochemical, physiological or ecological parameters that are a consequence of exposure to a toxicant [87], [88], [89], [90]. Terrestrial and aquatic plants can be early warning systems to measure the toxicity in natural freshwaters. When they are exposed to toxicants, the first effects are perceived at molecular and biochemical level and afterwards on level of the organism [81], [85], [87].

There are multiple potential biomarkers of exposure of which gene expression is one of them. Changes in gene expression give the first effects of exposure and are therefore highly sensitive information [16], [91], [92]. It can be used to study the mechanisms behind exposure and form a 'genetic signature' of the specific exposure pattern. Exposed plants can up- or downregulate specific genes dependent on the exposure [87]. The most commonly used techniques for gene expression analysis are RNA sequencing, Northern blot, microarray analysis and polymerase chain reaction (PCR). This last technique will be used in this project. Multiple genes at messenger RNA (mRNA) level can be simultaneously studied and potential molecular mechanisms and biomarkers can be identified. But there are also certain disadvantages such as variation of genes due to intern variability (physiologic state, cell types, environmental conditions…) [87], [92].

Another potential biomarker of exposure is DNA damage. As described above, genotoxic agents such as heavy metals or radiation can cause DNA damage. The genotoxicity of single genotoxic agents and mixtures can be evaluated by single-cell gel electrophoresis, also called "comet assay", whereby the effects of genotoxic agents are studied on individual cells [93]. Cells are embedded in agarose resulting in immobilized DNA. Subsequently an electrophoresis is performed whereby the DNA is migrated and the nuclei are fixated and afterwards indicated with a fluorescent staining [86]. In the past this technique was mainly

used with animal cells, but the use on plants gives new opportunities in environmental studies [93], [94], although using plants has an important disadvantage compared to the use of animal cells. The cellulose cell wall forms a barrier to prevent the release of DNA. By chopping the plant tissues with a razor blade, more optimal conditions are created for the release of the DNA [95]. The neutral version of the comet assay was first tested on plants, whereby the electrophoresis is performed under neutral conditions. However, the sensitivity decreased with the neutral version [95]. Thereby the alkaline version was created on *Vicia faba* to quantitatively measures DNA damage [96], [97], [98]. Hereby the treatment and electrophoresis is fulfilled under alkaline conditions which results in better visible comet tails and higher sensitivity [95]. This alkaline version of the comet assay has also some other advantages such as short analysis time and it is an inexpensive, non-specific method [31]. But, as described above, due to the plants cell wall and the absence of free cells difficulties arise with performing the comet assay on plants. Also the comet assay is known to be a technique that is difficult to standardize, which results in a lower reliability [96].

3 METHODS AND MATERIALS

In this chapter the methods and materials are given starting with the *L. minor* growth and exposure to U and the metal mixture. Subsequently the calculation of the specific growth rate and growth inhibition, the primer development and optimization and the measurements at molecular level are described. There are two types of measurements on molecular level: gene expression and the comet assay. At last the used statistical analysis is given.

3.1 *L. minor* growth and exposure to uranium and metal mixture

L. minor plants were cultivated in sterile Erlenmeyer flasks of 250 ml that consists of 100 ml sterile growth medium, also called Hutner's medium, and were closed with hydrophobic cotton and aluminum foil. The composition of the Hutner's medium is given in Table 3. Every 10 to 12 days the stock cultures were transferred to new Erlenmeyer flasks to maintain the plant culture. Therefore, three *L. minor* plants with 3 or 4 fronds were transferred with a sterile inoculation loop to a new sterile Erlenmeyer flask. All operations were executed in sterile conditions. According to the OECD guidelines the stock culture needs to be grown at 24°C with continuous light. But due to growth problems of the plants, they were grown under a day and night program (14h/10h) at respectively 24/18°C with a light intensity of ± 220 $\mu mol.s^{-1}.m^{-2}$. In addition, non-sterile L. minor plants were used. All the subsequent proceedings were performed under sterile conditions as described earlier.

Table 3: Final composition of Hutner's medium and P05-medium

	Hutner's medium ($mg.L^{-1}$)	**P05-medium ($mg.L^{-1}$)**
Macronutrients		
KNO_3	0.3	888.8
$Ca(NO_3)_2.4H_2O$	0.72	944
$MgSO_4.7H_2O$	0.0738	500
KH_2PO_4	0.0414	503.2
Na-EDTA	0.0029	9
Ferric acid	0.001	/
Tartaric acid	/	3
Micronutrients		
H_3BO_3	0.001	1.860
$ZnSO_4.7H_2O$	0.001	0.220
$Na_2MoO_4.2H_2O$	0.0001	0.120
$CuSO_4.5H_2O$	0.00003	0.080
$MnCl_2.4H_2O$	/	3.620
$FeCl_3.6H_2O$	/	5.400
$MnSO_4.H_2O$	0.0001	/

In total two experiments were performed which followed the standard 7-day growth inhibition test of the OECD using *L. minor*, with some modifications as described by Horemans et al. [99] and the alterations as described above. Six days before the experiments, a preculture of five *L. minor* plants with 3 or 4 fronds was set up in a sterile Erlenmeyer flasks of 250 ml that consists of 100 ml Hutner's medium.

The first experiment was performed with *L. minor* exposed in quadruplicate for 7 days to a metal mixture in combination with U. Five metal concentrations were used: M0, M1, M10, M100 and M1000. The metal concentration M1 is the concentration as it occurs in nature (0.0157 µM Cu, 0.017 µM Ni, 0.0765 µM Zn and 0.0145 µM Pb). M10, M100 and M1000 are respectively a tenfold, hundredfold and thousandfold concentrations and M0 is no addition of those metals. For U three concentrations were selected: 0, 2 and 10 µM, whereby 2 µM is the concentration occurring in the Beaverlodge Lake.

For the contamination a M10000 stock solution of Cu, Ni, Zn and Pb was prepared from separated 100 mM stock solutions of the metals ($CuSO_4.5H_2O$ (Sigma-Aldrich), $NiSO_4.6H_2O$ (Thermo Fisher Scientific), $ZnSO_4.7H_2O$ (Honeywell Fluka) and $Pb(NO_3)_2$ (Sigma-Aldrich)) and filter-sterilized with a 0.2 µm HT Tuffryn Membrane (Pall Corporation). An U stock solution of 10 mM was prepared from $UO_2(NO_3)_2.6H_2O$ (SPI-chem) and filter-sterilized with a 0.2 µm HT Tuffryn Membrane (Pall Corporation). During the exposure, P05-medium (Table 3) was used that is low in phosphate concentration to limit U precipitation [99]. Sterile 250 ml polycarbonate pots were filled with the contamination solution consisting of 0, 0.02 or 0.1 ml U stock solution and 0, 0.01, 0.1, 1 or 10 ml M10000 stock solution depending on the condition and afterwards filled with P05-medium until a total volume of 100 ml (exact composition of each condition is given in Appendix A). At each condition 1 ml of a filter-sterilized (0.2 µm HT Tuffryn Membrane (Pall Corporation)) 0.5 M 2-(*N*-morpholino)ethanesulfonic acid (MES) (Sigma-Aldrich) buffer was added and the pH of each solution was adjusted to 5 with filter-sterilized (0.2 µm HT Tuffryn Membrane (Pall Corporation)) 1 M HCl (VWR) or 1 M NaOH (Merck) to create the same experimental conditions. All *L. minor* plants of the preculture were transferred into a large polycarbonate pot of 1 L and afterwards three plants with 3 or 4 fronds were selected and transferred into the 250 ml polycarbonate pots filled with the P05-medium, U and/or metal contamination solution and MES buffer. Also a piece of sterile, floating ruler was added in each pot for later measurements. Pictures were taken at day 0, 4 and 7 for determinations of the number and surface of fronds (paragraph 3.2). After 7 days, plants were harvested, put onto an absorbent paper to remove any present medium and fresh weight (FW) was determined. Samples from 20-50 mg were snap frozen in liquid nitrogen for RNA extractions.

The second experiment was a time kinetics experiment whereby *L. minor* plants were exposed to three concentrations of the metal mixture (M0, M1, M100) in combination with the three U concentrations (0, 2 and 10 µM). The same experimental set-up as described above was used. Pictures were taken at day 0, 1, 4 and 7 for determinations of the number and surface of fronds (paragraph 3.2). After 1, 4 and 7 days, plants were harvested, FW was determined and samples of 20-50 mg for RNA extractions were frozen in liquid nitrogen to see how the gene expression of the possible biomarkers changes over time. The *L. minor* plants for day 1 of the harvest were exposed in sevenfold, whereas the plants for day 4 and 7 of the harvest were exposed in quadruplicate.

3.2 Specific growth rate and growth inhibition

Three measurements on cellular level were performed: FW (see paragraph 3.1), number of fronds and surface of fronds.

For both experiments pictures were taken to determine the number and surface of fronds at day 0, 4 and 7 (for the 7 days exposure experiment) and at day 0, 1, 4 and 7 (for the time kinetics experiment). The pictures were analyzed using the open source image processing program ImageJ. The total area was determined and the number of fronds was

also counted. The FW of the plants on each time point could be calculated based on the ratio FW-total area on day 0. The total area, FW or number of fronds was used to determine the average specific growth rate (μ) with the following formula [84]:

$$\mu_{i-j} = \frac{\ln(N_j) - \ln(N_i)}{t_j - t_i} \quad (1)$$

with μ_{i-j} as the average specific growth rate from time i to j, N_i the number of fronds observed, frond area or FW at time i, N_j the number of fronds observed, frond area or FW at time j, t_i the time point for the start of the period and t_j the time point for the end of the period.

With the average specific growth rate of the control and the contaminated conditions, the percentage of growth inhibition ($\%I_r$) could be calculated as [84]:

$$\%I_r = \frac{(\mu_C - \mu_T)}{\mu_C} . 100 \quad (2)$$

with μ_C the mean value for μ in the control condition and μ_T the mean value for μ in the contaminated condition.

Also the doubling time (t_d) was measured for the validation of the test. The t_d of frond number in the control condition must be less than 2.5 days (or the average specific growth rate must be higher than 0.275 d^{-1}) and could be calculated form the average specific growth rate with the following formula [84]:

$$t_d = \frac{ln(2)}{\mu} \quad (3)$$

The FW, average specific growth rate (μ) and percentage growth inhibition ($\%I_r$) were used as endpoints to compare the various conditions.

3.3 Primer development and optimization

Van Hoeck et al. (2015) [7] already studied the gene expression levels of *L. minor* plants exposed to U, beta (β) and γ radiation. This expression data was used to find potential U-biomarker genes that react specifically to U. Genes were selected based on their changed expression level for U with no changes after exposure to β and γ radiation. Genes with an increasing or decreasing fold change in function of increasing U concentration were selected for potential up- or downregulated U-biomarker genes. The mRNA of the selected genes along with the whole *L. minor* transcriptome was used for primer development with the primer-BLAST tool of National Center for Biotechnology Information (NCBI). Preprogrammed settings of NCBI were used, only the PCR product size was changed to 70-150 bp, maximum melt temperature difference was set on 2°C and targets were ignored that had four or more mismatches to the primer. Subsequently the properties and the possibility to form self-dimers of the potential primers were tested with the Oligo Analysis Tool (Eurofins Genomics). Only primers with a percentage of guanine and cytosine (%GC) between 50 and 60% and a melting temperature around 60°C were selected. The maximum annealing score, the number of overlay between two identical primers, should be maximum 14 for a potential good primer. Finally, a PCR check was executed with the Oligo Analysis Tool (Eurofins Genomics) to see if the forward and reverse primer can form cross-dimers during the PCR reaction. The formation of cross-dimers was the result of a homologous forward and reverse primer. If all the requirements were approved the primers

were ordered and further tested and validated within this project (paragraph 3.4.1). The developed primer pairs were given a number. This number (i.e. Primer "X") was used to describe the potential U-biomarker genes where the primers were linked to (see Table 4). Due to confidentiality of this thesis no primer sequences and gene numbers are given.

In addition, new housekeeping genes to improve the normalization of the gene expression data (paragraph 3.4.1) were selected from the RNA sequencing data [7]. Hereby genes with a constant fold change in function of all exposure conditions were selected. This mRNA of the new potential housekeeping genes along with the whole *L. minor* transcriptome was used for primer development as described above. The primers for the housekeeping genes are given in Table 4.

Table 4: Overview of the primers for the potential U-biomarker genes and the new housekeeping genes (used for the normalization of the data) with their related function

Primer number	Function
U-BIOMARKER GENES	
Primer 33	CYP76C2 Cytochrome P450 76C2
Primer 41	Negative regulation of growth
Primer 42	Negative regulation of growth
Primer 43	Negative regulation of growth
Primer 44	Negative regulation of growth
Primer 45	Response to oxidative stress
Primer 46	Response to oxidative stress
Primer 47	Response to oxidative stress
Primer 48	Response to oxidative stress
Primer 49	Metal ion transport
Primer 50	Metal ion transport
Primer 51	Response to auxin
Primer 52	Response to auxin
NEW HOUSEKEEPING GENES	
Primer 53	PAL
Primer 54	PAL
Primer 55	Cytochrome
Primer 56	Cytochrome
Primer 57	Fatty acid biosynthetic process
Primer 58	Fatty acid biosynthetic process
Primer 59	Flavin adenine dinucleotide binding
Primer 60	Flavin adenine dinucleotide binding
Primer 61	Flavin adenine dinucleotide binding
Primer 62	Flavin adenine dinucleotide binding
Primer 63	Ubiquitin protein ligase binding
Primer 64	Ubiquitin protein ligase binding
Primer 65	Cytochrome
Primer 66	Cytochrome
Primer 67	Photoreceptor
Primer 68	Photoreceptor

3.4 Measurements on molecular level

Two types of measurements on molecular level were performed in this thesis. First, the gene expression was analyzed whereby RNA was extracted from plant samples of the 7 days exposure experiment and time kinetics experiment. Subsequently copy DNA (cDNA or complementary DNA) was made and the real-time qPCR (quantitative polymerase chain reaction) reactions were executed. Secondly, the comet assay was performed whereby *L. minor* plants were exposed to MMS to see if induced DNA damage could be measured.

3.4.1 Gene expression

To analyze the gene expression of selected housekeeping genes and possible U-biomarkers, RNA was extracted. For this, frozen plant samples at -80°C were transferred to liquid nitrogen and then disrupted using the Mixer Mill MM400 (Retsch). At each sample approximately ten Zirconia (Biospec) beads (2.0 mm) were added. Afterwards the samples were placed in pre-cooled Mixer Mill adaptors and grounded for 3.5 minutes at 30 Hz. The RNA was extracted using the RNAeasy Plant Mini kit (Qiagen). The RNA content was measured with the Nanodrop ND-1000 (Thermo Fisher Scientific) and all samples were set on the same concentration of 10000 ng per 45 µl. RNA samples were stored at -20°C until further use.

Before synthesis of the cDNA, any present DNA in the RNA samples was removed using the Turbo DNA-free Kit (Invitrogen). Afterwards the RNA concentration was measured again with the Nanodrop ND-1000 (Thermo Fisher Scientific) and all samples were set on the same concentration of 1000 ng per 13 µl. Subsequently the cDNA was synthesized using the PrimeScript RT reagent Kit (Perfect Real Time) (TaKaRa) with standard protocol and the Peqstar Gradient thermocycler (VWR). cDNA samples were diluted with RNase and DNase free water (1:10) for real-time qPCR and can be stored at -20°C.

The qPCR was performed with the Rotor-Gene Q (Qiagen), using SYBR green chemistry and the developed primers of paragraph 3.3. For the qPCR the QIAgility (Qiagen) was used to fill each well of a Rotor-Disc with a total volume of 15 µl containing 11.25 µl qPCR MasterMix (7.5 µl Fast SYBR Green MasterMix (Applied Biosystems), 2.85 µl RNase and DNase free water, 0.45 µl forward primer and 0.45 µl reverse primer) and 3.75 µl cDNA. Subsequently the Rotor-Disc was sealed with the Rotor-Disc Heat Sealer (Qiagen) and placed in the Rotor-Gene Q (Qiagen) for the PCR reaction. During the qPCR, the following program was used: an initiation step at 95°C for 20 s followed by 45 cycles (3 s at 95°C and 30 s at 60°C). A melting curve was generated between 50 and 95°C to ascertain the specificity of the primers.

The amplification efficiency of the primers was tested with a mixed cDNA sample dilution series (1; 1/4; 1/16; 1/64; 1/256; 1/1024). The Rotor-Disc was filled with 11.25 µl qPCR MasterMix and 3.75 µl cDNA. The PCR reaction was performed with the Rotor-Gene Q (Qiagen). With the PCR products, a gel electrophoresis was performed to confirm the desired amplicon. The gel consists of 0.8 g agarose (Roche) and 5 µl GelRed (Biotium) per 100 ml TAE buffer. To load the samples on the gel, 1 µl loading dye was added to 5 µl PCR product. Per gel, 4 µl of this PCR product mixture and 2 µL ladder of 50 and 100 bp (Thermo Scientific) was loaded. The gel electrophoresis was ran for 50 min at 70 V and subsequently visualized with a gel imager (BioRad).

Various developed and optimized primers of the potential U-biomarker genes were tested together with multiple existing and new housekeeping genes (Table 4 and Table 5). The expression stability of the housekeeping genes was tested using GrayNorm [100]. Data

were normalized with a normalization factor from the housekeeping genes. Mean values of the gene expression of the U-biomarker genes was set relative to the control conditions by using the $2^{-\Delta Ct}$ method.

