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FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

Toxic effects induced in *Lemna minor* by an environmentally relevant mixture of uranium and metals: selection of potential uranium biomarker genes

Promotor :
Prof. dr. Ann CUYPERS

Promotor :
prof. dr. NELE HOREMANS

Elke Knaepen

Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen

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Samenvatting

Antropogene activiteiten hebben wereldwijd gezorgd voor uraniumniveaus die hoger zijn dan de natuurlijk voorkomende concentraties. In normale omstandigheden worden organismen blootgesteld aan een mix van verschillende chemicaliën. Het primaire doel van deze studie is daarom te onderzoeken hoe uranium bijdraagt aan de toxiciteit die geïnduceerd wordt in een mengsel bestaande uit uranium en andere metalen. Dit werd onderzocht met behulp van de vasculaire zoetwaterplant *Lemna minor*, ook wel gekend als klein kroos. Recent werd het *Lemna minor* genoom ontdekt waarna een RNAseq experiment uitgevoerd werd voor planten blootgesteld aan uranium, bèta- en gammastraling. Dit stelt de mogelijkheid open voor het ontwerpen van genen die mogelijk gebruikt kunnen worden als biomerkers voor uraniumblootstelling.

Voor deze studie werd er een *Lemna minor* groei-inhibitietest uitgevoerd om volledige dosis-respons curves op te stellen van een selectie individuele metalen, namelijk zink, nikkel, uranium, lood en koper. De concentraties hiervoor werden gekozen op basis van een literatuurstudie en een oriënterend experiment om te weten te komen in welke range ongeveer toxiciteit optreedt. Voor het mengselexperiment van uranium/metalen werd een experimentele setup gebruikt die gebaseerd is op stalen genomen uit Beaverlodge Lake (northern Saskatchewas, Canada), zodat het onderzoek zou gebeuren op relevante concentraties die voorkomen in de natuur nabij voormalige uraniummijnen. De concentraties in dit mengsel werd dan vermenigvuldigd met 10, 100 en 1000. De bijdrage van uranium aan de totale toxiciteit van het mengsel werd onderzocht door drie groeiparameters te vergelijken. Na optimalisatie van de primers werden de expressieniveaus van de mogelijke referentiegenen en biomerkers voor uranium geanalyseerd met behulp van real-time qPCR.

De dosis-respons curves voor zowel de individuele metalen als de metaalmengsels werden gebaseerd op drie groeiparameters van *Lemna minor*, namelijk de totale oppervlakte van de bladschijfjes, het aantal bladschijfjes en ook de totale biomassa van de bladschijfjes. De groei-inhibitie gebaseerd op het aantal bladschijfjes is minder dan die gebaseerd op de oppervlakte of biomassa. Na de primeroptimalisatie werden er zes referentiegenen en zes mogelijke biomerkers voor uranium geselecteerd. Van de zes biomerkergenen vertoonden er drie een up- en drie een downregulatie na blootstelling met uranium. Eén gen in het bijzonder reageerde zeer sterk na blootstelling met enkel uranium zowel individueel blootgesteld als in een mengsel, terwijl de andere genen ook een respons gaven na blootstelling met andere metalen.

Om te concluderen kan men zeggen dat het aantal bladschijfjes een minder gevoelige groeiparameter is dan de oppervlakte of biomassa. Ondanks het feit dat er maar een beperkt aantal genen getest werd in deze studie, kon er toch één gen geïdentificeerd worden dat veelbelovend is als mogelijke biomerker voor uranium. Het mengsel van uranium met metalen vertoonde aanwijzingen voor antagonistische groei-effecten in vergelijking met de blootstelling van individuele metalen door een mogelijke interactie op het niveau van uranium- en metaalopname.

Abstract

Introduction: Worldwide anthropogenic activities have caused increased uranium levels beyond naturally occurring concentrations. Under natural conditions, organisms are usually exposed to a mixture of chemicals. The aim of this study is to investigate on *Lemna minor* the contribution of uranium in the toxicity induced by a uranium/metal mixture in environmental relevant concentrations. Only recently a draft *Lemna minor* genome was obtained and an RNAseq experiment was conducted on plants exposed to uranium, beta and gamma radiation. This enabled the search for genes that can potentially be used as biomarkers for uranium exposure.

Material & methods: A *Lemna minor* growth inhibition test was used first to set up complete dose-response curves of a selection of individual metals. Concentrations were chosen based on a literature search and a range finding experiment. For the uranium/metal mixture, the set-up was mimicking existing conditions present at a uranium-mining site. The contribution of uranium to the mixture toxicity was compared based on growth parameters. After primer optimisation, expression levels of potential reference genes and uranium biomarkers were analysed with qPCR.

Results: Single dose-response curves and the multiple metal mixtures showed growth inhibition based on frond area, number of fronds and biomass. Based on number of fronds, growth inhibition seemed less than based on area or biomass. After primer optimisation, six reference genes and six uranium biomarkers were selected. Three biomarker genes showed up-, and three genes downregulation when exposed to uranium. One gene reacted more strongly after uranium exposure alone whereas the others were sensitive to several metals.

Discussion & conclusions: Frond number is a less sensitive growth parameter than frond area and biomass. Despite the limited number of genes tested, one gene could be identified with promising features as potential biomarker. The U/metal mixture showed signals for antagonist growth effects compared to the single exposures possibly due to interaction at the level of U/metal uptake.

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Abbreviations

$^{\bullet}OH$	Hydroxyl radical
$^{1}O_2$	Singlet oxygen
APX	Ascorbate peroxidase
As	Arsenic
AsA	Ascorbate
ATP	Adenosine triphosphate
ATSDR	Agency for Toxic Substances and Disease Registry
Ba	Barium
BER	Base excision repair
BIS	Biosphere Impact Studies
CA	Concentration addition
CAT	Catalase
CO_2	Carbon dioxide
Cu	Copper
DL	Dose level
DR	Dose ratio
DRC	Dose-response curve
DSB	Double-strand break repair
ECx	Effective concentration on x% of the population
EPA	Environmental Protection Agency
FANC	Federal Agency for Nuclear Control
FDA	Food and Drug Administration
Fe	Iron
G3P	Glyceraldehyde 3-phosphate
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
H_2O_2	Hydrogen peroxide
IA	Independent action
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma optical emission spectrometry
MES	2-(*N*-morpholino)ethanesulfonic acid
MMR	Mismatch repair
Mn	Manganese
Mo	Molybdenum
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NER	Nucleotide excision repair
NCBI	National Center for Biotechnology Information
Ni	Nickel
O_2	Oxygen
$O_2^{\bullet-}$	Superoxide
OECD	Organisation for Economic Co-operation and Development
Pb	Lead
qPCR	Quantitative real-time polymerase chain reaction
ROS	Reactive oxygen species
S/A	Synergism/antagonism
Se	Selenium
SE	Standard error
SOD	Superoxide dismutase
Sr	Strontium
Th	Thorium
U	Uranium
UF_6	Uranium hexafluoride
U_3O_8	Triuranium octoxide
UO_2^{2+}	Uranyl cation
UO_2CO_3	Uranyl carbonate
UO_2OH^{+}	Uranyl hydroxide
WHO	World Health Organization
Zn	Zinc

1. Introduction

Due to anthropogenic activities many regions worldwide have been contaminated with pollutants like metals, organic compounds, radioactive substances, etc. Uranium (U) is an example of a contaminating metal that is also radioactive. Although U is naturally present in the environment, contamination and hence increased levels compared to background finds its origin in diverse anthropogenic activities, such as U mining, milling, and processing of U-containing ores e.g. phosphate ores[1]. However, U is seldom the only contaminant at a certain location[2]. Usually an increase in a number of different contaminants e.g. U and other heavy metals is generally the case. Therefore, the main aim of this work is to investigate the effects on plants induced by the **co-occurrence of U with metals** in a freshwater environment. Contamination of freshwaters with metals can have harmful effects to aquatic plants and associated microbiota, which in turn can be disadvantageous for oxygen production, nutrient cycling or sediment stability[3]. To assess the adverse effects on plants in a freshwater environment, we used the **freshwater vascular plant** *Lemna minor* as test organism. This will be discussed in more detail below.

1.1 Metals

Metals are highly abundant in the environment due to natural occurrence and anthropogenic sources. **Natural sources** of increased metal levels include rock weathering, volcanic eruptions or windblown dust, whereas **anthropogenic sources** include agricultural (use of inorganic fertilizers, phosphate fertilizers or pesticides) and industrial activities (mining, milling or smelting)[4, 5]. Some metals, such as copper (Cu), zinc (Zn), iron (Fe), lead (Pb), nickel (Ni), manganese (Mn) and molybdenum (Mo), have important biochemical and physiological roles in plants and animals. They have for example indirect functions in redox reactions, or direct ones as a co-factor of important enzymes[5]. Although some metals are essential nutrients, excess concentrations are toxic to various organisms[6, 7]. Freshwaters can contain high concentrations of toxic metals and other pollutants, because agricultural, mining, urban and industrial wastes usually end up in aquatic environments[8]. Metals that end up in aquatic systems generally bind to particulate matter which deposits in the sediments. Therefore, surface sediment in an aquatic environment is the most important reservoir of metals and other pollutants[9, 10].

Determination of the **ecotoxicological risk** metals can pose to organisms is not possible by chemical analyses of water samples alone, because these tests do not provide information on toxicity to the environment. Therefore, ecotoxicological tests must be performed on living organisms to understand the genetic and physiological responses of heavy metals in exposed organisms[11]. Organisms that are used to evaluate the toxicity of a medium must be representative of one or more properties of the ecosystem in which they live in. These organisms are classified as ecotoxicological indicators[12]. *L. minor* is an example of such indicator because it is representative of a mesotrophic environment[13]. Although the ecotoxicological tests are indicative for metal exposure, and thus a measure of the quality of their environment, they are often not so sensitive or so specific for one metal[12]. This will be further explained in paragraph 1.6.