Table 5: Overview of the primers for the existing housekeeping genes (used for the normalization of the data) with their related function

EXISTING HOUSEKEEPING GENES	
Primer number	**Function**
Primer 4	BSL2 Serine/threonine-protein phosphatase BSL2
Primer 9	SBT3.3 Subtilisin-like protease SBT3.3
Primer 15	Mitogen-activated protein kinase kinase 1
Primer 18	TUBB5 Tubulin beta-5 chain
Primer 20	CYP71A25 Cytochrome P450 71A25
Primer 22	GCP3 Gamma-tubulin complex component 3

3.4.2 Comet assay

For the first time a comet assay on *L. minor* plants was performed within the BIS group. The comet assay was performed to optimize some of the parameters. Two conditions were fulfilled: plants exposed to MMS (Sigma-Aldrich) which is an inducer of DNA damage and a control condition. MMS serves as a positive control for DNA damage. In a time period of four hours, the *L. minor* plants were exposed to 5 mM MMS. Afterwards the comet assay handlings were executed.

At least one day before the experiment slides need to be coated with a 1% Normal Melting Point Agarose (NMPA) (Sigma-Aldrich). The NMPA was boiled and homogenated several times. Subsequently the slides were submerged into the NMPA for at least three times. The coated slides were dried overnight.

The comet assay was performed in the dark with only an inactinic red light to avoid the induction of DNA damage through actinic light. The first step was chopping of the plant material. For this, two *L. minor* plants were put into a petri dish and 400 µl extraction buffer (1/10 of 200 mM Na_2EDTA solution pH 10 (Sigma-Aldrich) and 9/10 of Phosphate Buffer Solution (PBS) pH 7 (consisting of 0.2 g KCl (Sigma-Aldrich), 0.2 g KH_2PO_4 (Merck), 8 g NaCl (Merck) and 1.78 g $Na_2HPO_4.2H_2O$ (Merck) per liter)) was added. Plants were chopped with a razor blade during 30 s. Hereafter, 225 µl of the nucleus solution was collected and mixed with 150 µl 2% Low Melting Point Agarose (Sigma-Aldrich). Subsequently, three gels of 10 µl were pipetted on the coated slide as shown in Figure 2. These operations were repeated until a total of 12 gels were pipetted on the coated slide.

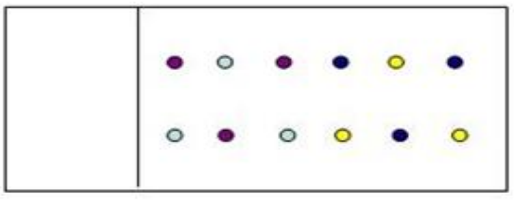


Figure 2: Coated slide for comet assay with a total of 12 gels

The slides were placed into the electrophoresis tank parallel to the border, filled with electrophoresis solution (3 ml of the Na_2EDTA solution and 18 ml of 10 N NaOH (Merck) in a total of 0.5 L). The denaturation took place during 15 min whereby the DNA in the gels

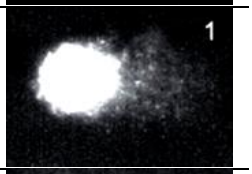
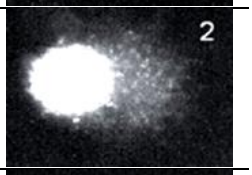
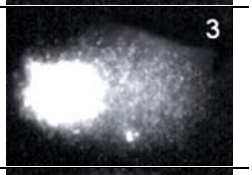
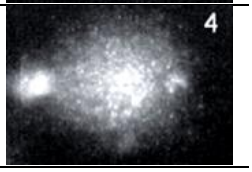
unwinds. Then an electrophoresis was ran at 300 mA and 16-18 V at 4°C. Three electrophoresis times were tested: 5, 10 and 15 min.

After the electrophoresis, the slides were neutralized for 1 min in demineralized water and twice for 5 min with 2 ml of the PBS. The gels were fixated overnight in absolute ethanol (Merck) until they were completely dry.

Slides were stained with 2 ml of a 1/10000 Sybr Gold solution (Invitrogen) after which the slides were washed twice with demineralized water to reduce the amount of background fluorescence. Two washing steps were tested: two washing steps of 5 min and a washing step of 5 min followed by a washing step of 10 min. Comets were visualized using the fluorescence microscope (Nikon Eclipse Ti with Lumencor Spectra X Chroma) with a 20x magnification.

A visual scoring was applied as described by Azqueta et al. (2011) [101] to determine the different levels of DNA damage. A scheme for visual scoring based on 5 recognizable classes of comets (Table 6) was used.

Table 6: Visual scoring of the comet assay [101], [102]

Class	Description	Picture
0	Undamaged, no discernible tail	
1	Very little damaged	
2	Normal damage, nice comet	
3	Much damage	
4	Almost all DNA in tail, insignificant head	

3.5 Statistical analysis

Statistical analyses were performed using the open source software package R (R Project for Statistical Computing, version 3.5.3). Outliers were detected with a Grubbs' test (GraphPad), also called the ESD method (Extreme Studentized Deviate), with a significance level of 0.05.

Data without outliers was used to detect differences between groups using two-way ANOVA. First a Shapiro-Wilk normality test and a Bartlett test were performed to test the normality of the data and homogeneity of the variances, respectively. If the assumption of normality of homogeneity of variances was not fulfilled, transformations of the data were applied where necessary. Four different transformations were performed: log(x), $(x)^{1/2}$, 1/x and e^x. The post-hoc Tukey HSD test was used for multiple comparison of the data.

If the assumptions for a parametric test were not met, a non-parametric Kruskal-Wallis test was performed, followed by a Pairwise Wilcoxon singed-rank test with no correction.

4 RESULTS

In this chapter, the results of this thesis are presented starting with the primer selection, optimization and testing their selectivity. Secondly, the growth and gene expression analyses of the 7 days exposure experiment to a metal mixture in combination with U are described followed by the time kinetics experiment of *L. minor* plants. Finally, the results of the comet assay performed on *L. minor* plants are presented.

4.1 Primer selection and optimization

As described above, the Beaverlodge Lake contains multiple contaminants, with the most important ones being U, Cu, Ni, Zn and Pb. The sequencing of the *L. minor* genome and transcriptome gives the possibility to study changes at the molecular level after exposure to those contaminants. Primers were developed using the primer-BLAST tool of NCBI and further tested with the Oligo Analysis Tool (Eurofins Genomics). Only primers with a %GC between 50 and 60% and a melting temperature around 60°C were selected. Due to the growth problems of the sterile plants, the efficiency of all new primers, 12 potential U-biomarker genes and 16 housekeeping genes (designed in paragraph 3.3) were in first instance tested on available cDNA of a previous U and metal mixture experiment [12]. For each primer the amplification plot and the melting curve are analyzed. A primer that reacts specific to one gene is given by one peak in the melting curve. In addition, the expression of the dilution series is concentration-dependent with the highest expression in the undiluted sample. The efficiencies of all primers is evaluated by setting the threshold in the linear phase of the amplification plot and using the correlated Ct values (Appendix B and C). Primers with an amplification efficiency of at least 90% and approved amplification plot and melting curve were selected and used for further testing.

4.2 Primer selectivity

The primers with a primer efficiency >90% were further tested as potential biomarker for U exposure. This was done with cDNA samples of a previous U and metal mixture experiment (*L. minor* plants exposed for 7 days to U (0, 2 or 10 µM) and/or metal mixtures (M0, M1, M10, M100 or M1000)) [12]. The tested primers were: primer 33, 41, 43, 44, 46, 47, 48, 50, 51 and 52. The expression of the potential U-biomarker genes was normalized with the new and existing housekeeping genes. Gene expression levels relative to the control condition (U0-M0) are given in Figure 3.

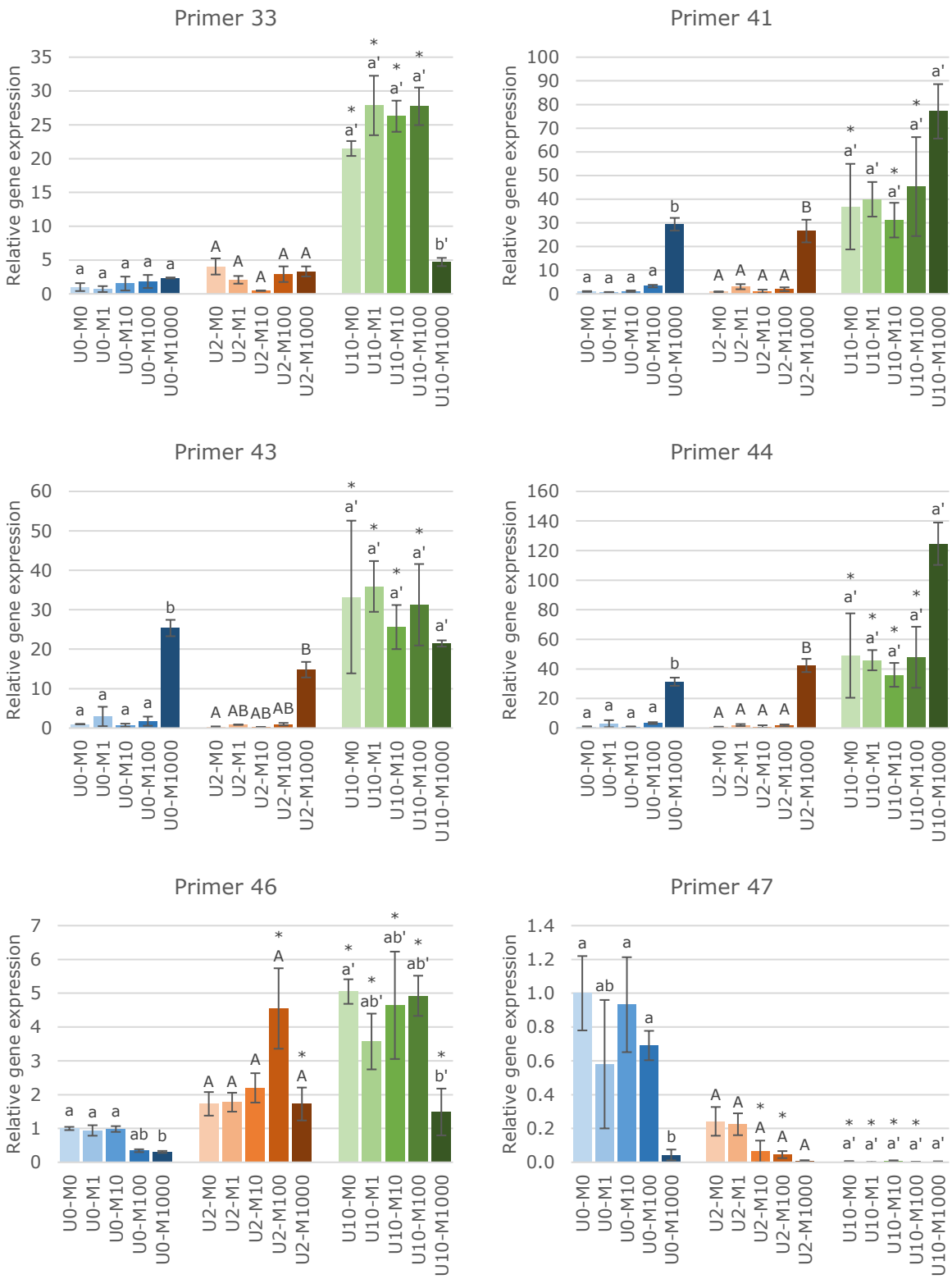


Figure 3: Gene expression levels for the primer optimization of potential U-biomarker genes for L. minor relative to control condition (U0-M0) after exposure of 7 days to U (0, 2 or 10 µM) and/or metal mixtures (M0, M1, M10, M100 or M1000). Mean values are given ± standard error (SE) of at least three biological independent replicates for each condition. Significant differences (two-way ANOVA, p-value <0.05) within the U0 condition are given with small letters, within the U2 condition with capital letters and within the U10 condition with small letters and an apostrophe. Significant differences (two-way ANOVA, p-value <0.05) between the U0 condition and U2/U10 condition are given with an asterisk.

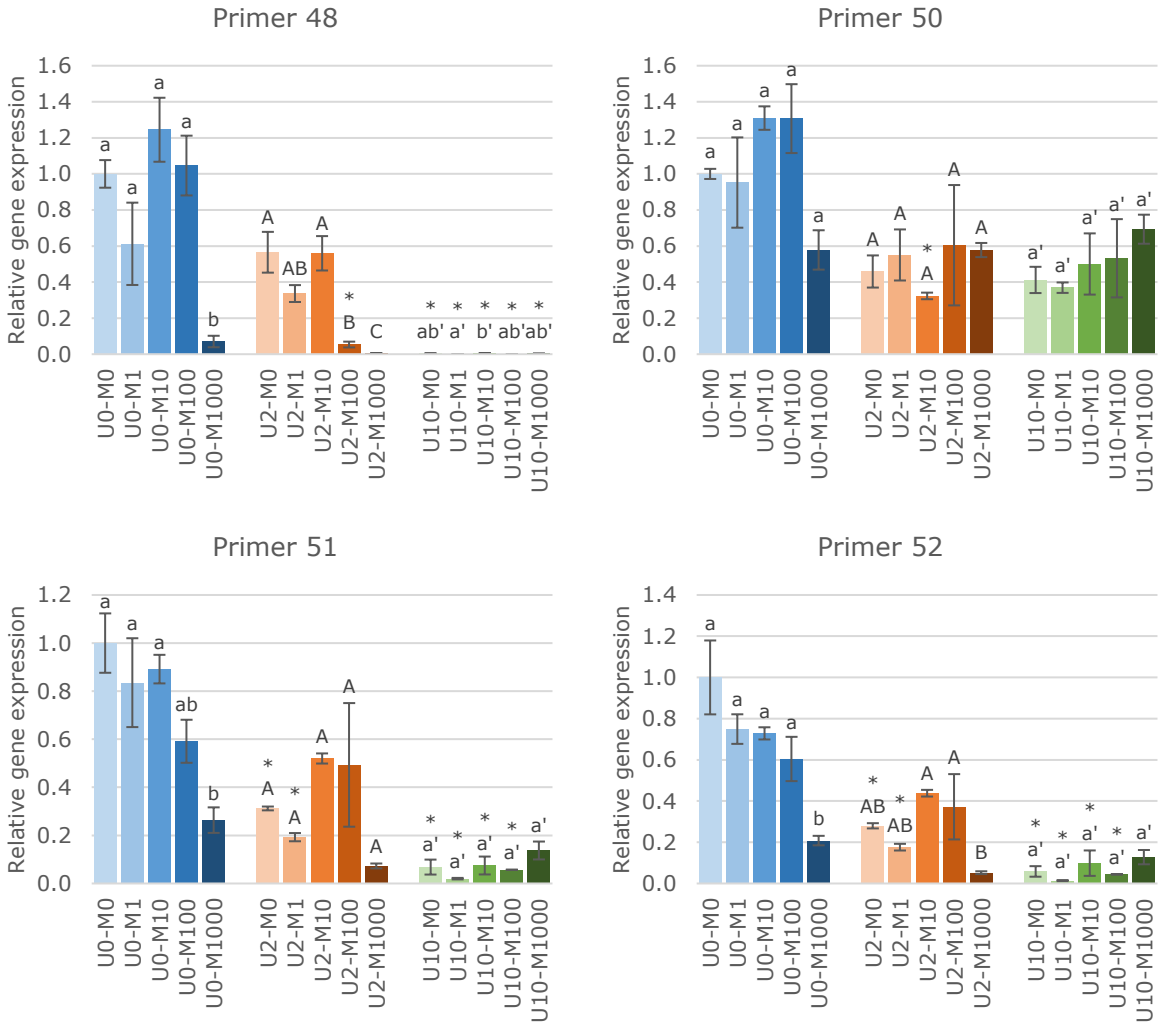


Figure 3 (continued).

In general, there is no significant effect of the different metal mixtures applied within one U concentration, except for the M1000 where significant changes are often observed. This can be explained by the fact that the M1000 conditions are highly concentrated and cause high toxicity to the plants [12]. These conditions cannot be used as early warning systems, which was the initial goal of this thesis. Therefore, the results of the M1000 conditions will not be further discussed in this thesis.

In the absence of U, no significant effects are observed for primer 33 between the different metal concentrations applied. The same trend is present in the U2 and U10 conditions. In addition, primer 33 shows a strong U-dependent response with an upregulation after exposure to 2 (non-significant) and 10 µM U (significant) in the absence of the metal mixture. At the U concentration of 10 µM the gene expression is approximately 22 times higher than the control condition. The M0, M1, M10 and M100 of the U10 condition are significant higher than their respective control without U.

For primer 41 no effect of the metal mixture is observed for the different U concentrations applied (0, 2 or 10 µM). In addition, no effects are present after exposure to U2. There is a significant increase in the gene expression after exposure to U10 as compared to U0 for all metal mixtures applied (except for U10-M1).

Primer 43 and primer 44, both primers for the same gene, show the same trend. No effect of the metal mixture is observed for the different U concentrations applied (0, 2 or 10 µM).

In addition, no effects are present after exposure to U2. There is a significant increase in the gene expression after exposure to U10 as compared to U0 for all metal mixtures applied.

For primer 46, no significant effects are observed between the different metal mixtures and control plants within one U concentration. An U-dependent up regulation is obtained with a significant effect after exposure to 10 µM U compared to the plants without U, but the upregulation is lower in comparison with the previous primers. An up regulation of approximately 5 is acquired for the U10 condition, whereas an up regulation of approximately 2 for the U2 condition.

Since primer 47 and primer 48 are both primers for the same gene, the results of both primers are similar. Within one U concentration, no significant effects are present, except for the downregulation in the U2-M100 condition compared to U2-M0 in primer 48. There is an U-dependent downregulation of the expression, whereby the expression level at U10 almost drops to zero.

Primer 50 does not give clear results. In general, no significant results are obtained.

Primer 51 and primer 52 are both primers for the same gene and the results show high similarities. For the different metal mixtures tested, no significant changes are observed within one U concentration. After exposure to U, a significant downregulation is obtained whereby M0 and M1 of the U2 condition and M0, M1, M10 and M100 of the U10 condition are significant lower than their corresponding control.

Based on the results of Figure 3, a selection of four primers is made, besides primer 33, to test in the 7 days exposure experiment (paragraph 4.3) and the time kinetics experiment (paragraph 4.4). Primer 44 and primer 46 as upregulated genes and primer 48 and primer 52 as downregulated genes.

4.3 7 days exposure experiment

During the 7 days exposure experiment *L. minor* plants were exposed to five concentrations of the metal mixture (M0, M1, M10, M100 and M1000) in combination with three U concentrations (0, 2 and 10 µM). The growth of the *L. minor* plants was followed by measuring the total area of the plants and counting the number of fronds (Appendix D). In this thesis the percentage of growth inhibition (%I_r) (Figure 4) is calculated from the FW of the plants linked to the total area.

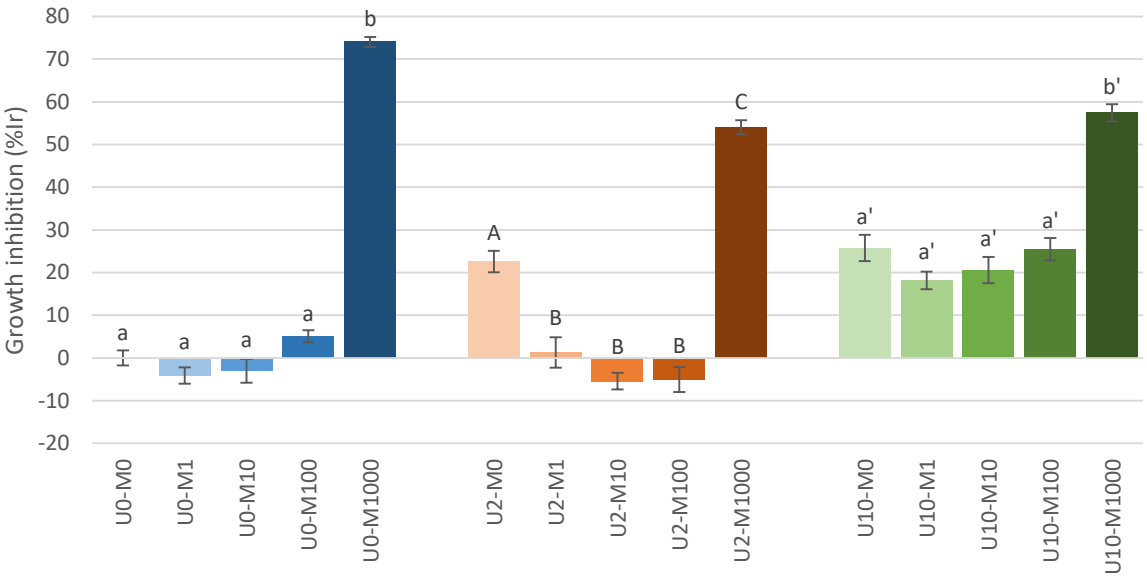


Figure 4: Percentage of growth inhibition (%I_r) calculated from the FW of the plants linked to the total area of L. minor plants exposed for 7 days to U (0, 2 or 10 µM) and/or metal mixtures (M0, M1, M10, M100 or M1000). The percentage of growth inhibition of the control condition (U0-M0) is 0%. Mean values are given ± standard error (SE) of at least four biological independent replicates for each condition. Significant differences (two-way ANOVA, p-value <0.05) within the U0 condition are given with small letters, within the U2 condition with capital letters and within the U10 condition with small letters and an apostrophe. Significant differences (two-way ANOVA, p-value <0.05) between the U0 condition and U2/U10 condition are given with an asterisk.

Exposing plants to M1000 causes >50% growth inhibition in all conditions, indicating high toxicity to the plants, as mentioned before in paragraph 4.1. These conditions cannot be used as early warning systems, which was the initial goal of this thesis. Therefore, the results of the M1000 conditions will not be further discussed.

In general, no significant differences are observed for the percentage of growth inhibition between the different U exposures. However, in the absence of the metal mixture, growth is reduced with >20% (non-significant effect). In addition, no significant effect is observed of the different metal mixtures applied within the U0 and U10 condition. In contrast, a reduced growth inhibition is present in the U2 condition after exposure to the metal mixture as compared to the U2-M0 condition.

The potential U-biomarker genes selected after the primer optimization (paragraph 4.1) (primer 33, primer 44, primer 46, primer 48 and primer 52) are further tested in this experiment. The expression of the genes is normalized with the new and existing housekeeping genes. Gene expression levels relative to the control condition (U0-M0) of *L. minor* plants exposed for 7 days to U (0, 2 or 10 µM) and/or metal mixtures (M0, M1, M10 or M100) are given in Figure 5.

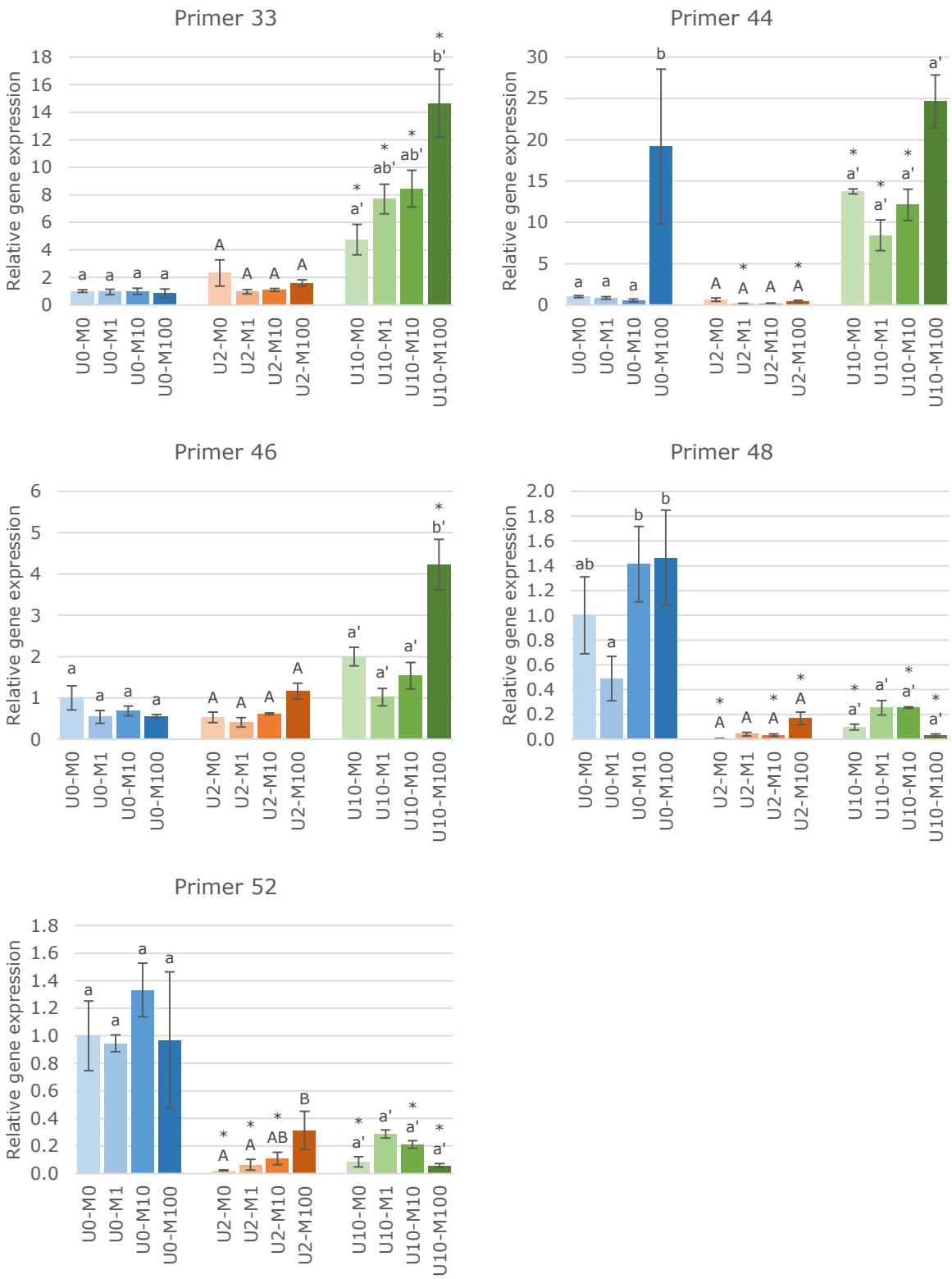


Figure 5: Gene expression levels of potential U-biomarker genes for L. minor relative to control condition (U0-M0) after exposure for 7 days to U (0, 2 or 10 µM) and/or metal mixtures (M0, M1, M10 or M100). Mean values are given ± standard error (SE) of at least three biological independent replicates for each condition. Significant differences (two-way ANOVA, p-value <0.05) within the U0 condition are given with small letters, within the U2 condition with capital letters and within the U10 condition with small letters and an apostrophe. Significant differences (two-way ANOVA, p-value <0.05) between the U0 condition and U2/U10 condition are given with an asterisk.

In the absence of U, no significant effects are observed between the different metal concentrations applied in primer 33. The same trend is present in the U2 condition. In contrast, the U10 condition shows a metal-dependent response, with a significant difference between M0 and M100. Furthermore primer 33 shows a strong U-dependent response with a 2-fold upregulation after exposure to 2 µM U (non-significant) and a 5-fold upregulation after 10 µM U (significant) in the absence of the metal mixture. The M0, M1, M10 and M100 of the U10 condition are significantly higher than their respective control without U.

In contrast to our expectations, a significant increase is observed in the expression of primer 44 after exposure to M100 in the absence of U. No significant effect of the metal mixture is observed when U is applied (2 or 10 µM). There is a significant increase in the gene expression after exposure to U10 as compared to U0 for M0, M1 and M10 metal mixtures and a significant decrease after exposure to U2 as compared to U0 for M1 and M100.

For primer 46, no significant effects are observed between the different metal mixtures within the U0 and U2 concentration. Within the U10 condition, exposure to the M100 concentration induced a significant increase in the transcript level compared to the other metal concentrations. By comparing the different U concentrations within the same metal mixture applied, a significant increase in gene expression is only present in the U10-M100 condition as compared to U0-M100.

No significant effects are present in the transcript levels of primer 48 for the different metal concentrations within one U concentration. After the application of U, there is a significant decrease in the expression of primer 48 for almost all the metal mixtures applied. However, this downregulation is not concentration-dependent. As such, the expression level at U2 almost drops to zero and increases slightly again at U10.

For the different metal mixtures tested for primer 52, no significant changes are observed within one U concentration, except within the U2 condition. The M100 concentration at 2 µM U had a higher expression level than the other metal concentrations. After exposure to U, a significant downregulation is obtained whereby the expression levels in M0, M1 and M10 of the U2 condition and M0, M10 and M100 of the U10 condition are significant lower than their corresponding control.

All of the primers were further tested in the time kinetics experiment whereby the gene expression is followed in function of time. This gives the opportunity to find fast-reacting biomarkers.

4.4 Time kinetics experiment

During the time kinetics experiment *L. minor* plants were exposed to three concentrations of the metal mixture (M0, M1, M100) in combination with the three U concentrations (0, 2 and 10 µM). Samples were taken after 1, 4 and 7 days and the growth of the *L. minor* plants is followed by measuring the total area of the plants and counting the number of fronds (Appendix E). The percentage of growth inhibition ($\%I_r$) is given as the FW of the plants linked to the total area. Table 7 gives the $\%I_r$ of the time kinetics experiment.

Table 7: Percentage of growth inhibition ($\%I_r$) calculated from the FW of the plants linked to the total area of L. minor plants exposed for 1, 4 or 7 days to U (0, 2 or 10 µM) and/or metal mixtures (M0, M1, M100). The percentage of growth inhibition of the control condition (U0-M0) is 0%. Mean values are given ± standard error (SE) of at least four biological independent replicates for each condition. Significant differences (two-way ANOVA, p-value <0.05) within the U0 condition are given with small letters, within the U2 condition with capital letters and within the U10 condition with small letters and an apostrophe. Significant differences (two-way ANOVA, p-value <0.05) between the U0 condition and U2/U10 condition are given with an asterisk.

		0 µM U	2 µM U	10 µM U
Day 1	**M0**	0.00% ± 3.37%^{a}	-6.51% ± 8.27%^{A}	-3.85% ± 4.58%$^{a'}$
	M1	-8.00% ± 4.36%^{a}	-15.18% ± 8.45%^{A}	7.38% ± 6.66%$^{a'}$
	M100	-10.01% ± 5.45%^{a}	-13.43% ± 8.32%^{A}	-5.42% ± 4.12%$^{a'}$
Day 4	**M0**	0.00% ± 7.68%^{a}	7.27% ± 2.25%^{A}	23.27% ± 0.85%$^{a'*}$
	M1	-5.73% ± 0.23%^{a}	-2.60% ± 4.35%^{A}	12.21% ± 0.93%$^{a'*}$
	M100	-3.11% ± 2.47%^{a}	9.08% ± 2.60%^{A}	15.90% ± 6.21%$^{a'*}$
Day 7	**M0**	0.00% ± 1.55%^{a}	0.55% ± 2.65%^{A}	16.54% ± 2.78%$^{a'*}$
	M1	5.95% ± 0.63%^{ab}	2.43% ± 1.91%^{A}	21.32% ± 1.11%$^{a'*}$
	M100	14.69% ± 2.22%^{b}	3.02% ± 3.18%^{A*}	23.41% ± 1.29%$^{a'}$

After 1 day of exposure, the different U concentrations and metal mixtures did not significantly affect the $\%I_r$. At day 4, no significant differences are obtained within one U concentration for the different metal mixtures. But exposure to 10 µM U leads to a significant growth inhibition compared to their respective controls exposed to 0 µM U. The same trend for the U10 condition is observed at day 7. No significant differences are obtained in the U2 condition for the different metal mixtures. However, plants grown in the absence of U showed a significant growth reduction after exposure to M100 as compared to U0-M0 after 7 days of exposure.

To find fast-reacting biomarkers, the expression levels of the potential U-biomarker genes was measured and normalized with the new and existing housekeeping genes. Gene expression levels relative to the control condition (U0-M0) of *L. minor* plants exposed for 1, 4 or 7 days to U (0, 2 or 10 µM) and/or metal mixtures (M0, M1, M100) are given in Figure 6 (primer 33), Figure 7 (primer 44), Figure 8 (primer 46), Figure 9 (primer 48) and Figure 10 (primer 52).

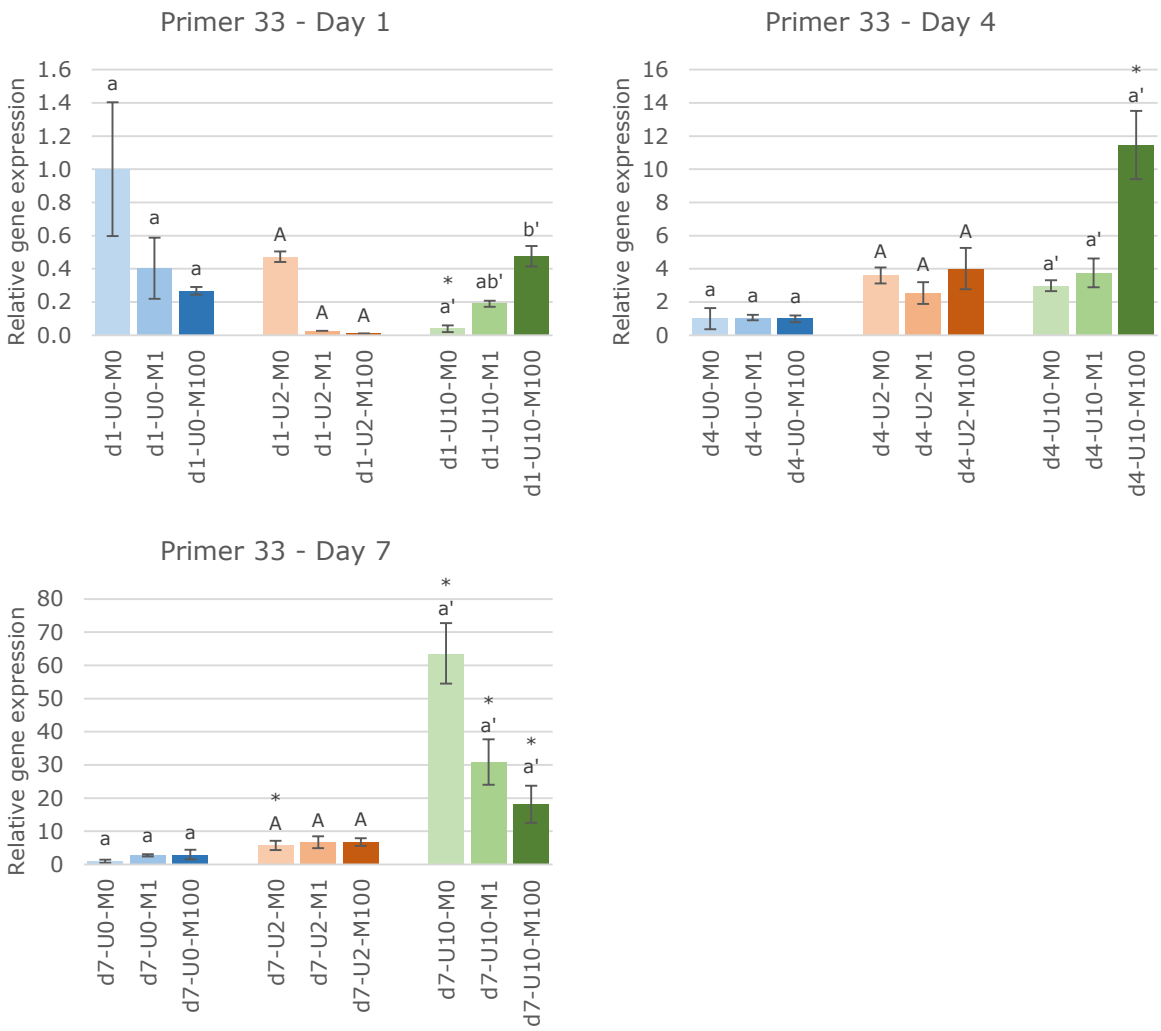


Figure 6: Gene expression levels of potential U-biomarker primer 33 for L. minor relative to control condition (U0-M0) after exposure for 1, 4 or 7 days to U (0, 2 or 10 µM) and/or metal mixtures (M0, M1, M100). Mean values are given ± standard error (SE) of at least three biological independent replicates for each condition. Significant differences (two-way ANOVA, p-value <0.05) within the U0 condition are given with small letters, within the U2 condition with capital letters and within the U10 condition with small letters and an apostrophe. Significant differences (two-way ANOVA, p-value <0.05) between the U0 condition and U2/U10 condition are given with an asterisk.