1.1.1 Uranium

U is a radioactive metal that is naturally present in rocks, soil and water. It can be released in the environment through wind and water erosion and volcanic eruptions. In addition to being a metal all U isotopes are radioactive. Naturally present U mostly occurs as uranium-238 (99.27%), uranium-235 (0.72%) and uranium-234 (0.006%)[14-16]. It slowly decays by **alpha particle emission** with a physical half-life of 4.5 billion years for uranium-238. Due to this very long half-life, natural U has a low level of radioactivity, and thus has greater **chemical toxicity** than radiological toxicity[17, 18]. The average concentration of U in soil and rock varies between 300 µg kg^{-1} and 4.2 mg kg^{-1} [19, 20]. In seawater, the average concentration is 3.3 µg L^{-1} [21]. In freshwater, concentrations of dissolved U vary between 0.03 and 2.1 µg L^{-1} [18, 22]. Due to (inactive) industries and other anthropogenic activities, U concentrations can exceed these normal background levels and hence pose potential health and **ecological problems**[18, 23, 24]. In the proximity of former U mines, U concentrations can rise to 450 µg L^{-1} [25]. Therefore, to enhance protection of the public, organisations such as the Agency for Toxic Substances and Disease Registry (ATSDR), the Food and Drug Administration (FDA), and the World Health Organization (WHO) have set **regulations and guidelines** to keep U concentrations below toxic levels[23]. However, according to the Federal Agency for Nuclear Control (FANC), no standard values have been established in Europe and Belgium regarding the maximum U concentration allowed in groundwater. In the Netherlands a value of 0.01 µg L^{-1} is the reference for U in groundwater. In comparison, in the U.S. applies a limit of 30 µg L^{-1} for U in groundwater. The differences in these values can be traced back to the assumption that U is less abundant in Dutch soils. The reference value for U in drinking water has been set at 15 µg L^{-1} by the WHO.

Toxicity on humans

Humans can be exposed to U via air, water, soil and food. Health effects, resulting from both uranium radiation and the chemical risk as a heavy metal, can be categorised in carcinogenic and non-carcinogenic effects[26]. Mammalian organ systems such as kidney, liver, respiratory, cardiovascular and central nervous system can be affected by U. The extent of the damage depends on dose, route of administration and form of U. The most dangerous compounds such as uranium hexafluoride (UF_6) are soluble in organic fluids, while the most commonly found forms such as uranyl cation (UO_2^{2+}) and triuranium octoxide (U_3O_8) are insoluble[16]. Insoluble reduced U compounds have a very low bioavailability, which makes them less toxic than soluble oxidised U[16].

Toxicity on plants

Although U is a non-essential element, it can be taken up into plants. The concentration of U depends on the plant species, but in general, the concentration in roots is much higher than in leaves or seeds[19, 27-30]. Various studies have investigated the uptake of U by terrestrial plants[19, 31-33]. However, limited studies have been conducted involving bioaccumulation of U in aquatic plants[34]. Plants can accumulate U during growth and development processes, but the rate of U uptake is strongly related to environmental factors, such as the pH[30]. The highest U accumulation occurs in plants growing in soils with a pH of 5.5 or less[35] [27]. Therefore, this acidic environment causes the highest U toxicity[36]. U can be present in a wide range of chemical species, but it mostly exist in 3 predominant species: uranyl cation (UO_2^{2+}), uranyl hydroxides (e.g., UO_2OH^+, $[UO_2]_3[OH]_7^-$), and uranyl carbonates (e.g.,

UO_2CO_3, $UO_2[CO_3]_2^-$)[27]. Under neutral conditions, the mainly formed U species are hydroxide complexes and phosphate complexes. The main U species found under acidic conditions is UO_2^{2+} [27]. Exposure to U can induce a variety of toxic effects such as, among others, induction of DNA damage, oxidative stress or inhibition of photosynthesis[18]. Evidence for U-induced **oxidative stress** in plants has been found for example in the upregulation of scavenging enzymes for reactive oxygen species (ROS)[37]. These ROS have both beneficial and harmful effects in plants, which will be further explained in paragraph 1.3.

1.1.2 Copper

Toxic metals such as Cu tend to accumulate in sediments[38]. In aquatic environments, an estimated 60% of Cu can be found in sediments which is in general not bio-available[39]. In general, Cu has an average concentration of 0.44 µg L^{-1} in freshwaters[40]. Moreover, the bioavailability and hence the degree of toxicity of the remaining Cu will further depend on different water characteristics like pH[39]. Cu is an essential nutrient to all organisms including plants, which plays important roles in cell physiology, such as taking part in photosynthesis, electron flow and catalysis of redox reactions[4, 5, 7]. It is also involved in different metabolic processes as a structural component of many proteins and enzymes. However, Cu can be toxic at high concentrations leading e.g. to the formation of free radicals. These free radicals can on their turn induce oxidative stress during for example the oxidative splitting of polyunsaturated fatty acids[39, 41]. Cu can also inhibit photosynthesis, which leads to plant growth retardation and leaf chlorosis[5, 39].

1.1.3 Nickel

Another essential micronutrient for plant growth and development is Ni. It serves for example as a cofactor for urease and is present in several Ni-containing enzymes in plants and bacteria. Therefore, it is indispensable for good health in living organisms[5]. However, contamination of water and soil with Ni has become a widespread problem due to anthropogenic activities[5, 42]. Ni concentrations in polluted soil can range from 200-26 000 mg kg^{-1}, which is 20- to 30-fold higher than the naturally occurring range (10-10 000 mg kg^{-1})[5]. In sea water, natural Ni concentrations range from 0.1-0.5 µg L^{-1}, and in unpolluted freshwater, dissolved nickel concentrations range from 1 to 3 µg L^{-1} [43, 44]. Excess Ni concentration in both aquatic environments and soil can cause toxic effects by several mechanisms, although these mechanisms are still poorly understood[45]. Ni can replace essential metal co-factors of metalloproteins, which assist in the uptake of metals by cells by controlling the import and/or export transport as well as storage of metal ions[45, 46]. Other toxic mechanisms include the binding of Ni to catalytic residues of non-metalloenzymes, the binding of Ni outside the catalytic site of an enzyme to inhibit allosterically and the indirect induction of oxidative stress[45]. Ni suppresses antioxidant enzyme activities of for example superoxide dismutase (SOD), catalase (CAT) or glutathione peroxidase (GPX)[47]. Previous studies also showed a decrease of photosynthesis rate and pigment contents, chlorosis, necrosis, and distortion of cell membrane functionality and ion balance in the cytoplasm[5, 42, 48, 49].

1.1.4 Zinc

The metal Zn is essential to all living organisms for its participation in physiological and metabolic processes, such as maintenance of ribosome structure and function[5, 50]. It is also a ligand of several

enzymes such as carbonic anhydrase, alcohol dehydrogenase, SOD and RNA polymerase. As a cofactor of RNA polymerase Zn also assists in formation of carbohydrates and in oxidation processes in plants[5]. Both Zn deficiency and elevated Zn levels are known to increase phytotoxicity and also oxidative stress as witnessed by expression of genes encoding antioxidative defence enzymes, such as ascorbate peroxidase (APX) and glutathione reductase (GR)[51, 52]. In freshwaters, Zn generally has an average concentration of 2.8 µg L^{-1}, but has become a common pollutant in aquatic environments[53]. This is caused by industrial or domestic effluents containing elevated Zn concentrations entering rivers and lakes[54]. Leaves start to develop phytotoxicity at concentrations exceeding 0.2 mg g^{-1} dry matter[52]. Raised Zn levels can also cause other adverse effects such as reduced growth of both root and shoot, malformed leaves, or chlorosis in the younger leaves and, after prolonged exposure, also in the older leaves[5, 55-59].

1.1.5 Iron

Iron is an essential element for all organisms for taking part in many metabolic processes, including photosynthesis, chloroplast development and chlorophyll biosynthesis. It is a constituent of haem-containing proteins such as haemoglobin (leghomoglobin in plants), myoglobin, cytochromes, CAT and peroxidase. It is the most abundant metal in the earth's crust, but it is mostly present as insoluble Fe^{3+}, and therefore unavailable to higher plants[39, 60, 61]. Only in an acid soil under anaerobic conditions, Fe can be easily taken up by plants[61]. It naturally exists in the minerals hematite, magnetite, taconite, and pyrite[62]. However, excess Fe concentrations in surface water can result from anthropogenic activities such as mining[63]. It is considered an essential element, but when a certain threshold concentration of Fe is reached, it becomes toxic[64]. The Environmental Protection Agency (EPA) has set a freshwater quality criterion for Fe of 1000 µg L^{-1}, however this criterion is very poorly validated due to a lack of tested organisms or environmentally irrelevant test conditions[65, 66]. According to Kinsman-Costello *et al.*, 48% of their tested sample sites had Fe concentrations higher than the EPA criterion[65].

In plants, exposure to high Fe concentrations can lead to an elevated Fe^{2+} uptake by roots and transportation to leaves. This can cause production of free radicals which irreversibly diminishes cellular structure and membranes. Furthermore, high Fe concentrations within plants and algae can reduce its growth because the Fe binding to the cell wall can inhibit nutrient uptake[64, 67].

1.1.6 Lead

The metal Pb is considered a non-essential metal, because it has no biological role in organisms[68, 69]. It is one of the most pervasive toxic elements in the soil. The EPA has set a Maximum Permissible Concentration of 5 µg L^{-1} for Pb in freshwaters[66]. Many Pb compounds are water-insoluble, but Pb contamination in water can occur due to corrosion of Pb-bearing materials used in the water supply system[70, 71]. Excess of Pb concentrations affects morphology, growth and photosynthetic processes in plants by interfering with important enzyme activities, water imbalance, membrane permeability and mineral nutrition. This can lead to inhibition of seed germination, reduced root and shoot growth and a decreased leaf expansion; Furthermore, elevated Pb concentrations also induce the production of ROS in plants, leading to oxidative stress. The degree to which Pb affects plants depends on the concentration of Pb, ionic composition, pH, and bioavailability[5, 72]. Only desorbed Pb is bioavailable, while absorbed Pb is not[72].