At day 1 no significant effects are observed for primer 33 in the U0 and U2 condition between the different metal concentrations applied. Within the U10 condition a metal dependent increase in expression is observed, with a significant increase after exposure to M100 as compared to M0. In general no significant differences are obtained between the different U concentrations for the same metal concentration, with the exception of U10-M0 compared to the non-exposed controls. At day 4, no significant effects are observed within one U concentration. A slight non-significant increase in the gene expression of the U2 and U10 is present compared to the U0 condition, with a significant increase in the U10-M100 condition compared to U0-M100. After 7 days of exposure, no significant effects are observed within one U concentration for the different metal concentrations. Exposing the plants to 2 µM U leads to an increased expression of the genes (approximately 4 times higher than the control) with a significant increase in the U2-M0 condition. In the U10-M0 condition, the transcript level is 60 times higher. When metals are present with 10 µM U, the expression rises up to 30 times higher for M1 and 20 times higher for M100. The expression levels in all of the U10 conditions are significant increased compared to their corresponding control conditions.

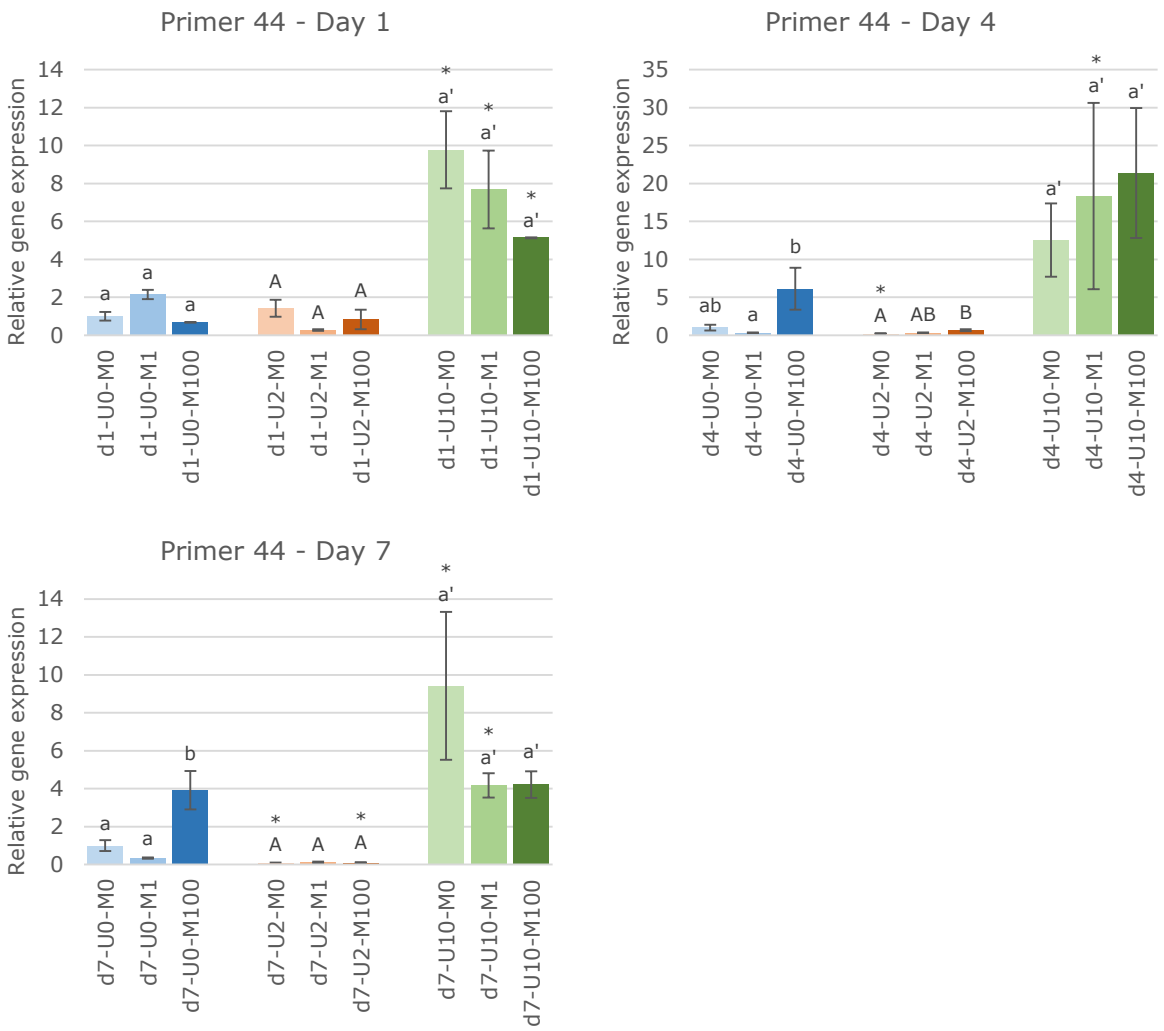


Figure 7: Gene expression levels of potential U-biomarker primer 44 for L. minor relative to control condition (U0-M0) after exposure for 1, 4 or 7 days to U (0, 2 or 10 µM) and/or metal mixtures (M0, M1, M100). Mean values are given ± standard error (SE) of at least three biological independent replicates for each condition. Significant differences (two-way ANOVA, p-value <0.05) within the U0 condition are given with small letters, within the U2 condition with capital letters and within the U10 condition with small letters and an apostrophe. Significant differences (two-way ANOVA, p-value <0.05) between the U0 condition and U2/U10 condition are given with an asterisk.

At day 1 no significant differences are observed for primer 44 within one U concentration for the different metal mixtures. A significant upregulation in the gene expression is obtained in all the U10 conditions compared to their respective controls in U0. In contrast no significant differences are present in U2 condition compared to U0. At day 4 significant differences are obtained within the U0 and U2 condition for the different metal mixtures. As such, an increased expression is present after exposure to M100 in both conditions. This increase is not shown in the U10 condition. The transcript level of primer 44 is significantly lower after exposure to U2-M0 compared to U0-M0. In contrast, a 10-fold increase in expression is present in the U10 conditions, with a significant upregulation in U10-M1 compared to their corresponding control. At day 7, no significant differences are observed within one U concentration, except for the M100 of U0 which is significant higher than the other metal concentrations for U0. There is significant decrease in the gene expression after exposure to U2 as compared to U0 for M0 and M100 metal mixtures and a significant increase after exposure to U10 as compared to U0 for M0 and M1.

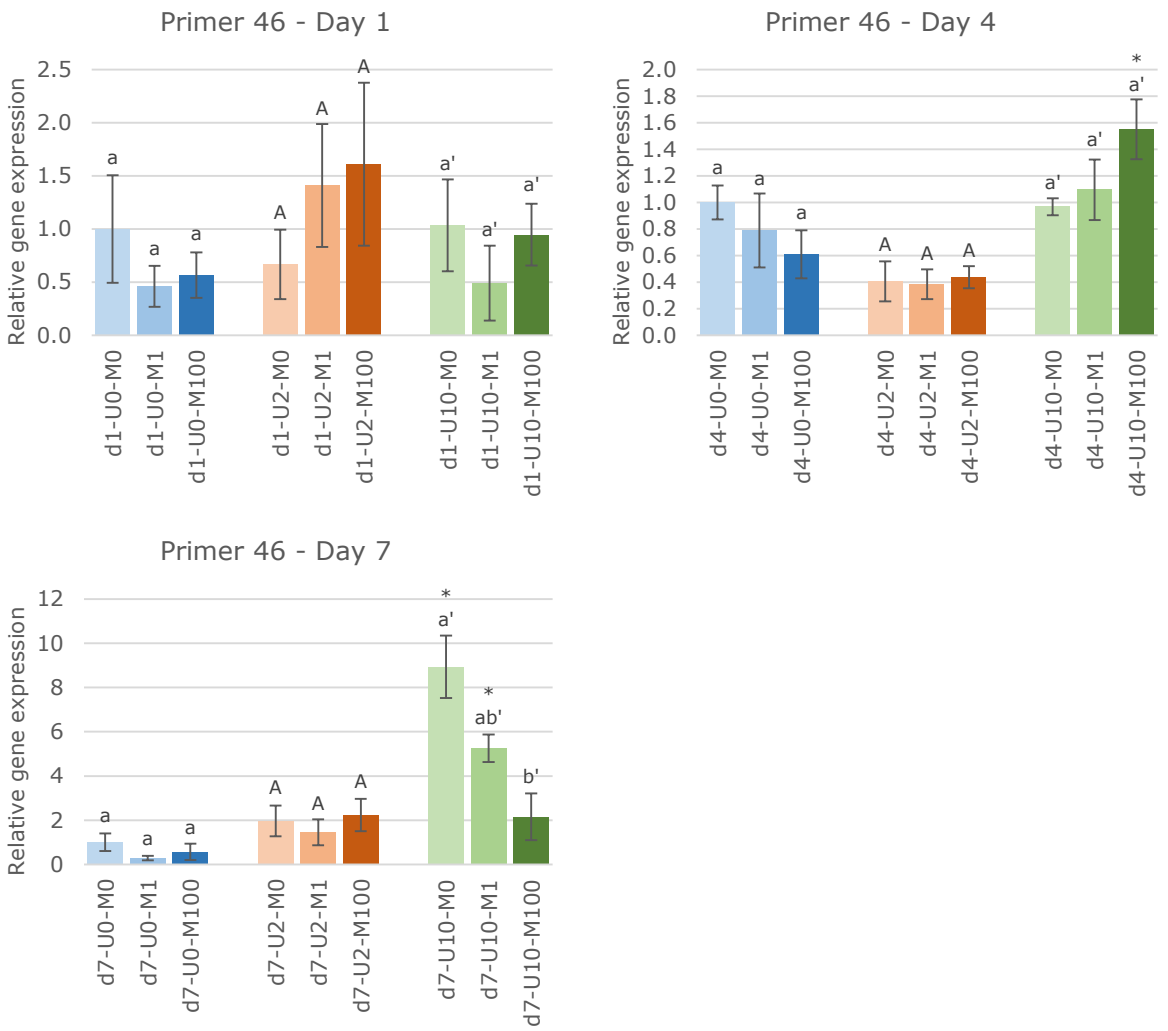


Figure 8: Gene expression levels of potential U-biomarker primer 46 for L. minor relative to control condition (U0-M0) after exposure for 1, 4 or 7 days to U (0, 2 or 10 µM) and/or metal mixtures (M0, M1, M100). Mean values are given ± standard error (SE) of at least three biological independent replicates for each condition. Significant differences (two-way ANOVA, p-value <0.05) within the U0 condition are given with small letters, within the U2 condition with capital letters and within the U10 condition with small letters and an apostrophe. Significant differences (two-way ANOVA, p-value <0.05) between the U0 condition and U2/U10 condition are given with an asterisk.

At day 1 and 4 no significant differences are observed for primer 46 within one U concentration. In general no significant differences are obtained between the U concentrations for the same metal concentration, with the exception of U10-M100 of day 4 compared to its control. At day 7, no significant differences are observed within the U0 and U2 condition. For the U10 condition, the transcript level in the M0 metal concentration is significant higher than in the M100 concentration. Between the different U concentrations, the transcript levels in the M0 and M1 of the U10 conditions are significantly upregulated as compared to their controls in the U0 condition.

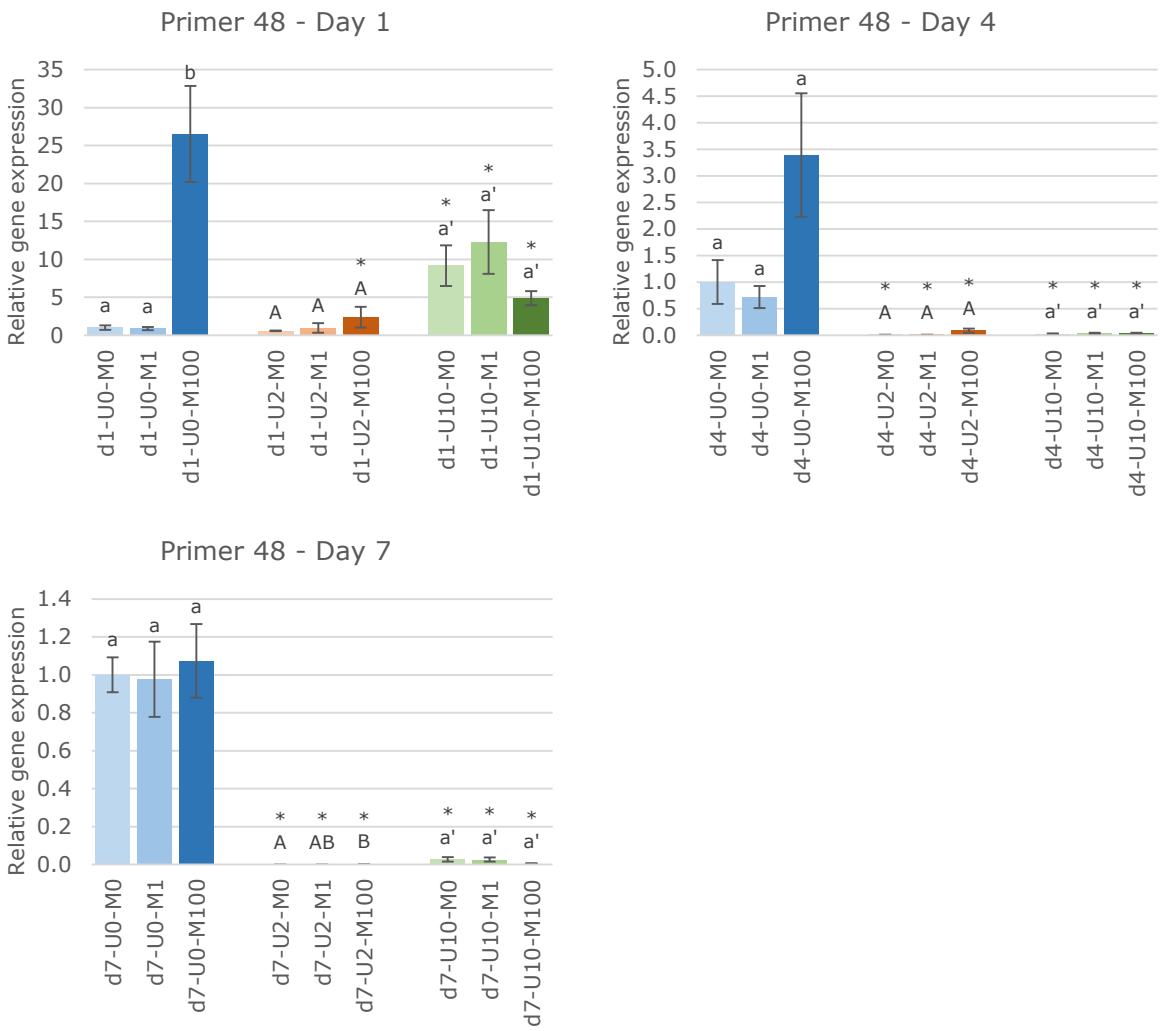


Figure 9: Gene expression levels of potential U-biomarker primer 48 for L. minor relative to control condition (U0-M0) after exposure for 1, 4 or 7 days to U (0, 2 or 10 µM) and/or metal mixtures (M0, M1, M100). Mean values are given ± standard error (SE) of at least three biological independent replicates for each condition. Significant differences (two-way ANOVA, p-value <0.05) within the U0 condition are given with small letters, within the U2 condition with capital letters and within the U10 condition with small letters and an apostrophe. Significant differences (two-way ANOVA, p-value <0.05) between the U0 condition and U2/U10 condition are given with an asterisk.

At day 1, almost no significant differences are obtained for primer 48 for the different metal mixtures within one U concentration, except for the significant upregulation at M100 of the U0 condition. Between the U concentrations there is significant increase in gene expression after exposure to U10 as compared to U0 for the M0 and M1 metal mixtures. This in contrast to the significant decrease in gene expression after exposure to both U2 and U10 as compared to U0 for the M100 metal mixture. At day 4 there are no significant differences observed within one U concentration. All the metal concentrations of the U2 and U10 condition show a significant decrease in gene expression as compared to U0. At day 7, a similar trend as at day 4 is observed with no significant differences within one U concentration, except for the expression level in the U2-M100 condition that is significant lower than in U2-M0. In addition, all the metal concentrations of the U2 and U10 condition show a significant decrease in gene expression as compared to U0.

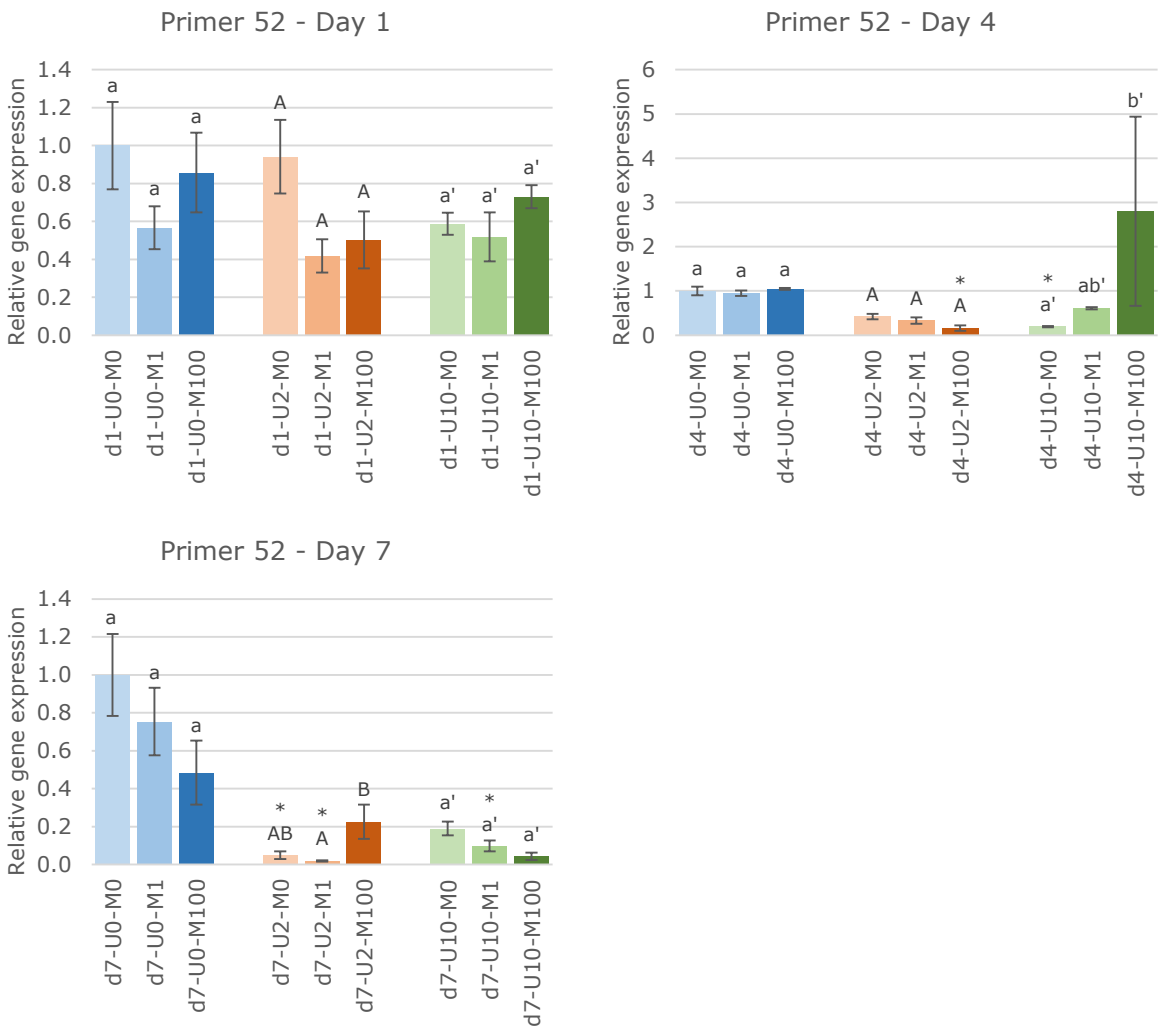


Figure 10: Gene expression levels of potential U-biomarker primer 52 for L. minor relative to control condition (U0-M0) after exposure for 1, 4 or 7 days to U (0, 2 or 10 µM) and/or metal mixtures (M0, M1, M100). Mean values are given ± standard error (SE) of at least three biological independent replicates for each condition. Significant differences (two-way ANOVA, p-value <0.05) within the U0 condition are given with small letters, within the U2 condition with capital letters and within the U10 condition with small letters and an apostrophe. Significant differences (two-way ANOVA, p-value <0.05) between the U0 condition and U2/U10 condition are given with an asterisk.

At day 1 no effects are observed for primer 52 within one U concentration and between the different U concentrations. At day 4 there are no significant differences within the U0 and U2 condition for the different metal mixtures. In contrast, a significant upregulation in transcript level is present for the U10-M100 condition compared to U10-M0. There is a significant decrease in the gene expression after exposure to U2 as compared to U0 for the M100 metal mixture and a significant decrease after exposure to U10 as compared to U0 for M0. At day 7, no significant effects are obtained within the U0 and U10 condition for the different metal mixtures. In contrast, there is a significant downregulation in gene expression in the U2-M1 condition compared to U2-M100. Between the U concentrations there is a significant decrease in the gene expression after exposure to U2 as compared to U0 for the M0 and M1 metal mixtures, and for the exposure to U10 as compared to U0 for the M1 metal concentration.

4.5 Summary of the gene expression results

All of the gene expression results given in paragraph 4.3 and 4.4 are analyzed on a number of criteria: exposure dose-response relationship, exposure time-response relationship, sensitivity and chemical specificity.

The exposure dose-response relationship is the up- or downregulation of the primer in function of rising U concentrations. If the gene expression is ascending or descending with the U concentration, this criterion is approved. The exposure time-response relationship is the degree to react in function of time. This information will be obtained from the time-kinetics experiment where the changes in relative gene expression are followed in function of time. The earlier a primer shows an up- or downregulation, the better the time-response relationship. The sensitivity is the responding of the primer to a low concentration of U. A reaction at low U concentrations facilitated the search for an U-biomarker. At last there is the chemical specificity, which is the responding of the primer to the metal mixtures. These are the observed significant differences within one U concentration, i.e. a response to the metal mixture. Since the objective of this thesis is to find biomarkers that react specific to U, primers are approved if no significant effects are observed to the metal mixture [103]. Table 8 gives the overview of the potential U-biomarker primers analyzed with the four biomarker criteria. A check mark indicates that the primer is accepted for this specific criterion, a cross indicates rejection.

Table 8: Overview of potential U-biomarker primers analyzed with the four biomarker criteria. A check mark indicates that the primer is accepted for this specific criterion, a cross indicates rejection.

	Exposure dose-response relationship	**Exposure time-response relationship**	**Sensitivity**	**Chemical specificity**
Primer 33	✓	✓	✓	✓
Primer 44	×	✓	×	×
Primer 46	×	×	×	×
Primer 48	×	✓	✓	✓
Primer 52	×	✓	✓	✓

Primer 33 matches all four criteria. Primer 44 is only approved by the exposure time-response relationship since it has a fast reaction in gene expression just after one day (Figure 7). Primer 46 did not meet any of the criteria. Primer 48 and primer 52 score exactly the same. Only the exposure dose-response relationship gives negative advice.

4.6 Comet assay

For the first time a comet assay on *L. minor* plants was performed within the BIS group to detect DNA damage. Since it was the aim to test the feasibility of this technique on *L. minor* plants and to optimize some of the parameters, the comet assay was performed on control plants and on plants exposed for four hours to 5 mM MMS, which is an inducer of DNA damage. In total three electrophoresis times were tested: 5, 10 and 15 min. Also two washing steps were tested: two washing steps of 5 min and a washing step of 5 min followed by a washing step of 10 min. An overview of the results is given in Table 9.

Table 9: Overview of different electrophoresis times and washing steps for the performed comet assay

		OK?
Electrophoresis time	5 min	✓
	10 min	✓
	15 min	×
Washing steps	5 min and 5 min	✓
	5 min and 10 min	✓

From the results in Table 9, it can be concluded that an electrophoresis time of 5 and 10 min can be used and that both applied washing steps can be used. An electrophoresis time of 15 min does not give optimal results. As shown in Figure 11 there is some visual DNA damage in the control condition. The comets show tails, which indicates that damaged DNA had been migrated during the electrophoresis, which is not ideal. Based on this results the optimization is continued with 5 and 10 min electrophoresis.

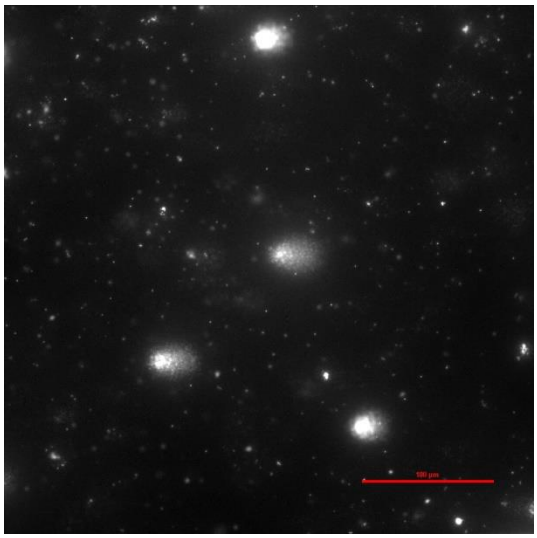


Figure 11: Visual comets of control condition at 15 min of electrophoresis

The visual scoring to determine the different levels of DNA damage is applied on both the control condition and the MMS condition, independently of the electrophoresis time and applied washing steps. Figure 12 gives an example of the results of the comet assay of the control and MMS condition. As shown in the figure, the control condition has mostly comets of class 0 and 1 whereas the MMS condition has comets more spread over the different classes. Table 10 gives a summary of the percentages of the classes from both conditions.

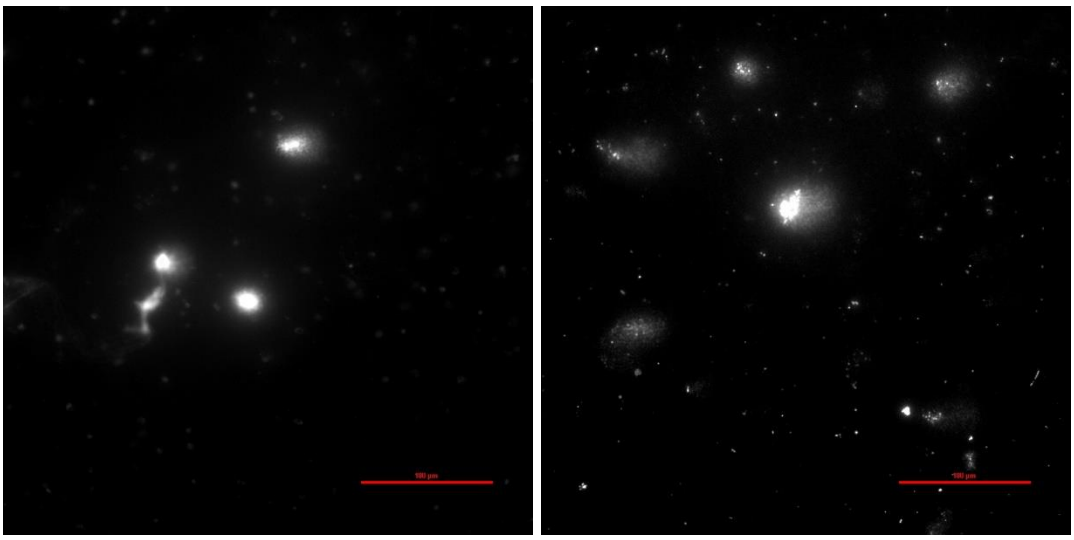


Figure 12: Results of comet assay. Left control condition, right MMS condition both performed with 10 min of electrophoresis and two washing steps of 5 min.

Table 10: Overview of results from the visual scoring of the comet assay. The percentage of each class is given of the control condition and the MMS condition.

Condition	Class	% of each class
Control	0	62.73
	1	27.59
	2	9.67
	3	0.00
	4	0.00
MMS	0	2.50
	1	29.58
	2	19.58
	3	16.25
	4	32.08

In the absence of MMS, most comets are scored within class 0 and 1 and a few in class 2. No comets are scored in class 3 and 4. In contrast, the MMS condition has comets more spread over the different classes, with >30% in the highest class.

5 DISCUSSION

The aim of this study was divided into two. Firstly, there was the validation of the potential U-biomarker gene (primer 33) and the selection and validation of other potential U-biomarker genes for the freshwater plant *L. minor* when they were exposed to U in combination with an environmentally relevant metal mixture. The environmentally relevant metal mixture was based on concentrations that were measured in the Beaverlodge Lake in Canada [11] and the most important co-contaminants (Cu, Ni, Zn and Pb) were selected and used in this research. The selection of the potential U-biomarker genes was done based on available RNA-sequencing data [7]. Secondly, the feasibility of the comet assay on *L. minor* plants was tested and some of the parameters were optimized using MMS as an inducer of DNA damage. The comet assay can be used as a possible biomarker for detecting DNA damage after exposure to radiation or radionuclides.

5.1 Growth effects induced by exposure to U and the metal mixture

Due to the growth problems of the sterile *L. minor* plants, experiments were not performed according to the OECD guidelines. As such, non-sterile *L. minor* plants were used and plants were grown in a day and night program (14h/10h) at respectively 24/18°C, causing a slight reduction in the growth of the plants. However, the growth of the non-exposed plants (U0-M0) of the 7 days exposure experiment still met the requirements by the OECD (guideline 221) [84], i.e. an average specific growth rate higher than 0.275 d^{-1} (experimental result of 0.298 d^{-1}). By this, the validity of the experiments is achieved. For the time kinetics experiment an average specific growth rate of 0.272 d^{-1} is obtained. This indicates that the plants did not met the requirements by the OECD (guideline 221). Therefore, it is important to repeat the time kinetics experiments to validate the results obtained in this project.

During the 7 days exposure experiment and the time kinetics experiment, the growth of *L. minor* is measured. Following to the OECD guidelines, the total area of the plants and the number of fronds is counted. The FW of the plants can be linked to the total area. Those three endpoints do not give the same results. According to Horemans et al. (2016) [99] the frond number is the least reliable growth parameter considering it not being directly related to the total biomass. Under U stress the frond size will decrease more rapidly than the frond number. As a result the most sensitive endpoint is the total area. Within this thesis the percentage of growth inhibition is calculated from the FW linked to the total area.

In all U concentrations used (0, 2 and 10 µM), plants exposed to M1000 metal concentrations have >50% growth inhibition. This indicates high toxicity at these extremely high metal concentrations, which is a thousandfold higher than environmentally relevant concentrations. As the initial goal of this thesis was to find biomarkers that can be used as early warning systems, i.e. limited effects at the cellular and individual level, this high metal concentration will be excluded from further discussion.

The growth of the *L. minor* plants in the 7 days exposure experiment was inhibited with approximately 20-30% when exposed to 2 and 10 µM U in the absence of metals. While these conditions have a limited growth inhibition at 2 µM U and 15% inhibition at 10 µM U in the time kinetics experiment. As such, there seems to be some differences in growth inhibition in the two experiments, which cannot be explained at the moment. The growth inhibition of the time kinetics experiments is similar to the results obtained by Horemans et al. (2016) [99], at which a 30% growth inhibition was obtained at 10 µM U exposure.

Since the results of Horemans et al. were obtained using the OECD guidelines, a real comparison can only be made when the current experiments are repeated under the same growth conditions.

In accordance with previous experiments [12] a hormesis-like effect is obtained in the U2 condition of the 7 days exposure experiment with all of the metal concentrations. This can be explained by the fact that some of the metals in the metal mixture (Cu, Ni and Zn) are essential for the plant growth. In the absence of these metals, the plants experience toxicity of U. Vanhoudt et al. (2011) [44] proved that U induces a reduction of Cu and Zn concentrations in *A. thaliana* plants. Although this reduced element concentration has not been shown before for *L. minor* plants, it seems that adding those metals to the medium reduces the U toxicity. Therefore, a possible hypothesis is that also in *L. minor* plants, U will influence the nutrient composition of the plants leading to a shortage of essential metals. The effects of the 7 days exposure experiment are not present at day 7 of the time kinetics experiment. Currently there are no explanations for these different results, but studying the uptake of the various elements would help to provide an explanation. In the U10 condition, U shows an adversely effect on the growth of *L. minor* with or without the metal mixtures, in both experiments. No hormesis-like effect is observed, which indicates toxicity.