1.2 Mixtures

The not-to-exceed levels for U mentioned above are based on laboratory experiments testing single toxicant exposure conditions. The adverse effects of individual metals are generally well studied, and include oxidative stress and inhibition of enzymes[7]. In the environment, however, organisms are usually exposed to a mixture of chemicals rather than to only one substance[2]. Interactions in naturally occurring aquatic mixtures of metals are not yet completely understood, although toxicity tests with metal mixtures have been carried out for more than 30 years[73]. The combination of several metals can have other combined or interacting effects than the effects of an individual metal. Metals in mixtures can have additive, synergistic, or antagonistic effects, or they can act independently, depending on their concentration or external factors such as temperature, pH and light (Figure 1)[6]. There exist theoretical toxicological models that can determine whether a mixture is additive, antagonistic or synergistic, but complex mixture toxicity remains a challenge for ecotoxicology[74].

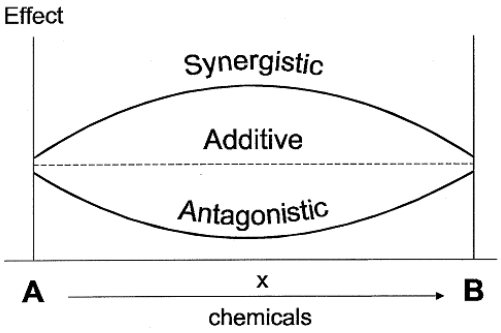


Figure 1: Synergism and antagonism in toxicity. If two substances A and B are administered together in proportion x, the sum of each separate effect is the additive effect. The effect is synergistic when their combined effect is larger than the sum, which means they enhance each other's effect. The effect is antagonistic when their combined effect is smaller than the sum, which means they counteract each other's effect[75].

Mixture toxicity can thus be very complicated and difficult to model. There is still no clear standard pattern for a qualitative prediction of the expected effects of a metal mixture[73]. The necessity to accurately investigate different mixtures remains, because there is no simple straightforward model that can be used for every condition[76, 77].

One of the regulation strategies in European law regarding chemical mixtures is to treat every component in a mixture evenly, for example using the regulatory not-to-exceed levels for each individual chemical in the mixture, and dividing it by the total number of considerable toxicants to obtain a theoretical mixture effect. An additional safety factor is used to make up for uncertainties in data and evaluation processes, and to add a margin of safety to observed effects, for example to account for interspecies differences[74]. Usually, a safety factor of 10 or 100 is applied, meaning if a certain dose of a toxic substance is safe for a test animal, one tenth or one hundredth of that dose would be assumed to be safe for humans[73]. This approach is not very accurate and poses problems for mixtures containing essential metals as due to the conservative approach some metals need to be below levels that are beneficial for organisms. Additionally, this approach does not take synergistic or antagonistic interactions into account. As such, to reduce the uncertainty on mixture approaches, specific mixture effects still need to have additional profound testing. The question arises how much complexity needs to be integrated in a model to be a reliable and accurate representation of the reality[74]. Two models are commonly used to predict mixture effects, the concentration addition (CA)

principle and independent action (IA). When it is assumed that noninteracting chemicals cause toxicity by acting on the same molecular site within the organism, it is referred to as CA. When it is assumed that each chemical in a mixture causes an effect independent of the other chemicals and by a different mode of action, it is referred to as IA[78].

1.3 Oxidative stress

Exposure to metals can induce a variety of toxic effects of which induction of oxidative stress or genotoxicity are important common reactions in plants and other organisms[79]. Transformation of oxygen (O_2) into ROS occurs by two distinct mechanisms. Reduction of O_2 leads to the formation of ROS, such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$), whereas energy transfer to O_2 leads to the formation of singlet oxygen ($^1O_2$) (Figure 2). Oxidative stress is defined as an imbalance between the cellular redox status caused by a decreased production of antioxidative enzymes and metabolites and/or an increased production of ROS. These ROS are continuously formed in plants as by-products of their aerobic metabolism and are the most common, endogenous toxicants in aerobic organisms[80]. Some ROS, such as H_2O_2, also have a beneficial role in plant function as key regulators involved in growth, development and defence pathways[27]. Plants have evolved efficient defence mechanisms to rapidly remove ROS and hence keep ROS levels within a biologically active but harmless range[81]. It has been described that as a reaction to the oxidative stress, metal toxicity increases antioxidant activity by increasing the activity of enzymes involved in the antioxidative defence mechanism[5, 82]. The antioxidative defence system of plants contains both antioxidative enzymes and metabolites.

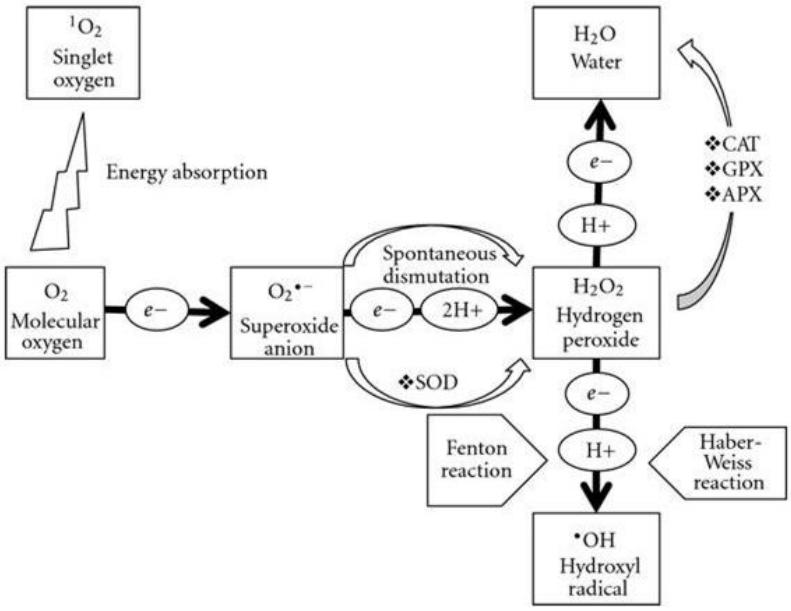


Figure 2: Formation and detoxification of reactive oxygen species (ROS) in plants. Oxygen (O_2) can be transformed into several ROS, such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and the hydroxyl radical ($^{\bullet}OH$). Superoxide is easily dismutated to H_2O_2 either nonenzymatically or by the action of superoxide dismutase (SOD). Catalase (CAT), glutathione peroxidase (GPX), and ascorbate peroxidase (APX) then react with H_2O_2 to catalyse the formation of water (H_2O) and O_2 (Adapted from Sharma *et al.*, 2012[83]).

Examples of important **antioxidative enzymes** are SOD, CAT, GPX, and APX. Superoxide dismutase act as the primary line of defence against ROS by catalysing the dismutation of O_2^- into O_2 and H_2O. Subsequently, CAT, GPX and APX detoxify H_2O_2. Glutathione (GSH) is used by GPX as an electron donor to reduce H_2O_2 to water, whereas APX utilises ascorbate (AsA) as the electron donor[84, 85]. The **antioxidative metabolites** can be categorized into lipid- and water soluble compounds. Lipid soluble antioxidative metabolites include tocopherols (vitamin E) and carotenes. Examples of water soluble antioxidative metabolites are AsA and GSH, which are kept in their reduced form by the AsA-GSH cycle[81].

1.4 Genotoxicity

An important target of environmental and oxidative stress in both aquatic and terrestrial organisms is known to be DNA[86]. In normal circumstances, DNA damage is repaired during the arrest of cell cycle progression at the G_2-M boundary. Single-strand damage can be repaired by the DNA base excision repair (BER) pathway, the nucleotide excision repair (NER) or the DNA mismatch repair (MMR) depending on the nature of the induced damage[80]. As such, BER repairs damage to single nitrogenous bases, NER repairs helix-distorting damage such as pyrimidine dimerization and the MMR repairs errors that are not corrected by proofreading. Double-strand damage is repaired by double-strand break (DSB) repair[87]. If DNA damage persists, however, permanent heritable changes in somatic cells or germ cells might occur of chromosome fragments or whole chromosomes may be unable to engage with the mitotic spindle during mitosis, resulting in micronuclei[88]. Damage to DNA can be induced directly through interactions of mutagens e.g. including some metals or ionising radiation. Direct effects include single- or double-strand breaks, cross linking, point mutations and misrepair of strand breaks in DNA, resulting in chromosome abnormalities[88, 89]. On the other hand, indirect mechanisms such as lipid peroxidation and protein adducts or ROS can also induce DNA damage[90]. If both repair processes and cellular apoptosis fail, damaged DNA can lead to alterations in the genetic information, which is known as genotoxicity. In plants, these stress factors can affect cell cycle control via induction of **endoreduplication**[81, 91, 92]. Endoreduplication is a common variant of the typical cell cycle and includes a complete genome replication during synthesis but lacks mitosis[93]. During endoreduplication, the cells replicate their genomic DNA without undergoing mitosis and/or cytokinesis, resulting in polyploid cells[93]. Endoreduplication has important functions in the growth and development of various plant organs such as petals and leaves, including trichomes, although the physiological significance of endoreduplication and its underlying mechanisms are still poorly understood[94].