5.2 Gene expression of potential U-biomarkers

Within this project 10 primers for potential U-biomarker genes were developed and their selectivity was tested on available cDNA of a previous U and metal mixture experiment [12] using real-time qPCR. Based on the results in Figure 3, a selection of five potential U-biomarkers was made and the gene expression levels were measured during the 7 days exposure experiment and the time kinetics experiment. All primers were analyzed on four criteria: 1) exposure dose-response relationship; 2) exposure time-response relationship; 3) sensitivity; and 4) chemical specificity (Table 8).

The function of the gene related to primer 46 is "response to oxidative stress". Although the first screening (Figure 3) showed a dose-related increase in transcript levels and a chemical specificity to U, those results could not be validated in further experiments (Figure 5 and Figure 8). As such, primer 46 did not meet any of the criteria which indicates that this primer can probably not be used as an ideal U-biomarker. In addition, the expression levels in both the 7 days exposure experiment and the time kinetics experiment showed a lower upregulation than results obtained from the cDNA from the previous experiment, indicating the need for the repetition of the experiments with sterile *L. minor* plants according to the OECD guidelines.

Primer 44 is linked to a gene with the function "negative regulation of growth". The transcript levels of this gene were already significantly upregulated after 1 day of exposure to U10, although this was not accompanied yet with a reduced growth of the plants (Table 7). Furthermore, the transcript levels remain upregulated at day 4 and day 7, although non-significant, indicating a fast and long-lasting response to U exposure (exposure time-response relationship). This corresponds to the reduced growth of the plants at day 4 and day 7 after exposure to the highest U concentration, although there is no direct relationship between gene expression and higher levels of biological complexity. In contrast no significant differences were present in U2 condition compared to U0, excluding the dose-response relationship and sensitivity criteria. The expression levels of primer 44 in the 7 days exposure experiment and the time kinetics experiment are also much less upregulated compared to the results obtained from the cDNA from a previous experiment after exposure

to U10. In addition, no significant effects of metal exposure were present in the previous experiment, while in the 7 days exposure experiment and the time kinetics experiment a significant upregulation was observed after exposure to U0-M100, excluding the chemical specificity criteria. The increased expression at U0-M100 was followed by a significant reduction in growth at day 7 in this condition, indicating that an increased expression in this gene precedes a reduction in growth. Thus, primer 44 may not be considered as an ideal U-biomarker according to the analysis in Table 8, but it is definitely a fast-reacting gene.

Primer 48 had only negative advice for the exposure dose-response relationship. The gene related to this primer has as function "response to oxidative stress". It is known that U causes oxidative stress in *L. minor* plants, which was shown by an increased activity of different antioxidative enzymes [21]. In addition, Horemans et al. (2014) [21] has shown that also Cd provokes oxidative stress in *L. minor* plants. However, the antioxidative responses to U and Cd are not identical. For example, the antioxidative enzyme guiaicol peroxidase was strongly induced by Cd whereas it was inhibited by U. Therefore, a possible hypothesis is that due to a different antioxidative response, primer 48 is specific towards U stress. However, more research is necessary with other metals in the environmentally metal mixture, including Cd, to further investigate the chemical specificity of this gene.

The gene related to primer 52 has a function in the response to auxin. As for primer 48, only the exposure dose-response relationship gives negative advice for this primer to become an U-biomarker. It has been hypothesized before that U disrupts the transport and gradient of auxin in the root in *A. thaliana* plants [104]. However, also other metals (e.g. Cd) are known to influence the expression of auxin-related genes. Therefore, it would be interesting to include more metals in the metal mixture to further investigate the chemical specificity of this potential U-biomarker.

Primer 33 is the only primer that matches all four criteria, a primer which is related to a gene encoding for a cytochrome P450 family protein. Similar results are obtained in all the experiments after 7 days of exposure (Figure 3, Figure 5 and Figure 6). The expression levels of the U10 conditions varies from approximately a 25-fold upregulation after 10 µM U in the previous experiment, an 8-fold upregulation in the 7 days exposure experiment and a 60-fold upregulation in the time kinetics experiment. Although the differences in gene expression levels between the different experiments cannot be explained at this moment, it is clear that this gene reacts specifically and consistent to U-exposure. Since this primer was already selected in previous experiments, primer 33 could be validated as biomarker for U exposure in the current experiments. However, a repetition of the experiments according to the OECD guidelines is advised.

5.3 Comet assay optimization on *L. minor*

The comet assay on *L. minor* was performed for the first time within the BIS group to detect DNA damage. The aim was to test the feasibility of this technique on *L. minor* plants and to optimize some of the parameters. From the first results, it can be concluded that an electrophoresis time of 5 or 10 min can be used to perform the comet assay on *L. minor*, and also both washing steps are approved because similar results are obtained after both tested washing steps. An electrophoresis time of 15 min is considered to be too long. This time was only tested on control plants, but all of the cell nuclei were damaged probably due to the long electrophoresis time. They all had a longer comet tail in comparison with the control plants after 5 or 10 min electrophoresis. The visual scoring based on 5 recognizable classes of comets (Table 6) clearly shows induction of DNA damage in the

MMS treated samples, with almost 68% of the nuclei in class 2, 3 and 4. However, this visual scoring requires some training. If this training is not provided, this technique seems to be quite subjective. A better way to score the comets is with the use of a specialized program such as Comet Assay IV (Score comets). By this, more reliable/consistent results are obtained. The comet assay can definitely be used in further investigations on *L. minor* and in the repetition of the 7 days exposure experiment and time kinetics experiment

CONCLUSION

The aim of this research was to validate the potential U-biomarker gene (primer 33) and to select and validate other potential U-biomarker genes for the freshwater plant *L. minor* when they are exposed to U in combination with an environmentally relevant metal mixture of metals (Cu, Ni, Zn and Pb) based on concentrations that were measured in the Beaverlodge Lake in Canada. Primers for potential U-biomarker genes were developed and tested on available cDNA of a previous U and metal mixture experiment. Five primers with an efficiency >90% and that reacted specifically to U were selected and further tested in two experiments, a 7 days exposure experiment and a time kinetics experiment.

In the 7 days exposure experiment *L. minor* plants were exposed for 7 days to a metal mixture in combination with U. In all U concentrations used, plants exposed to M1000 metal concentrations have >50% growth inhibition. This indicates high toxicity at these extremely high metal concentrations. As the initial goal of this thesis was to find biomarkers that can be used as early warning systems, this high metal concentration was excluded from further discussion.

All five primers were also tested in the time kinetics experiment whereby *L. minor* plants were exposed for 1, 4 or 7 days to three U concentrations (0, 2 and 10 µM) and/or three metal concentrations (M0, M1, M100). Fast-reacting biomarkers could be selected within this experiment.

The primers were analyzed on four criteria: 1) exposure dose-response relationship; 2) exposure time-response relationship; 3) sensitivity; and 4) chemical specificity. The earlier discovered primer 33 matches all four criteria and could be validated as biomarker for U exposure from both experiments. In addition, two new U-biomarker genes were selected within this project, i.e. primer 48 and primer 52 that matches three of the four criteria. Both primers reacted specifically to U already after 4 days of exposure and to the lowest tested U concentration (2 µM). The gene related to primer 48 has as function "response to oxidative stress". U causes oxidative stress in *L. minor* plants by an increased activity of different antioxidative enzymes [21]. But also Cd provokes oxidative stress. However, the antioxidative responses to U and Cd are not identical. More research with other metals in the environmentally metal mixture, including Cd, is necessary to further investigate the chemical specificity of this gene. The gene related to primer 52 has as function in the response to auxin. A hypothesis was made for *A. thaliana* plants, that U disrupts the transport and gradient of auxin in the root of the plants [104]. Also other metals, such as Cd, are known to influence the expression of auxin-related genes. The chemical specificity of this potential U-biomarker under U stress must be further investigated.

A comet assay on *L. minor* was also performed within this project as a possible biomarker for DNA damage after exposure to U. The feasibility of this technique was tested on *L. minor* plants exposed to MMS as a positive control for DNA damage and some of the parameters were optimized. An electrophoresis time of 5 or 10 min was approved. In both the control and the MMS condition cell nuclei were observed. Within the MMS condition, DNA damage was perceived with >30% in the highest class of the visual. In contrast, the control condition has most comets within class 0 and 1 and a few in class 2. However, the used visual scoring based on 5 recognizable classes seems to be quite subjective if no training is provided. A specialized program such as Comet Assay IV (Score comets) could give more reliable/consistent results. But the comet assay can definitely be used in further research on *L. minor*.

Future research should focus on further testing the selected genes under different environmental relevant scenarios, such as the addition of Cd in the metal mixture. This gives possibilities to study the chemical specificity of the genes. It could also be interested to study higher levels of biological complexity. Hereby the reaction of the proteins coded by these genes will be investigated to check if the effects are more than just a change in gene expression. A repetition of the experiments according to the OECD guidelines must be accomplished. This will possibly provide an explanation for the different growth results and could be used as validation of primer 48 and primer 52. However, U-biomarkers based on gene expression are not the only solution. A multiparametric approach is better to predict the outcome and is highly recommended in ecotoxicology. The ideal combination includes 'physiological' biomarkers, biomarkers of general stress and more specific biomarkers [105]. So a combination of the U-biomarker genes and the comet assay can be more applicable in various scenarios.

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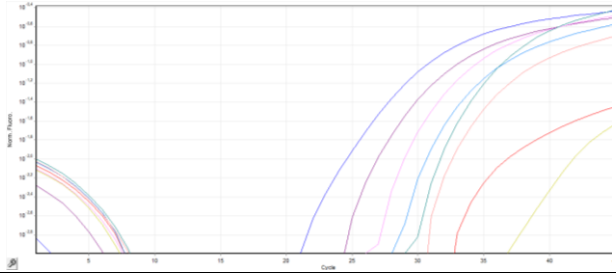
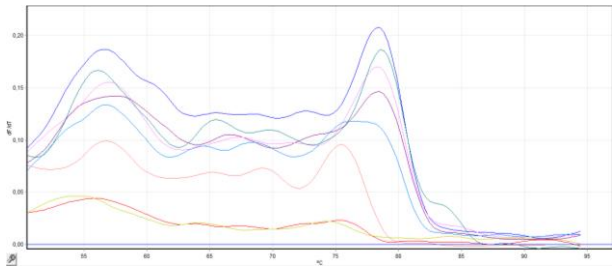
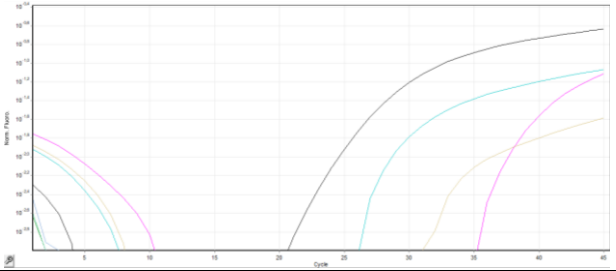
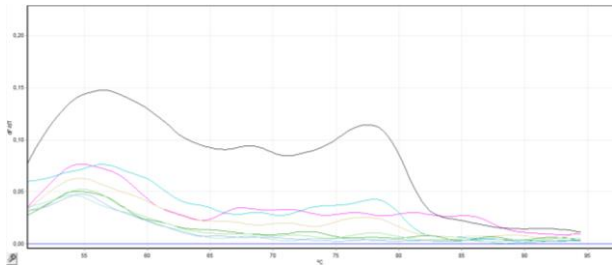
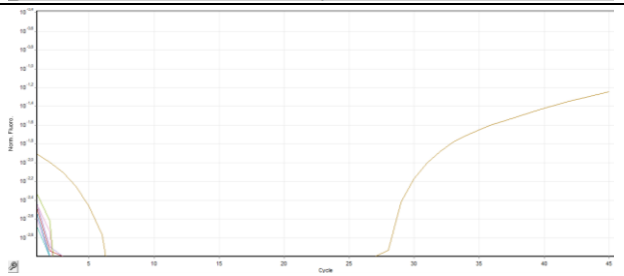
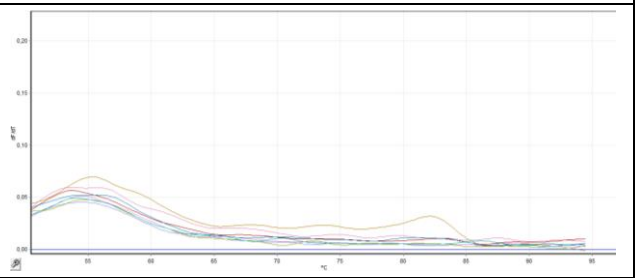
APPENDIX A: COMPOSITION OF THE CONTAMINATION SOLUTION OF THE 7 DAYS EXPOSURE EXPERIMENT AND THE TIME KINETICS EXPERIMENT

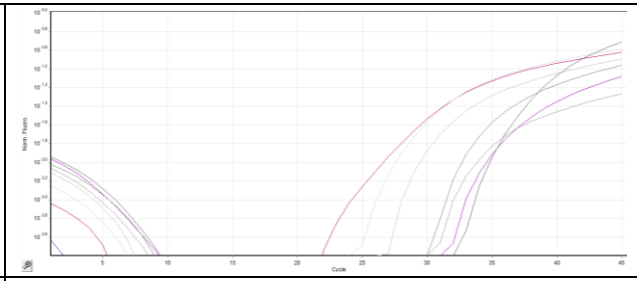
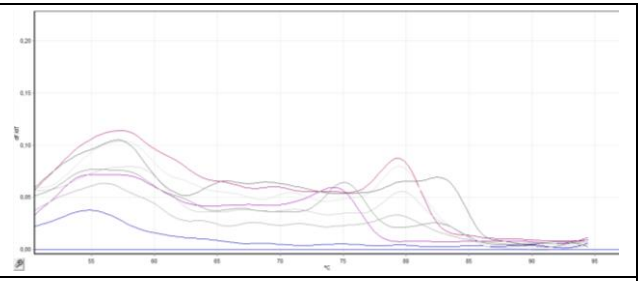
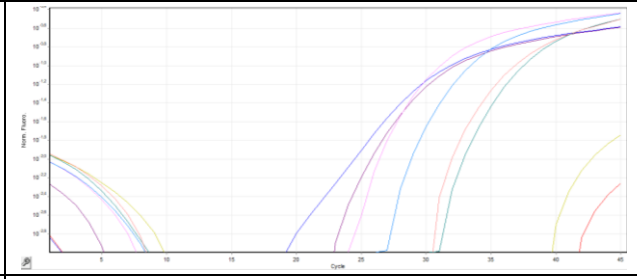
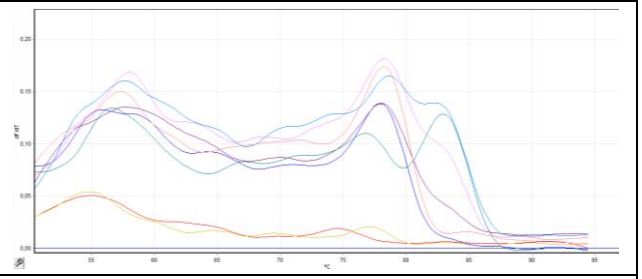
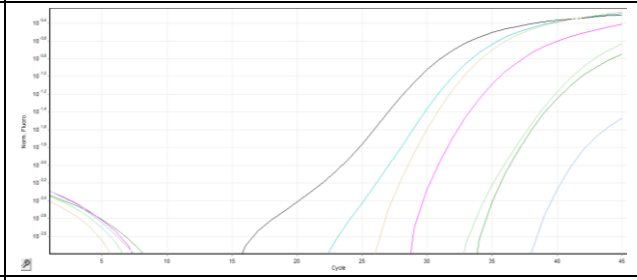
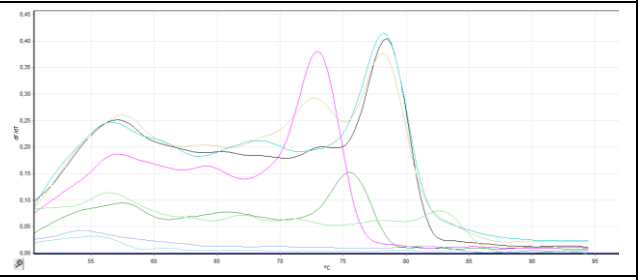
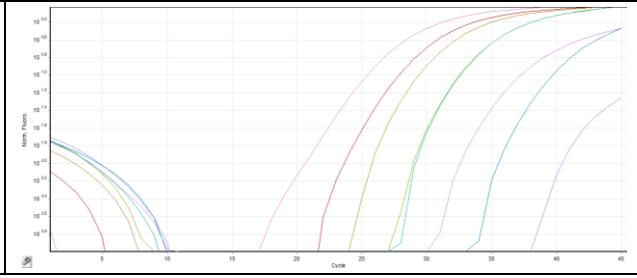
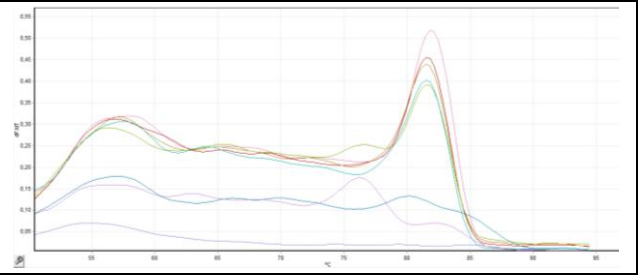
Table 11: Exact composition of the contamination solution used in the 7 days exposure experiment and the time kinetics experiment