1.5 *Lemna minor*

Duckweed and other water plants are highly exposed to aquatic pollutants, which makes them very sensitive to their toxic effects, and therefore suitable to measure the extent of toxicity in natural freshwaters[39, 41]. As such *L. minor* (common duckweed) is widely used in ecotoxicity tests for xenobiotic substances to examine the consequences of metals on freshwater vascular plants. *Lemna minor* is a floating macrophyte and the smallest vascular plant. It is an easy to use species due to its simple structure, small size and rapid growth rate[18]. It also has a high vegetative reproduction rate, resulting in genetically identical clones. *Lemna minor* can easily be grown in sterile and

controlled laboratory conditions[95]. It is to date the standard species for toxicity testing on freshwater plants using a growth inhibition test for which detailed guidelines are available and approved by e.g. the Organisation for Economic Co-operation and Development (OECD)[96]. The plants have a surface of only a few square millimetres, with one, two or three leaves, which are called fronds. The plant has a dominant vegetative growth with a high growth rate. On average, one adult plant splits into two new plants every 2.5 days when more fronds are formed[97].

1.6 Experimental aim

In order to assess the impact of U on the environment, further research is needed in environmentally relevant conditions to investigate toxicity of organisms to a mixture of chemicals of which U is one of the co-contaminants. To address possible interactions of U with other contaminants under environmentally relevant conditions, we exposed *L. minor* to a complex mixture of U with **multiple metals** based on samples taken on U-mining sites as described by Lofts *et al*.[98]. Our mixture is primarily based on samples taken from the Beaverlodge Lake (Table 1), which is the largest water body close to Uranium City in northern Saskatchewas, Canada[98].

Table 1: Concentrations of trace metals, metalloids, uranium and thorium in Beaverlodge Lake.
nm = not measured

Contaminant	As	Cu	Mn	Fe	Ni	Zn	Se	Mo	Ba	Pb	U	Th
Concentration	1.8 µg dm^{-3}	1 µg dm^{-3}	nm	65 µg dm^{-3}	1 µg dm^{-3}	5 µg dm^{-3}	4.8 µg dm^{-3}	nm	0.56 µg dm^{-3}	3 µg dm^{-3}	483.6 µg dm^{-3}	0.079 pg dm^{-3}

Because data of individual metals is needed for experimental design as well as analysis of possible interactions between the metals, a literature search (Paragraph 3.1) was done previous to the experiments. This literature search identified as far as possible toxicity levels in *L. minor*, and served to set up a dose-response curve (DRC) for each individual toxicant and the specific set-up used in this study. As such effect concentration (EC) inducing growth inhibition on 30% or 50% of the population (EC30, or EC50 respectively) for several components of interest from table 1 were defined. Based on these toxicity levels, the multiple mixture was composed of the most important toxic components. The aim of this part of the work was to compare the extent of toxicity induced by the **mixture with U and the mixture without U** on different endpoints. These endpoints include **growth** (frond number, biomass production and frond area) and **uptake** of U and metals.

As described above, ecotoxicological tests such as the *L. minor* growth inhibition test can be indicative for metal exposure but are often not so sensitive or so specific for one metal. To resolve this problem, studies started focussing on **biomarkers**, which provide more complete and biologically relevant data. There are many different kinds of biomarkers, such as biomarkers of exposure, of effects, of stress and of alteration, where the biomarker most used is the stress biomarker[12]. However, there can be doubt about the specificity toward a given xenobiotic, because co-exposure to multiple toxicants generally is the case[99]. In this project, we therefore try to identify a set of biomarkers comprised of distinguishing genes of *L. minor* that are specific to U exposure. Real-time polymerase chain reaction (qPCR) is a sensitive and reliable method to analyse expression of specific genes of any species as long as it is feasible to design primers that are a measure for the production and stability of transcripts[99]. The genome of *L. minor* was unknown until recently an RNA

sequencing analysis was carried out within the Biosphere Impact Studies (BIS) group to study the transcriptome response of *L. minor* after exposure to U, beta or gamma radiation[100]. Several strongly responsive genes involved in endoreduplication and antioxidative responses were found (Van Hoeck *et al.*, unpublished results). Based on this RNA sequencing analysis, our goal is to identify a selection of genes that are specific to U exposure, and do not react after exposure to beta or gamma radiation. Our aim is to use this set of genes as distinct biomarkers for U stress. Using quantitative qPCR, the **expression levels of a selection of genes** was monitored between the different U, metal and U+metal exposed plants. This U specificity is studied by comparing plants exposed to a mixture of U and several heavy metals.

2. Materials and methods

2.1 Plant materials and growth conditions

Lemna minor cv. Blarney plants, obtained from Dr M. Jansen (University College Cork, Cork, Ireland, Serial number 1007, ID number 5500), were aseptically cultured in a growth chamber using 250 mL glass Erlenmeyer flasks containing half-strength Hutner medium under continuous light (Osram 400 W HQI-BT daylight, OSRAM GmbH, Augsburg, Germany, 65 ± 1 µmol m^{-2} s^{-1}) at 24.0 ± 0.5 °C. The plant culture was maintained by transferring three plants to 100 mL of fresh growth medium every 10-12 days.

The experiment protocols were adapted from the standard 7-day inhibition test of the OECD using *L. minor*, with some modifications as described by Horemans *et al.*[96, 101]. To gain a consistent and uniform plant population, sterile *L. minor* plants were precultured prior to experiments for seven days. This was achieved by transferring five mature plants, each consisting of three or four fronds, to 250 ml glass Erlenmeyer flasks containing 100 ml half-strength Hutner medium (Table 2).

After the preculture, experiments were run in polycarbonate pots containing 100 ml of a sterile K-medium with a low KH_2PO_4 concentration of 0.5 mg L^{-1} (Table 2). This low phosphate concentration was justified because phosphate reduces U bioavailability to plants[102]. A pH of 5 was obtained by adding the sufficient amount of 1 M filter-sterilised (0.22 µm) sodium hydroxide (NaOH) to the medium. To stabilize the pH, 5 mM filter-sterilised (0.22 µm) of 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (Sigma) was added. After adding the filter-sterilised metal solutions to the medium, three healthy looking plants, with a total of 9-12 fronds, were transferred aseptically from the preculture to each pot containing the K-medium, MES buffer and metal solution. For image calibration purposes, a 1-cm surface-sterilised floating ruler was added to each pot. The pots were then covered with a 9-cm plastic petri dish and experiments were run for seven days.

Table 2: Composition of modified K-medium and half-strength Hutner medium.

	Modified K-medium (mg L^{-1})	Hutner medium (mg L^{-1})
Macronutrients:		
KH_2PO_4	5.032	0.0414
KNO_3	888.8	0.3
$Ca(NO_3)_2 \cdot 4H_2O$	944	0.72
$MgSO_4 \cdot 7H_2O$	500	0.0738
Na_2-EDTA·2 H_2O	9	0.0029
Tartaric acid	3	
Ferric citrate		0.001
Micronutrients:		
H_3BO_3	1.86	0.001
$ZnSO_4 \cdot 7H_2O$	0.22	0.001
$Na_2MoO_4 \cdot 2H_2O$	0.12	0.0001
$CuSO_4 \cdot 5H_2O$	0.08	0.00003
$MnCl_2 \cdot 4H_2O$	3.62	
$FeCl_3 \cdot 6H_2O$	5.4	
$MnSO_4 \cdot H_2O$		0.0006

2.2 Exposure of *Lemna minor* to metals and metal mixtures

First, a range finding experiment was performed. To set up the experimental design, a literature search was performed to find published toxicity levels in *L. minor*, such as the EC10, EC30 or EC50, for several metals and possible interactions (Paragraph 3.1). Using these toxicity levels, a range finding experiment was carried out to select relevant concentrations for a complete DRC. *L. minor* plants were exposed in triplicate to 50, 200, 400 or 1000 µM $FeSO_4.7H_2O$, to 2, 10, 50 or 200 µM $NiSO_4 \cdot 6H_2O$ (Merck), $ZnSO_4 \cdot 7H_2O$ (UCB) or $Pb(NO_3)_2$ (Sigma) or to 0.1, 0.5, 5 or 10 µM $CuSO_4 \cdot 5H_2O$ (Sigma). For each experiment six controls were included, which were not exposed to any metal. A range finding for U was unnecessary because enough information was available from previous experiments performed within the BIS group[18, 101]. The metal concentration of the medium was verified at day 0 and day 7 using the 932 atomic absorption spectrometer (GBC).

To carry out the full dose-response toxicity tests, *L. minor* plants were exposed to one of five metals. The plants exposed to $NiSO_4 \cdot 6H_2O$ (Merck), $ZnSO_4 \cdot 7H_2O$ (UCB) or $Pb(NO_3)_2$ (Sigma) were exposed to concentrations of 10, 25, 50, 100, 200, 500 or 750 µM. The plants were exposed to $CuSO_4 \cdot 5H_2O$ (Sigma) in concentrations of 5, 10, 15, 20, 30, 40 and 50 µM, or to $UO_2(NO_3)_2 \cdot 6H_2O$ (SPI chemicals) in concentrations of 0.5, 4, 6.5, 10, 25 µM. Concentrations were chosen based on the literature study described above and the range finding experiments. Each concentration was tested in triplicate. Six controls were grown in only modified K-medium.

For the metal mixtures, plants were exposed in triplicate to 14 different combinations based on the composition of the Beaverlodge Lake. The original sample of Beaverlodge Lake consisted of 0.0157 µM Cu, 0.0145 µM Pb, 0.017 µM Ni and 0.0765 µM Zn. Plants were exposed to this onefold combination, and also tenfold, hundredfold and thousandfold concentrations. These combinations were then combined with 0 µM, 2 µM or 10 µM U to study the influence of U on the heavy metal mixtures. Six controls were grown in modified K-medium without metals or U. Additionally, plants were exposed to 2 or 10 µM U in modified K-medium without the Beaverlodge combination.