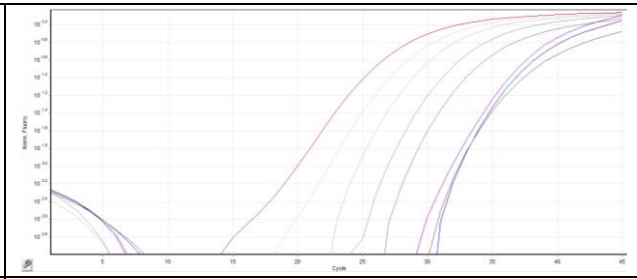
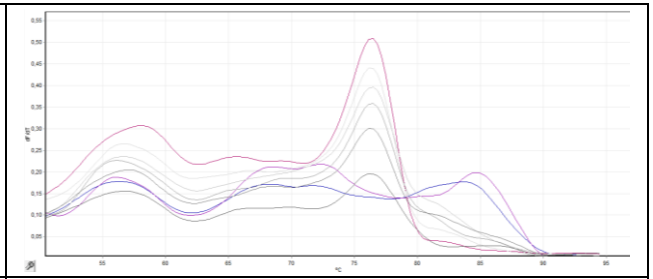
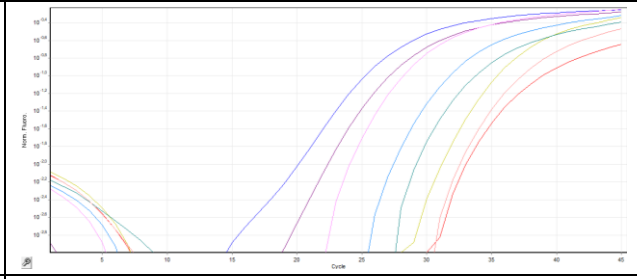
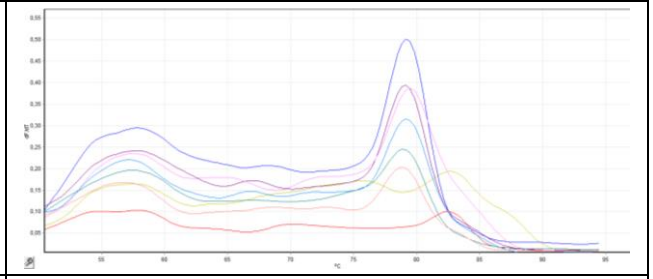
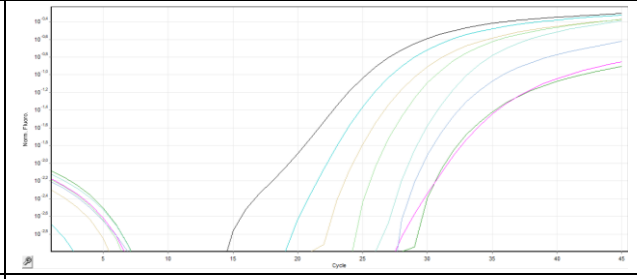
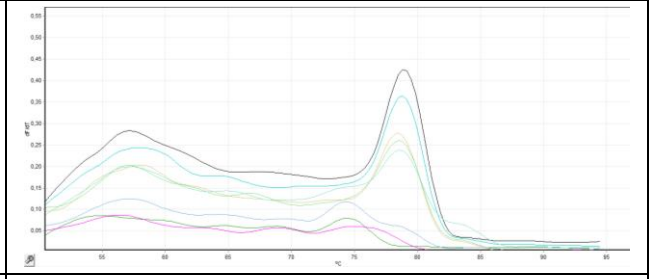
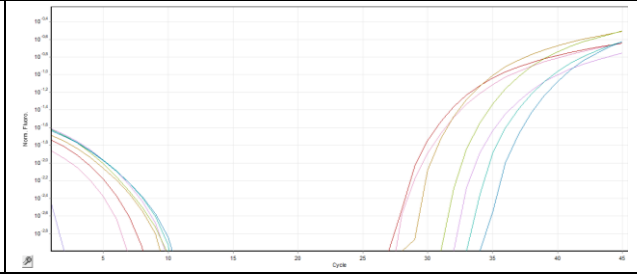
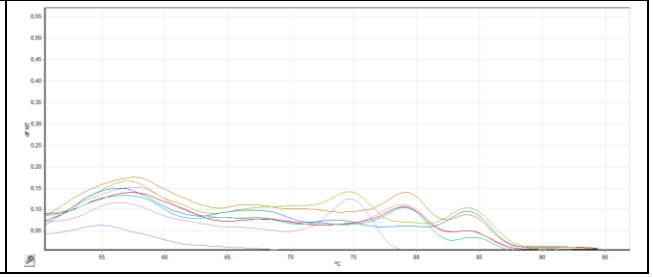
Condition	V M10000 stock solution (ml)	V U stock solution (ml)	V P05-medium (ml)	V MES (mL)
U0-M0	0	0	100	1
U0-M1	0.01	0	99.99	1
U0-M10	0.1	0	99.90	1
U0-M100	1	0	99.00	1
U0-M1000	10	0	90.00	1
U2-M0	0	0.02	99.98	1
U2-M1	0.01	0.02	99.97	1
U2-M10	0.1	0.02	99.88	1
U2-M100	1	0.02	98.98	1
U2-M1000	10	0.02	89.98	1
U10-M0	0	0.1	99.90	1
U10-M1	0.01	0.1	99.89	1
U10-M10	0.1	0.1	99.80	1
U10-M100	1	0.1	98.90	1
U10-M1000	10	0.1	89.90	1

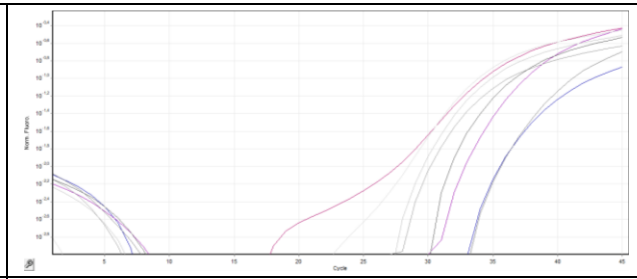
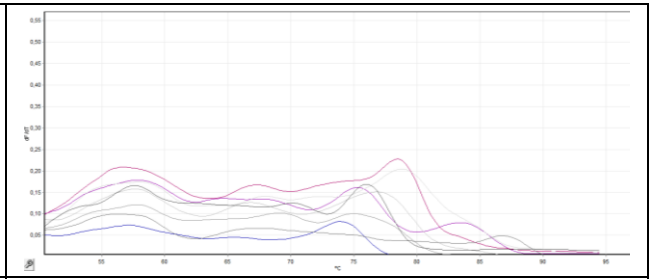
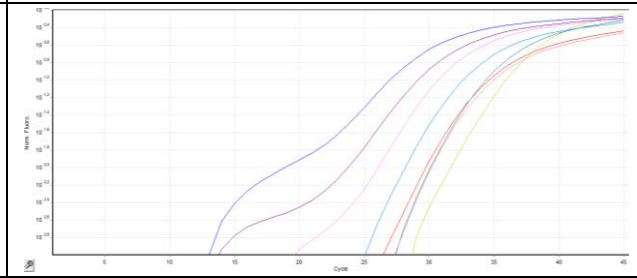
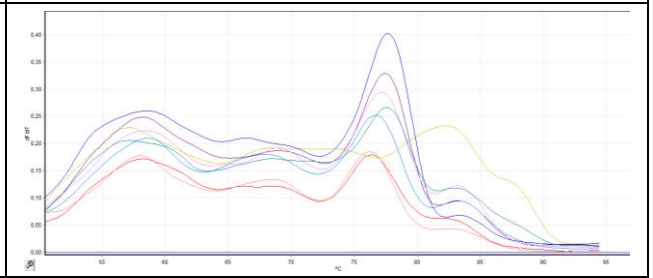
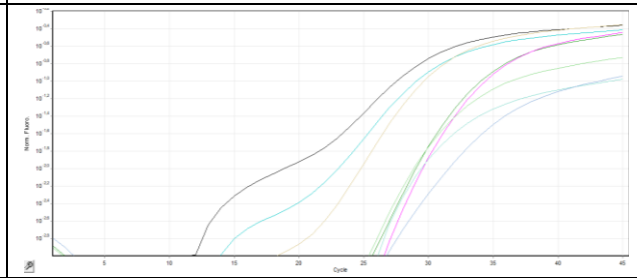
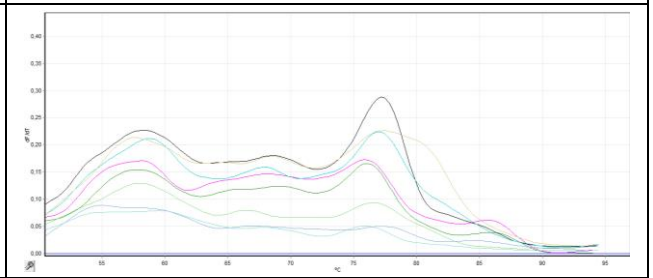
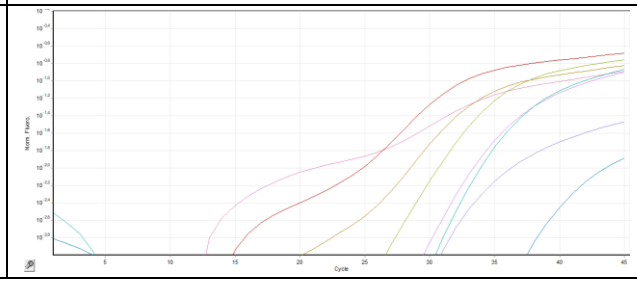
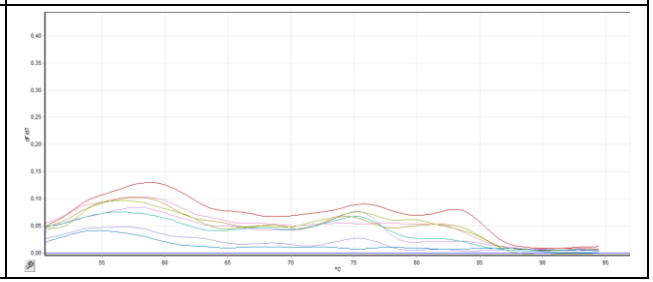
APPENDIX B: SPECIFICITY TESTING OF POTENTIAL HOUSEKEEPING GENES FOR *L. MINOR*

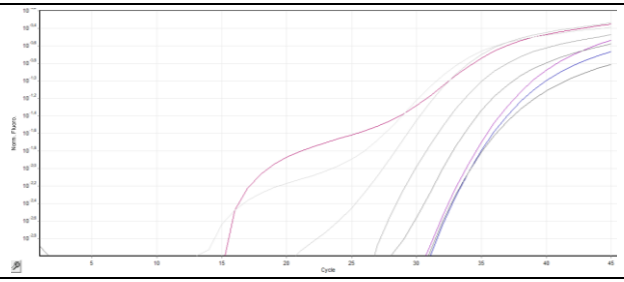
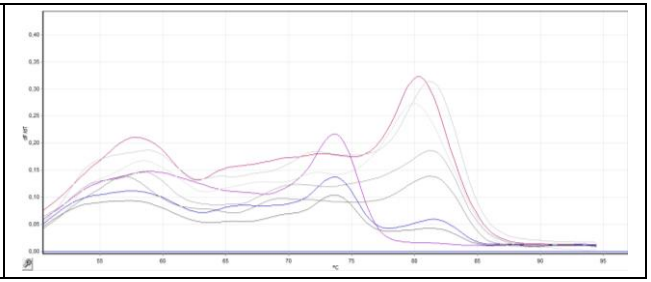
Table 12: Specificity testing of potential housekeeping genes for L. minor based on real-time qPCR (amplification plot and melt curve) and primer efficiencies (%) (NA = not applicable). All primers with a primer efficiency >90%, one peak in the melting curve and the concentration dependent expression of the dilution series starting from the highest concentration were further tested within this project.

Primer number	Primer efficiency (%)	Amplification plot	Melting curve
Primer 53	103.07		
Primer 54	NA		
Primer 55	NA		

Primer 56	97.96		
Primer 57	96.28		
Primer 58	NA		
Primer 59	92.90		

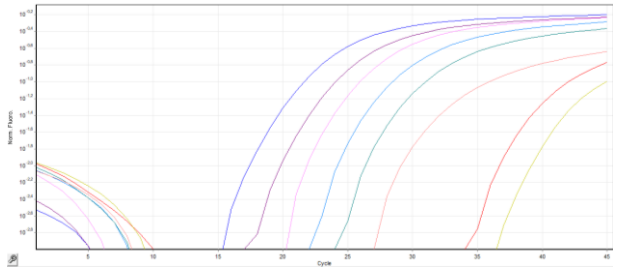
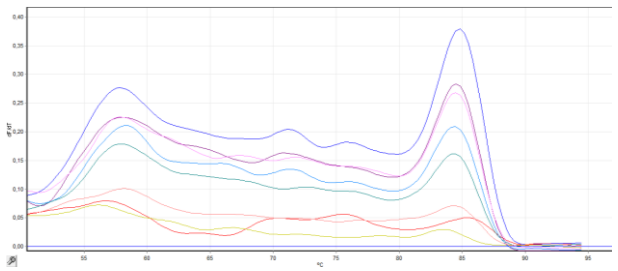
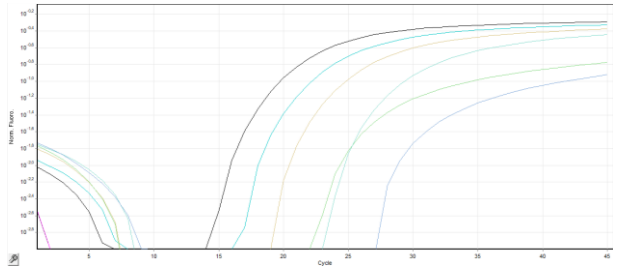
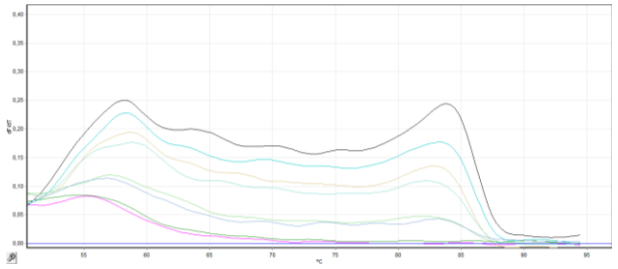
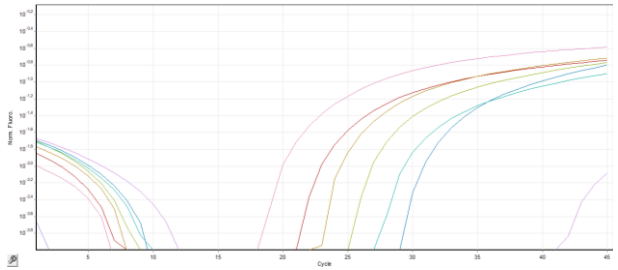
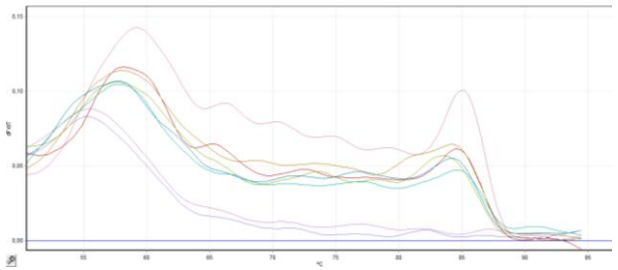
Primer 60	93.93		
Primer 61	86.30		
Primer 62	100.52		
Primer 63	143.82		