Concentration of the metals in the 0.22 µm-filtered growth media were verified for every experiment before exposure and after seven days of exposure. Medium concentrations of Cu were verified at day 0 and day 7 using the 932 atomic absorption spectrometer (GBC). Medium concentrations at day 0 and day 7 of Ni, Pb and Zn were determined using inductively coupled plasma optical emission spectroscopy (ICP-OES) (Agilent 700 Series; Agilent Technologies) at the Centre for Environmental Sciences (CMK, Hasselt University) as described by Krznaric *et al.*[103]. Concentrations at day 0 and day 7 of U were determined at the Chemistry building from SCK•CEN using inductively coupled plasma mass spectrometry (ICP-MS, (XSeries II; Thermo Scientific) equipped with a PFA-ST Nebulizer (Elemental Scientific) as described by Horemans *et al.*[18].

After 7 days, plants were harvested and fresh weight was measured to determine frond biomass after which the samples were dried at 70°C for one week to determine dry weight. For gene expression, samples were snap frozen in liquid nitrogen and stored at -80°C after determining fresh weight.

2.3 Relative growth rate and growth inhibition

In addition to fresh weight, growth of the plants was determined using total frond area or number of fronds as endpoints. Total area covered by fronds and the number of fronds was determined on day 0, 3, 5 and 7 using Image J open source software (version 1.49)[104]. The average specific growth rate (μ) was calculated using following formula:

$$\mu = \frac{ln\, x_{t_7} - ln\, x_{t_0}}{t_7 - t_0}$$

where x_{t_7} and x_{t_0} are the values of examined parameters (biomass, dry weight, frond area and number of fronds) at day 7 (t_7) and day 0 (t_0) respectively. The percentage growth inhibition is calculated using:

$$\%I_r = \frac{(\mu_c - \mu_t)}{\mu_c} \times 100$$

where $\%I_r$ is the percentage inhibition in average specific growth rate (μ) and μ_c and μ_t are the mean values for average specific growth rate for control and treated samples, respectively. Validation criteria were checked prior to toxicity tests, as described in guideline 221 of the OECD (2006). These criteria include a doubling time (t_d) of frond number in control conditions of 2.5 days (60 hours) or less, which means plant growth is adequate and according to standards. The doubling time (t_d) was determined by:

$$t_d = \frac{\ln 2}{\mu}$$

where μ represents the average specific growth rate of the controls. The DRCs were then established by plotting the log-logistic function as closely as possible through the growth inhibition data using the Solver tool in Excel.

2.4 Metal uptake

Plant samples for U analysis were harvested by washing for 10 minutes in 1 mM $Pb(NO_3)_2$ and twice in distilled H_2O for two minutes to remove surface-bound U as described by Horemans *et al.*[18]. Fresh weight was determined to calculate frond biomass after which the samples were dried at 70°C during one week. After determination of their dry weight, samples were calcinated in a muffle furnace at 550°C for 24 hours and digested using 0.1 M HCl. Samples were filtered with Acrodisc 25 mm syringe filters with 0.45 μm HT Tuffryn (Pall Corporation) to exclude any remaining particles. Subsequently, Cu, Ni, Pb an Zn contents of the samples were determined by ICP-OES (Agilent 700 Series; Agilent Technologies) at CMK, Hasselt University as described by Krznaric *et al.*[103]. The U contents of the samples were determined at the Chemistry building of SCK•CEN by ICP-MS (XSeries II; Thermo Scientific) equipped with a PFA-ST Nebulizer (Elemental Scientific) as described by Horemans *et al.*[18].

2.5 Primer optimisation

Housekeeping genes were selected based on the standard deviation of gene expression levels measured in a RNAseq experiment previously performed in the BIS group in which *L. minor* was exposed to different U concentrations or beta- or gamma radiation. The genes with the lowest overall standard deviation and a high overall expression for all exposure concentrations were considered. The U primer markers were also selected based on their gene expression level. Genes were considered if they only reacted after uranium exposure and not after beta or gamma radiation, in a

dose-dependent manner, and with the highest difference compared to controls (Appendix 1). Primers were designed using the primer-BLAST tool from the National Center for Biotechnology Information (NCBI). Primer melting temperatures were set between 57 and 63°C, with an optimal temperature of 60°C. The maximum difference between the melting temperature of forward and reverse primer was 3°C. Primer length was ideal between 85 and 150 base pairs. The minimal number of bases that must anneal to the template at the 5' and 3' side of the exon-exon junction is 7 and 4 respectively. Primer GC content is specified between 20 and 80%. Maximum 3' stability is set at 9. The higher this number, the more stable the 3' end. A selection of six housekeeping genes and six U markers was obtained (Table 3).

Table 3: Overview of the primer sequences (5'-3') used to determine the expression of the housekeeping genes and potential uranium markers using real-time PCR.

Housekeeping genes		
Gene		**Sequence (5' > 3')**
Lminor_003273	Forward	GCGCCGATCCTAGATTTGAA
	Reverse	CAGGCCCATCCTCCTTCTTC
Lminor_014887	Forward	GAGACGGGGCAAGAGTTCAA
	Reverse	CATCCACCGGCTAACCCATT
Lminor_009513	Forward	TTGGATCATGAGGGGAAGGAG
	Reverse	CGTCCTGAAAGACGCCACAA
Lminor_004034	Forward	TTCGTCCGGATAGCTTCGTG
	Reverse	GCCATTTTCGGATTCCTCGC
Lminor_012610	Forward	TTGAGCTTGCTCTTGCTGGT
	Reverse	TCCTGCGAATGGTAAAGCCC
Lminor_009166	Forward	TCGTCAGCCAGCCAGTAAAG
	Reverse	AGTCTCCCTGTCCGAGAAGA
Uranium markers		
Gene		**Sequence (5' > 3')**
Lminor_020037	Forward	ACTACAACAATGGCTGGCCC
	Reverse	ATCGGTCTGCTTGATGCTCG
Lminor_010222	Forward	CGACACTCTCATGTCCCTGC
	Reverse	ATATCGTGATCCAACGCCCG
Lminor_020596	Forward	TGATGGACATCGACAGCGAG
	Reverse	ATCGTTCGTACCTGTGTCGC
Lminor_016305	Forward	AGACAGTGGCTGGCTTCATC
	Reverse	CGACCAGTCTCCACTCTCCA
Lminor_009276	Forward	CCTCGAACACCCTTCCTTCC
	Reverse	ACGAAGTAGTCCTCTCCGCA
Lminor_014505	Forward	GCAGCACAACCAAGATGTGA
	Reverse	CTGACTCACCGCGTTCTTGA

2.6 Gene expression

Plant samples that were snap frozen in liquid nitrogen and stored at -80°C were shred while frozen using the Mixer Mill MM400 (Retsch). This was done by placing plant samples containing three chrome steel beads (2.3 mm diameter, Retsch) in pre-cooled Mixer mill adapters and shredding for 3.5 minutes at 30Hz. RNA was then extracted using the RNeasy plant mini kit (Qiagen) following the standard protocol. The concentration and the quality of the extracted RNA were evaluated with the Nanodrop ND-1000 (Thermo Fisher Scientific). RNA integrity was checked using the Agilent 2100 Bioanalyzer (Agilent Technologies) and the Agilent RNA 6000 Nano kit (Agilent Technologies) following the standard protocol published by the company in 2013. The RNA samples were then

stored at -80°C until further use. Subsequently, cDNA synthesis was performed using the TaKaRa PrimeScript™ RT Reagent Kit for real time PCR (Clontech Laboratories). For each RNA sample, 1 µg sample was diluted in 9.75 µL RNase-free H_2O. An additional DNA removing step was included using the Ambion® TURBO DNA-*free*™ Kit (Applied Biosystems). After the DNase step, 5.25 µL of a cDNA mastermix was added to each sample. This mastermix was composed of 3 µL 5x PrimeScript Buffer, 0.75 µL PrimeScript RT Enzyme Mix I, 0.75 µL Oligo dT Primer and 0.75 µL random hexamers per sample. After cDNA synthesis, another 75 µL RNase-free H_2O was added to the 15 µL cDNA samples to obtain a 1:6 dilution of the cDNA samples to minimise potential contamination.

Prior to real-time PCR experiments, primer efficiencies were tested against a four-fold cDNA dilution series (1; 1/4; 1/8; 1/16; 1/64; 1/256; 1/1024; 0). In each well, 7.5 µL of a mastermix was added to 2.5 µL of cDNA. The mastermix consisted of 5 µL Fast Sybr® Green Mastermix (Thermo Fisher Scientific), 0.3 µL forward primer, 0.3 µL reverse primer and 1.9 µL RNase-free H_2O. All PCR reactions were performed with 96-well plates in the 7500 Fast Real-Time PCR system (Applied Biosystems). As an additional primer efficiency verification, a gel electrophoreses (Mupid gel electrophoresis apparatus, Kem-En-Tec) was carried out with the resulting PCR products. For the agarose gel, 0.9 g agarose was added to 100 mL Tris/Borate/EDTA (TBE) buffer together with 5 µL GelRed™ Nucleic Acid Gel Stain (Biotium), which was then dissolved using a microwave. To load the samples to the agarose gel, 5 µL PCR product was mixed together with 1 µL 6x DNA Loading Dye (Thermo Fisher Scientific) and 5 µL of this mixture was then loaded to the gel. Additionally, 1 µL of a 50 base pair GeneRuler DNA ladder (Fermentas by Thermo Fisher Scientific) was loaded. Gel electrophoresis was run for 55 minutes at 75 V. The agarose gels were visualised with a Fusion FX Spectra (Vilber Lourmat).

2.7 Statistical analysis

The data obtained from the experiments described above are presented as mean values ± standard error (SE) and statistically analysed using a one-way ANOVA. A two-way ANOVA was used to test significant differences between the metal mixtures, using U concentration and metal concentration as two variables. the normality and homoscedasticity were tested using a Shapiro-Wilk test and a Bartlett's test respectively. When necessary, data were transformed (square root, inverse, exponent or logarithm). The Tukey-Kramer test was applied as a post-hoc test for multiple comparison of the data. In case the assumptions for a parametric test were not met, a non-parametric Kruskal-Wallis test and a post hoc Wilcoxon signed-rank test were performed. All statistical analyses were performed using the open source software package R (R Project for Statistical Computing; version 3.2.2). To prove there is interaction between components in the mixtures, the program "Mixtox" was used as described by Jonker *et al*.[105]. Mixtox generates a predicted mixture DRC that fits through as much data points as possible. It then checks by means of a chi-square test whether the measured combined effects follow concentration addition or independent action predictions or whether deviation of these two general concepts exist. To use MixTox, the M10 combination without U had to be used as control, because the program expects a univariate response and therefore could not deal with the negative growth inhibition values of the lower metal concentrations (M0 and M1).