Primer 64	NA		
Primer 65	101.19		
Primer 66	138.75		
Primer 67	NA		

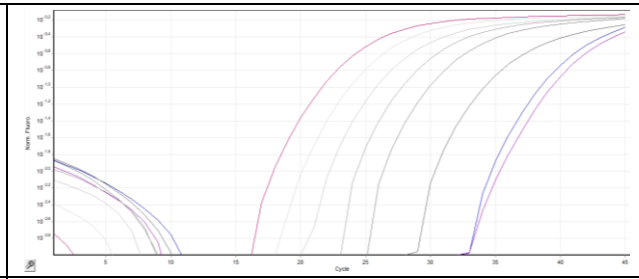
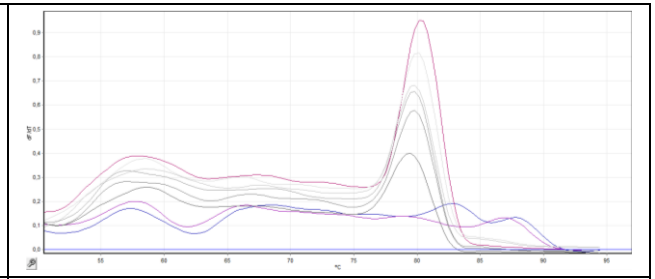
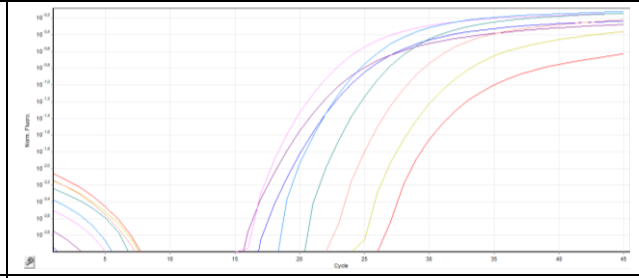
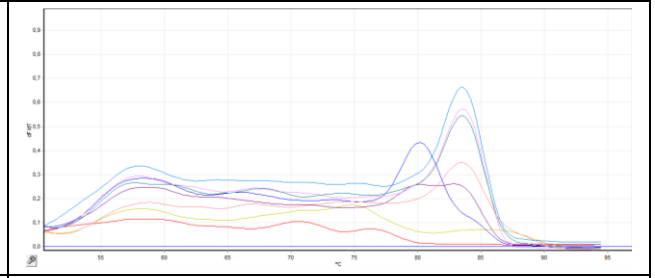
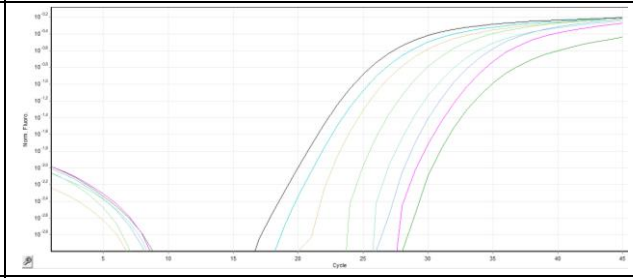
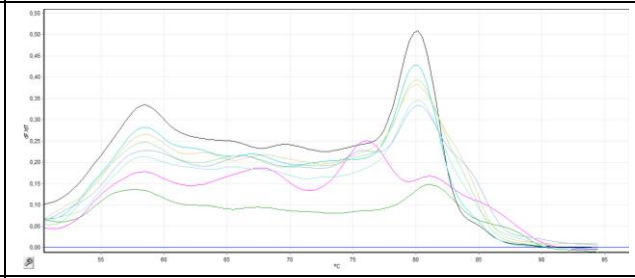
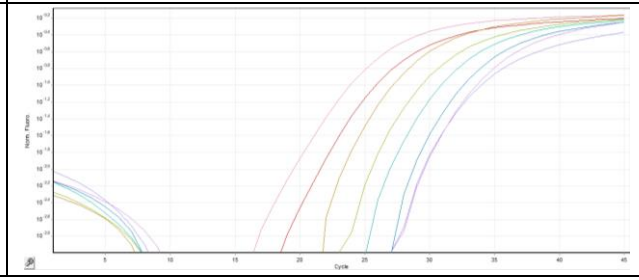
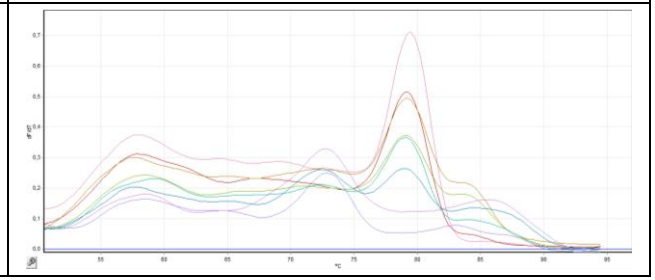
Primer 68	NA		

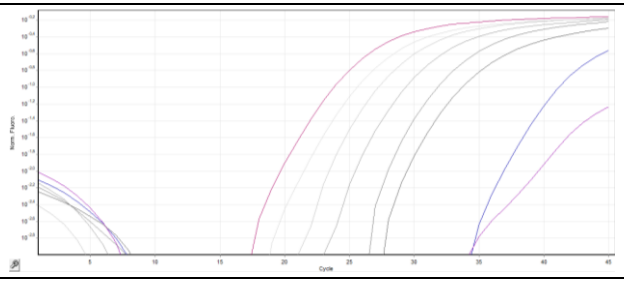
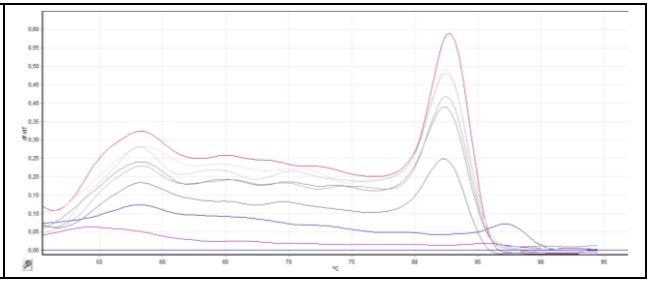
APPENDIX C: SPECIFICITY TESTING OF POTENTIAL U-BIOMARKER GENES FOR *L. MINOR*

Table 13: Specificity testing of potential U-biomarker genes for L. minor based on real-time qPCR (amplification plot and melt curve) and primer efficiencies (%) (NA = not applicable). All primers with a primer efficiency >90%, one peak in the melting curve and the concentration dependent expression of the dilution series starting from the highest concentration were further tested within this project.

Primer number	Primer efficiency (%)	Amplification plot	Melting curve
Primer 41	97.23		
Primer 42	75.47		
Primer 43	90.79		

Primer 44	98.13		
Primer 45	NA		
Primer 46	110.54		
Primer 47	97.94		

Primer 48	90.44		
Primer 49	NA		
Primer 50	118.93		
Primer 51	110.18		

Primer 52	97.98		

APPENDIX D: GROWTH OF *L. MINOR* PLANTS IN 7 DAYS EXPOSURE EXPERIMENT

Table 14: Growth measurements (total area and number of fronds) of L. minor plants at day 0, 4 and 7 in 7 days exposure experiment

Condition	Day 0		Day 4		Day 7	
	Total area (mm^2)	Number of fronds	Total area (mm^2)	Number of fronds	Total area (mm^2)	Number of fronds
U0-M0-a	37.990	13	108.595	37	283.547	76
U0-M0-b	32.652	11	110.985	32	273.573	66
U0-M0-c	39.010	12	123.751	34	294.527	74
U0-M0-d	33.694	9	107.830	26	246.426	59
U0-M0-e	38.403	12	140.146	35	326.685	74
U0-M0-f	35.465	12	140.065	38	326.848	76
U0-M1-a	39.580	13	136.389	33	342.587	78
U0-M1-b	38.315	14	123.249	39	310.609	80
U0-M1-c	36.214	12	108.544	31	311.444	76
U0-M1-d	39.203	15	161.345	46	383.685	88
U0-M10-a	31.297	12	120.972	31	307.545	72
U0-M10-b	30.225	9	99.111	26	227.154	50
U0-M10-c	34.417	10	122.559	31	282.028	65
U0-M10-d	36.448	14	128.441	36	324.491	75
U0-M100-a	37.030	13	113.383	35	262.652	76
U0-M100-b	31.614	11	106.329	31	248.923	68
U0-M100-c	32.828	11	113.168	29	235.286	63
U0-M100-d	34.178	10	113.204	28	234.256	63
U0-M1000-a	38.998	13	55.448	22	70.973	34
U0-M1000-b	37.019	12	51.246	23	63.747	33
U0-M1000-c	31.895	10	44.805	16	54.826	25
U0-M1000-d	37.334	14	49.154	20	60.286	30

U2-M0-a	35.297	12	89.136	31	166.802	64
U2-M0-b	29.913	10	66.646	24	133.978	51
U2-M0-c	33.726	12	85.585	27	180.523	57
U2-M0-d	36.386	11	90.106	34	204.485	68
U2-M1-a	34.087	11	130.638	36	329.801	76
U2-M1-b	38.194	11	120.453	35	290.320	73
U2-M1-c	35.989	10	106.873	32	246.996	64
U2-M1-d	40.983	12	110.645	34	304.928	73
U2-M10-a	45.572	11	155.195	34	385.397	78
U2-M10-b	38.628	13	129.951	40	323.422	80
U2-M10-c	35.983	9	126.551	27	354.121	62
U2-M10-d	32.463	9	116.844	28	307.260	62
U2-M100-a	35.629	9	108.131	27	274.349	63
U2-M100-b	30.417	12	111.576	34	312.452	76
U2-M100-c	35.274	11	127.282	30	328.770	69
U2-M100-d	32.505	12	116.794	32	281.766	67
U2-M1000-a	37.218	9	57.664	22	96.502	43
U2-M1000-b	33.427	10	55.212	23	89.873	40
U2-M1000-c	35.529	11	59.450	26	99.219	51
U2-M1000-d	34.511	12	57.997	31	81.804	45
U10-M0-a	34.247	13	94.004	28	193.699	54
U10-M0-b	29.850	9	63.853	22	128.737	43
U10-M0-c	29.478	11	69.262	27	126.405	46
U10-M0-d	31.643	12	74.270	28	147.527	52
U10-M1-a	33.669	13	83.013	31	167.590	53
U10-M1-b	37.728	11	107.597	31	225.677	60
U10-M1-c	33.004	12	92.044	29	193.413	54
U10-M1-d	34.793	11	92.138	33	183.757	59
U10-M10-a	38.013	14	102.978	34	209.783	59

U10-M10-b	34.244	9	84.773	25	148.753	47
U10-M10-c	36.258	11	99.114	28	196.432	54
U10-M10-d	43.689	14	129.580	42	252.552	66
U10-M100-a	24.291	9	54.722	18	98.767	33
U10-M100-b	34.262	9	80.938	25	164.025	49
U10-M100-c	29.672	11	76.487	25	144.005	43
U10-M100-d	38.341	12	111.254	29	202.471	61
U10-M1000-a	26.992	10	42.672	23	58.852	34
U10-M1000-b	35.610	13	55.269	29	89.314	43
U10-M1000-c	42.205	11	69.859	27	112.423	49
U10-M1000-d	35.000	11	57.765	27	83.784	40

APPENDIX E: GROWTH OF *L. MINOR* PLANTS IN TIME KINETICS EXPERIMENT

Table 15: Growth measurements (total area and number of fronds) of L. minor plants (sampled at day 1) at day 0 and 1 in time kinetics experiment

Condition	Day 0		Day 1	
	Total area (mm^2)	Number of fronds	Total area (mm^2)	Number of fronds
U0-M0-a	38.645	12	47.436	13
U0-M0-b	42.230	14	55.664	17
U0-M0-c	30.760	9	38.576	13
U0-M0-d	34.460	10	44.288	13
U0-M0-e	36.618	10	47.181	13
U0-M0-f	28.211	8	35.199	10
U0-M0-g	34.458	10	41.602	14
U0-M0-h	35.372	10	44.656	14
U0-M0-i	41.774	12	54.062	16
U0-M0-j	44.797	12	57.662	18
U0-M0-k	31.558	9	38.446	11
U0-M0-l	37.098	11	47.840	12
U0-M1-a	45.147	14	59.223	16
U0-M1-b	34.924	10	45.730	11
U0-M1-c	35.673	11	47.038	14
U0-M1-d	49.410	15	62.184	20
U0-M1-e	42.327	12	52.873	16
U0-M1-f	44.052	13	58.067	14
U0-M1-g	34.907	10	43.862	13
U0-M100-a	45.078	14	60.228	17
U0-M100-b	38.387	12	47.915	16
U0-M100-c	39.517	13	51.850	17
U0-M100-d	41.678	12	54.735	15

U0-M100-e	44.090	12	54.174	18
U0-M100-f	30.868	10	40.535	12
U0-M100-g	41.652	13	54.761	14
U2-M0-a	41.705	12	56.361	15
U2-M0-b	44.858	11	59.744	15
U2-M0-c	49.923	13	60.431	17
U2-M0-d	44.113	13	58.856	16
U2-M0-e	34.623	11	45.116	12
U2-M0-f	35.247	10	42.433	12
U2-M0-g	43.208	11	54.534	15
U2-M1-a	37.002	11	47.711	14
U2-M1-b	47.699	11	65.215	20
U2-M1-c	43.847	14	57.481	16
U2-M1-d	39.193	13	52.297	14
U2-M1-e	34.009	10	46.755	14
U2-M1-f	47.436	13	56.403	16
U2-M1-g	46.091	15	60.675	19
U2-M100-a	41.325	13	57.863	15
U2-M100-b	46.084	13	59.769	18
U2-M100-c	43.160	11	56.291	14
U2-M100-d	44.025	12	58.445	15
U2-M100-e	30.934	9	37.549	12
U2-M100-f	40.961	12	55.405	15
U2-M100-g	39.233	11	49.006	14
U10-M0-a	37.399	12	47.621	15
U10-M0-b	38.076	11	50.807	15
U10-M0-c	43.354	12	55.969	17
U10-M0-d	46.816	13	60.261	16
U10-M0-e	41.036	13	52.316	16
U10-M0-f	30.374	9	37.705	12

U10-M0-g	47.957	13	59.124	16
U10-M1-a	37.604	10	46.925	13
U10-M1-b	36.961	13	44.480	15
U10-M1-c	37.373	10	47.690	12
U10-M1-d	30.163	10	35.648	13
U10-M1-e	43.245	13	56.284	17
U10-M1-f	43.749	13	56.447	16
U10-M1-g	35.948	14	43.299	15
U10-M100-a	39.735	12	50.216	16
U10-M100-b	32.053	9	40.64	11
U10-M100-c	36.192	10	46.783	13
U10-M100-d	34.342	11	43.081	14
U10-M100-e	40.593	10	53.628	13
U10-M100-f	41.767	10	52.105	13
U10-M100-g	44.897	13	59.268	16

Table 16: Growth measurements (total area and number of fronds) of L. minor plants (sampled at day 4) at day 0 and 4 in time kinetics experiment

Condition	Day 0		Day 4	
	Total area (mm^2)	Number of fronds	Total area (mm^2)	Number of fronds
U0-M0-a	35.462	13	110.848	34
U0-M0-b	40.221	11	119.838	32
U0-M0-c	39.186	9	107.017	25
U0-M0-d	36.285	11	131.712	30
U0-M0-e	41.389	8	83.468	21
U0-M0-f	32.460	10	102.674	26
U0-M1-a	41.188	11	108.949	32
U0-M1-b	39.974	11	123.076	29
U0-M1-c	32.723	10	101.077	25
U0-M1-d	40.981	13	125.547	34
U0-M100-a	38.077	10	117.813	27
U0-M100-b	41.441	13	130.784	32
U0-M100-c	31.841	9	92.787	24
U0-M100-d	40.108	12	113.035	28
U2-M0-a	49.443	13	130.572	34
U2-M0-b	40.706	11	116.471	23
U2-M0-c	36.201	10	96.757	26
U2-M0-d	40.030	11	102.216	26
U2-M1-a	44.582	13	135.932	33
U2-M1-b	36.798	9	98.539	23
U2-M1-c	38.033	10	126.808	28
U2-M1-d	39.184	10	112.979	25
U2-M100-a	39.666	10	99.009	24
U2-M100-b	43.908	12	112.134	33
U2-M100-c	43.977	14	124.573	34

U2-M100-d	39.002	10	103.167	25
U10-M0-a	35.183	11	77.643	26
U10-M0-b	37.560	12	85.684	29
U10-M0-c	30.195	9	69.489	22
U10-M0-d	36.732	11	82.818	24
U10-M1-a	39.969	12	100.567	26
U10-M1-b	32.646	9	68.531	21
U10-M1-c	38.308	11	99.344	29
U10-M1-d	31.559	9	79.507	23
U10-M100-a	43.554	11	104.572	29
U10-M100-b	39.766	11	110.332	28
U10-M100-c	32.750	9	67.103	22
U10-M100-d	40.885	12	107.054	30

Table 17: Growth measurements (total area and number of fronds) of L. minor plants (sampled at day 7) at day 0, 4 and 7 in time kinetics experiment

Condition	Day 0		Day 4		Day 7	
	Total area (mm^2)	Number of fronds	Total area (mm^2)	Number of fronds	Total area (mm^2)	Number of fronds
U0-M0-a	39.566	10	125.635	30	336.561	69
U0-M0-b	33.151	9	111.078	25	300.449	66
U0-M0-c	38.830	10	134.340	31	362.986	75
U0-M0-d	31.610	12	113.007	33	324.381	82
U0-M0-e	32.113	9	107.216	24	258.433	58
U0-M0-f	38.535	11	121.310	27	358.259	75
U0-M1-a	35.868	11	100.131	25	279.348	63
U0-M1-b	39.714	11	114.549	29	323.787	75
U0-M1-c	30.456	11	84.528	26	247.468	61
U0-M1-d	36.700	9	104.170	24	283.789	62
U0-M100-a	33.150	8	100.748	25	234.099	62
U0-M100-b	31.571	10	91.670	27	217.478	65
U0-M100-c	42.407	11	114.217	29	240.993	73
U0-M100-d	37.837	11	106.558	28	252.673	63
U2-M0-a	36.343	11	115.509	28	363.913	72
U2-M0-b	41.672	11	118.884	27	338.230	63
U2-M0-c	33.365	10	104.276	26	326.692	66
U2-M0-d	35.968	10	96.876	24	290.136	58
U2-M1-a	40.674	10	131.801	29	395.376	70
U2-M1-b	32.662	9	95.781	23	269.224	59
U2-M1-c	38.112	11	104.510	28	307.885	68
U2-M1-d	34.135	8	95.846	22	286.781	53
U2-M100-a	35.103	10	107.651	26	332.524	67
U2-M100-b	41.965	14	104.273	31	293.226	73
U2-M100-c	34.143	11	101.362	26	318.465	61

U2-M100-d	42.096	12	120.781	33	352.153	79
U10-M0-a	38.921	11	109.693	28	276.513	65
U10-M0-b	43.266	11	100.790	28	255.229	60
U10-M0-c	42.467	12	101.195	28	232.440	60
U10-M0-d	36.017	12	97.437	31	246.129	63
U10-M1-a	29.125	9	67.677	23	162.284	51
U10-M1-b	39.804	12	98.059	24	232.670	52
U10-M1-c	40.491	13	104.923	31	240.265	66
U10-M1-d	37.399	10	84.016	24	199.188	53
U10-M100-a	36.378	10	87.272	26	211.715	53
U10-M100-b	41.936	11	94.021	25	217.070	55
U10-M100-c	33.779	10	75.070	27	185.724	53
U10-M100-d	37.701	10	80.342	26	194.800	56