3. Results

3.1 Literature search

Information of the toxicity of the individual metals to *L. minor* was needed to be able to design a proper experimental set-up using metal mixtures. As most of these metals had not been tested before within the BIS group, first a literature survey was carried out to find existing data (Table 4). The focus was to find information on metals Fe, Ni, Zn, Pb, Cu, arsenic (As), Mn, selenium (Se), Mo, barium (Ba) and thorium (Th) as these are present in the mining samples as described in table 1.

This literature search identified toxicity levels in *Lemnaceae*, such as the effective concentration on 50% of the population (EC50). Preferably, only studies that involved *L. minor* were taken into account. However, other *Lemnaceae*, such as *L. gibba*, were mentioned if not sufficient information about *L. minor* was found. Additionally, a comparison was made between published articles based on exposure dose, exposure duration, plant species and which salt was used to expose the plants. Comparing based on metal species was important since different molecules can yield different results. Then, the concentrations and metal species were selected that were most commonly used to expose *L. minor*.

Thorium was excluded because of very low occurrence (0.079 pg dm^{-3}) in our Beaverlodge sample. Additionally, only one article could be identified in the literature search, in which a very high and environmental irrelevant exposure dose of 100 000 µM over a period of 24 hours did not induce any toxic effects. No other relevant articles were found on Th.

Finally, only Fe, Ni, Zn, Pb and Cu were selected as relevant metal species for the study here. Due to time restrictions it was not possible to study all metals from table 1, but these investigations will be conducted in the future. In spite of the defined ECs in the literature search, it remained necessary to test the toxicity of the selected metals in the modified growth medium (Table 2) routinely used in the BIS group. This modified growth medium, called K-medium has been optimised for U ecotoxicity and has a low KH_2PO_4 concentration (Table 2)[101]. Low phosphate concentrates are used since phosphate easily precipitates with U and thus reduces U bioavailability to plants as previously described[37, 101]. A range finding experiment for all the metals under investigation had to be carried out, as it was questionable if the metals in this modified growth medium would have the same amount of toxicity as described in literature.
It was decided to expose *L. minor* plants in this study to sulphate salts ($FeSO_4$, $NiSO_4$, $ZnSO_4$) or nitrate salts ($Pb(NO_3)_2$, $UO_2(NO_3)_2$) of the metals to gain consistent results with as little variation as possible, and because these salts were mostly used in the articles from the literature search.

Table 4: Literature search. Heavy metal exposure tests using *Lemnaceae*.

Iron (Fe)			
Article	Ecotoxicological effects of heavy metals on duckweed plants (*Lemna minor*). III. Tests for growth rate reducing by copper and iron[39]	Effects of Superparamagnetic Iron Oxide Nanoparticles on Photosynthesis and Growth of the aquatic plant *Lemna gibba*[106]	Preliminary tests on phytotoxicity of heavy metals Cu, Zn, Cd and Fe on aquatic plants of duckweed (*Lemna minor*)[97]
Species	*L. minor*	*L. gibba*	*L. minor*
Exposed to	Unknown	- Fe_3O_4 (SPION-1) - $Co_{0.2}Zn_{0.8}Fe_2O_4$ (SPION-2) - $Co_{0.5}Zn_{0.5}Fe_2O_4$ (SPION-3)	$FeSO_4 \cdot 7H_2O$
Exposure dose	66, 132, 198, 264, 330 µM	12.5, 25, 50, 100, 200, 400 µg mL^{-1}	20, 80, 320, 1280, 5120 µM
Exposure duration	7 days	7 days	14 days
Remarks	EC_{50} = 401.3 - 545.3 µM	$EC_{50,\ SPION\text{-}1}$ = 244 µg mL^{-1} $EC_{50,\ SPION\text{-}2}$ = 183 µg mL^{-1} $EC_{50,\ SPION\text{-}3}$ = 237 µg mL^{-1}	Recommended concentration domain for testing is 0 - 320 µM.

Nickel (Ni)				
Article	Growth response of the duckweed *Lemna gibba* L. to copper and nickel phytoaccumulation[107]	Heavy metal toxicity to *Lemna minor*: studies on the time dependence of growth inhibition and the recovery after exposure[7]	Growth rate based dose–response relationships and EC-values of ten heavy metals using the duckweed growth inhibition test[108]	Effects of nickel on the chloroplasts of the duckweeds *Spirodela polyrhiza* and *Lemna minor* and their possible use in biomonitoring and phytoremediation[42]
Species	*L. gibba*	*L. minor*	*L. minor*	*L. minor*
Exposed to	$NiCl_2 \cdot 6H_2O$	$NiSO_4 \cdot 6H_2O$	$NiCl_2$	$NiCl_2 \cdot 6H_2O$
Exposure dose	0.5, 1, 2, 3, 4, 8 µM	81.8 µM	0.1, 1, 10, 100, 1000 µM	0.4, 1.0, 4.0, 10, 40, 100 µM
Exposure duration	4 days	7 days	7 days	7 days
Remarks	- Ni tolerated at ≤ 2 µM - low accumulation of Ni (0.5 mg g^{-1} DW) - LCI = 4 µM	EC_{50} = 56.3 µM	EC_{50} = 6.2 µM	EC_{50} = 6.6 µM

Zinc (Zn)					
Article	Frond development gradients are a determinant of the impact of zinc on	Uptake and partitioning of zinc in *Lemnaceae*[57]	Heavy metal toxicity to *Lemna minor*: studies on the time dependence	Growth rate based dose–response relationships	Preliminary tests on phytotoxicity of heavy metals Cu, Zn, Cd and Fe

	photosynthesis in three species of *Lemnaceae*[109]		of growth inhibition and the recovery after exposure[7]	and EC-values of ten heavy metals using the duckweed growth inhibition test[108]	on aquatic plants of duckweed (*Lemna minor*)[97]
Species	*L. minor*	*L. minor*	*L. minor*	*L. minor*	*L. minor*
Exposed to	$ZnSO_4 \cdot 7H_2O$	- $^{65}ZnCl2$ (0.02 mg L^{-1} of total [Zn]) - $ZnSO_4 \cdot 7H_2O$ (remaining Zn)	$ZnCl_2$	$ZnCl_2$	$ZnSO_4 \cdot 7H_2O$
Exposure dose	10, 35, 100, 350 µM	0.7, 10, 35, 100, 350 µM	1026.4 µM	0.1, 1, 10, 100, 1000 µM	20, 80, 320, 1280, 5120 µM
Exposure duration	7 days	10 days	7 days	7 days	14 days
Remarks	/	0.7 µM is the concentration of zinc in the Hutner medium on which duckweeds were cultured and is in the range of low zinc levels in the environment.	- EC_{50}: 46.1 µM - Zn showed the lowest toxicity to the growth of *L. minor*	EC_{50}: 10.4 µM	A maximum concentration of 320 µM is recommended for toxicity tests.

Lead (Pb)			
Article	Mercury and lead: Assessing the toxic effects on growth and metal accumulation by *Lemna minor*[110]	Growth and lead accumulation capacity of *Lemna minor* and *Spirodela polyrhiza* (*Lemnaceae*): interactions with nutrient enrichment[111]	Lead toxicity to *Lemna minor* predicted using a metal speciation chemistry approach[112]
Species	*L. minor*	*L. minor*	*L. minor*
Exposed to	$PbCl_2$	$PbCL_2$	$Pb(NO_3)_2$
Exposure dose	0.35, 0.7, 1.79, 3.5, 7, 18, 28, 36 µM	3.6, 18, 36, 90, 180 µM	2.77, 7.5, 8.75, 11.11, 11.5, 13, 29.5 µM
Exposure duration	7 days	1, 3, 5 and 7 days	7 days
Remarks	EC_{50} = 19 µM	Pb accumulates in a time- and concentration-dependent manner.	/

Copper (Cu)								
Article	Growth response of the duckweed *Lemna gibba* L. to copper	Heavy metal toxicity to *Lemna*	Effect of copper on the toxicity and	Growth, photosynthesis and respiratory	Effects of copper and cadmium on	Growth rate based dose–	Preliminary tests on phytotoxicity	Ecotoxicological effects of heavy metals

	and nickel phytoaccumulation[107]	*minor*: studies on the time dependence of growth inhibition and the recovery after exposure[7]	genotoxicity of cadmium in duckweed (*Lemna minor* L.)[6]	response to copper in *Lemna minor*: a potential use of duckweed in biomonitoring[113]	heavy metal polluted waterbody restoration by duckweed (*Lemna minor)*[4]	response relationships and EC-values of ten heavy metals using the duckweed growth inhibition test[108]	of heavy metals Cu, Zn, Cd and Fe on aquatic plants of duckweed (*Lemna minor*)[97]	on duckweed plants (*Lemna minor*)[39]
Species	*L. gibba*	*L. minor*	*L. minor*	*L. minor*	*L. minor*	*L. minor*	*L. minor*	*L. minor*
Exposed to	$CuSO_4 \cdot 5H_2O$	$CuCl_2$	$CuCl_2$	Unknown	$CuSO_4 \cdot 5H_2O$	$CuCl_2$	$CuSO_4 \cdot 5H_2O$	$CuSO_4$
Exposure dose	0.5, 1, 2, 3, 4, 8 µM	12.1 µM	2.5 and 5 µM	0.2, 0.3, 0.5, 1.0 mg/L	0.2, 2, 20, 40, 80 µM	0.1, 1, 10, 100, 1000 µM	10, 40, 160, 640, 2560 µM	32, 64, 96, 128, 160 µM
Exposure duration	4 days	7 days	4 and 7 days	4 days	4 days	7 days	14 days	7 days
Remarks	I_{50} = 1.8 µM LCL = 2 µM	EC_{50}: 9.7µM	/	Threshold toxicity: 0.3 mg/L	At 40 µM, the antioxidant system of plants began to break down.	EC_{50}: 2.7 µM	A maximum concentration of 160 µM is recommended for toxicity tests.	EC_{50} = 18 – 21.4 µM

Arsenic (As)

Article	Biological responses of duckweed (*Lemna minor* L.) exposed to the inorganic arsenic species As(III) and As(V)[114]		Comparative phytotoxicity of methylated and inorganic arsenic- and antimony species to *Lemna minor*[115]		Limitations of growth-parameters in *Lemna gibba* bioassays for arsenic and uranium under variable phosphate availability[116]	Exposure of *Lemna minor* to Arsenite: Expression Levels of the Components and Intermediates of the Ubiquitin/Proteasome Pathway[117]
Species	*L. minor*		*L. minor*		*L. gibba*	*L. minor*
Exposed to	- $NaAsO_2$ (As(III)) - $Na_2HAsO_4 \cdot 7H_2O$ (As(V))		- $NaAsO_2$ (As(III)) - $AsHNa_2O_4 \cdot 7H_2O$ (As(V)) - $(CH_3)AsJ_2$ (MMAs(III))		$NaH_2AsO_4 \cdot 7H_2O$	$NaAsO_2$
Exposure dose	1, 4, 16, 64 µM		Max. concentration of 400 mg L^{-1}		0.17, 0.35, 1.72, 3.5 µM	50 µM

Exposure duration	1, 2, 4, 6 days	4, 7 days	21 days	6 hours
Remarks	*L. minor* can accumulate high levels of As from polluted water, and thus can be used as a phytoremediator.	- MMAs(III): $EC_{50,\ 4\ days}$: 2 mg/L - MMAs(III): $EC_{50,\ 7\ days}$: 2 mg/L - As(V): $EC_{50,\ 4\ days}$: 87 mg/L - As(V): $EC_{50,\ 7\ days}$: 82 mg/L - As(III): $EC_{50,\ 4\ days}$: 3 mg/L - As(III): $EC_{50,\ 7\ days}$: 2 mg/L	/	/

Manganese (Mn)	
Article	Metal Uptake and Physiological Changes in *Lemna gibba* exposed to manganese and nickel[8]
Species	*L. gibba*
Exposed to	Unknown
Exposure dose	0.25, 1, 4, 16 mg L^{-1}
Exposure duration	24, 48, 72 hours
Remarks	*L. gibba* can be used as a phytoremediant for excess Mn due to its more efficient Mn uptake and accumulation potential compared to Ni.

	Molybdenum (Mo)	**Selenium (Se)**	**Barium (Ba)**	**Thorium (Th)**
Article	The chronic toxicity of molybdate to freshwater organisms. I. Generating reliable effects data[118]	Toxicity of selenium to *Lemna minor* in relation to sulfate concentration[119]	Site-specific barium toxicity to common duckweed, *Lemna minor*[120]	Uranium and thorium uptake on hydrophilic plants[121]
Species	*L. minor*	*L. minor*	*L. minor*	*L. minor*
Exposed to	$Na_2MoO_4 \cdot 2H_2O$	- Na_2SeO_3 - Na_2SeO_4	Ba is various water samples, encompassing lake and stream waters with a wide variation in water quality	$Th(NO_3)_4$
Exposure dose	110, 225, 438, 814, 1624, 3335, 6505 µM	1, 2, 4, 8, 16, 32, 64, 128, 256 µM	/	100 000 µM
Exposure duration	7 days	12 days	/	24 hours
Remarks	- EC_{10}: 1000 µM - NOEC: 100 µM - LOEC: 214 µM	/	Ba toxicity to duckweed was highly dependent on site-specific water quality.	High uptake of Th^{4+} ions in the absence of ionic competition.

3.2 Exposure of *Lemna minor* to individual metals

Using toxicity levels defined from the literature search (see above), a range finding experiment was first set up to gain insight on the amount of metal toxicity that could be expected. The range finding experiments enabled us to select relevant concentrations for a complete DRC. This range finding experiment was performed for Ni, Pb, Zn, Cu and Fe. The growth inhibition (relative to control) after seven days of exposure was calculated on three different endpoints: frond area, number of fronds and the frond biomass (Figure 3). For all metals a dose-dependent growth inhibition was observed, although none of the curves reached a growth inhibition of 100%.

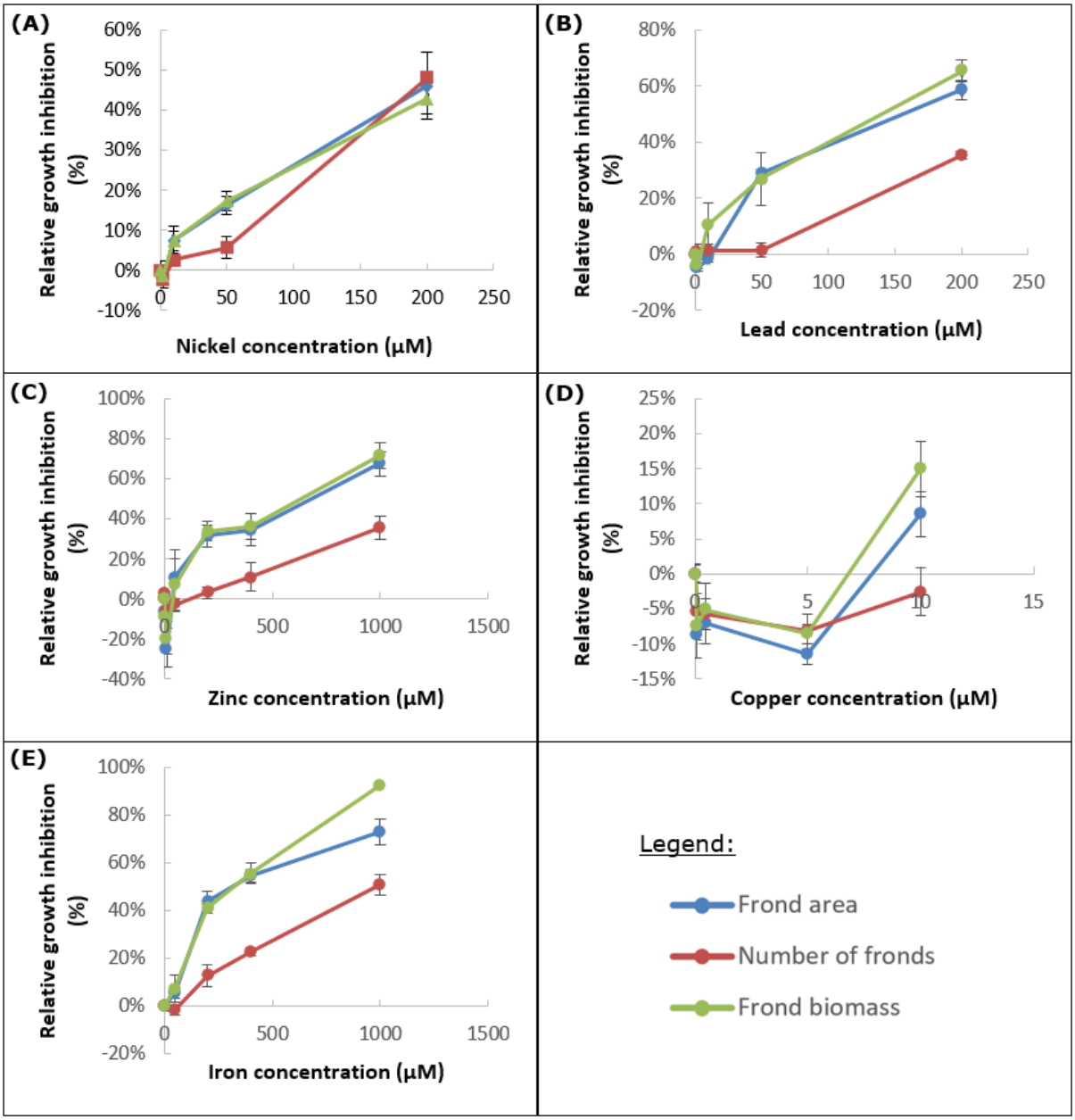


Figure 3: Range finding experiments. Growth inhibition (relative to control) based on frond area, number of fronds and frond biomass induced in *Lemna minor* plants exposed for seven days to (A) 2, 10, 50 or 200 µM nickel; (B) 2, 10, 50 or 200 µM lead; (C) 2, 10, 50, 200, 400 or 1000 µM zinc; (D) 0.1, 0.5, 5 or 10 µM copper; (E) 50, 200, 400 or 1000 µM iron. Data represent the mean ±SE of at least three biological independent replicates for each metal concentration, and six replicates for the control. The x-axis represents the metal exposure concentrations.

Of all tested metals Fe reached the highest growth inhibition of approximately 90%. The toxicity of the other metals was much lower. The maximum relative growth inhibition of Ni was only 50% (Figure 3A), while Pb (Figure 3B) and Zn (Figure 3C) both reached a relative growth inhibition of approximately 60%. Especially for Cu (Figure 3D) the observed maximum growth inhibition of 15% was much lower than expected based on the literature search. Most of the defined EC50 values for Cu in the literature search were 10 µM or less, while in this experiment only 15% growth inhibition was reached after exposure to 10 µM. At low concentrations, Cu (Figure 3D) and Zn (Figure 3C) showed a negative growth inhibition. It can be noted that, for every metal, the curve for number of fronds showed less growth inhibition than both frond area and frond biomass.

High concentrations of the Fe dissolved in the growth medium partially precipitated, which means the fraction of Fe available to the plants is uncertain. Therefore, it was impossible to draw conclusions between exact Fe concentration and growth inhibition observed. Hence, Fe was excluded from further studies. A range finding for U was unnecessary because enough information regarding toxicity was available from previous experiments performed within the BIS group[18, 101]. Based on these results, a range of concentrations was identified that were expected to cover the entire DRCs between 0-100% growth inhibition. The concentrations for Ni and Zn ranged between 10 µM and 750 µM, Cu between 5 µM and 50 µM and U between 0.5 µM and 25 µM.

Dose-response curves for single metals

A DRC is used to plot the effects on an organism caused by increasing levels of exposure to a chemical after a certain exposure time. In this experiment, *L. minor* plants were exposed for seven days to a broad range of concentrations of Ni, Zn, Pb, Cu or U, after which a log-logistic function was fitted through the data (Figure 4). As described above in the range finding experiment growth inhibition (relative to control) after seven days of exposure was based on three different growth-related endpoints: frond area, number of fronds and frond biomass.

Based on the log-logistic curves, the EC10, EC30 and EC50 were estimated for each metal (Table 5). The standard error could not be displayed because this experiment was only conducted once. The goodness of fit, which summarises the discrepancy between observed and the expected values, is shown as the residual sum of squares and should be as low as possible.

The ECx values based on frond area and frond biomass were correspondent to each other, whereas the endpoints based on number of fronds showed higher ECx values for most of the metals. This was especially noticeable after exposure to Ni, of which the ECx values of number of fronds were twice as high (EC50 of 252 µM) as the ECx values of frond area (EC50 of 140 µM) and frond biomass (EC50 of 164 µM). However, after exposure to U the EC10 based on the endpoint number of fronds was lower (0.85 µM) than both frond area (1.99 µM) and frond biomass (2.57 µM), while the EC30 (10.24 µM) and EC50 (48 µM) based on number of fronds were higher than the endpoints based on area and biomass. Based on these results, Cu had the highest toxicity because the EC50 was reached after exposure to only 30 µM.

Table 5: Concentrations of copper, nickel, lead, uranium and zinc inducing 10% (EC10), 30% (EC30) and 50% (EC50) growth inhibition based on three endpoints in *Lemna minor* after 7 days of exposure. The goodness of fit is shown as the residual sum of squares and should be as low as possible.

Metal	Endpoint	Goodness of fit	EC10 (µM)	EC30 (µM)	EC50 (µM)
Copper	Frond area	0.21	14.8	19.7	23.3
	Frond biomass	0.30	18.1	21.4	23.8
	Number of fronds	0.04	21.7	26.7	30.3
Nickel	Frond area	0.09	15.6	60.2	140
	Frond biomass	0.10	27	81.9	164
	Number of fronds	0.10	103	179	252
Lead	Frond area	0.06	6.28	19	38
	Frond biomass	0.07	8.49	29.9	65.8
	Number of fronds	0.08	16.8	36	57.9
Uranium	Frond area	0.06	1.99	5.72	11.1
	Frond biomass	0.09	2.57	5.54	8.98
	Number of fronds	0.05	0.85	10.2	48
Zinc	Frond area	0.20	39.3	198	546
	Frond biomass	0.30	62.3	725	1444
	Number of fronds	0.09	237	725	1444

As can be concluded from the low goodness of fit values (Table 5), the log-logistic functions in figure 4 fitted all data relatively well. All curves were sigmoid curved and showed a dose-dependent trend. However, not all curves reached a growth inhibition of 100%. The DRCs of Zn reached a maximum growth inhibition of only 60%, the DRCs of Ni only 80%.

For each metal, the three curves from the growth-based endpoints were more or less similar to each other. The curves were close to each other, meaning they had the same conclusion about toxicity. Except the DRC of number of fronds for each metal showed less growth inhibition than the DRCs of frond area and frond biomass for the same metal. This is visible in figure 4B in which the DRC based on number of fronds after exposure to Pb reached only 30% growth inhibition, while the DRCs based on area and biomass reached 70% growth inhibition. This is analogous to the data in table 5, where the ECx values of number of fronds were the most divergent compared to frond area and frond biomass. In figure 4A, the DRC based on number of fronds underestimated the toxicity after exposure to Ni when compared to the DRCs based on area and biomass, but eventually this DRC reached the same maximum growth inhibition of 80% as the DRCs based on frond area and biomass.

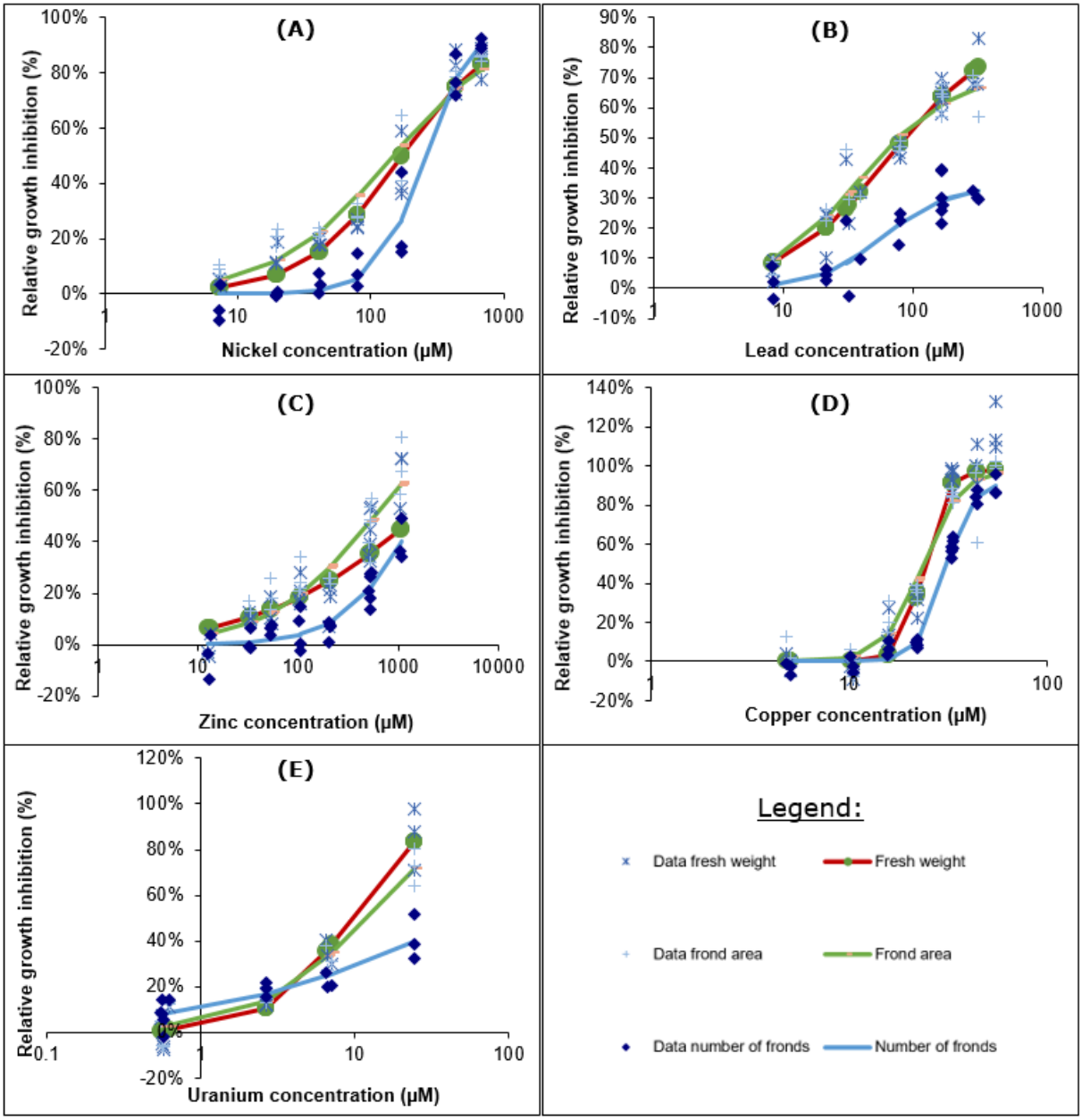


Figure 4: Dose-response curves (DRCs). Growth inhibition (relative to control) based on frond area, number of fronds and frond biomass induced in *Lemna minor* plants exposed for seven days to (A) 7, 20, 40, 80, 170, 435 or 680 µM nickel; (B) 8, 21, 35, 80, 170 or 308 µM lead. (C) 10, 25, 50, 100, 200, 500 or 1000 µM zinc; (D) 5, 10, 15, 20, 30, 40 or 50 µM copper; (E) 0.5, 4, 6.5, 10 or 25 µM uranium. Data points represent three biological independent replicates for each metal concentration, and six replicates for the control, with a log-logistic curve based on the EC50 fitted through the data points. The x-axis represents the medium concentrations of the metals measured at the start of each experiment. Crosses (X), plus signs (+) and diamonds (◆) represent measured data of fresh weight, frond area and number of fronds respectively.

3.3 Exposure of *Lemna minor* to metal mixtures

To investigate the effects of U on mixtures composed of several metals, a metal mixture experiment was conducted in which *L. minor* plants were exposed to several different combinations of a mixture. This metal mixture was composed of Cu, Pb, Ni and Zn in the same amounts they were present in a sample from Beaverlodge Lake, in the proximity of a former U mining site. This mixture was then combined with or without U to identify U-induced effects. The experiment was conducted twice and relative growth inhibition was based on frond area, number of fronds and frond biomass (Table 6). The combination that naturally occurs in the Beaverlodge sample is the M1 combination with 2 µM.

No statistical differences were found between the two experiments. Therefore, the results of the two experiments will be counted together. A dose-dependent growth inhibition could be seen with increasing U concentration or with increasing metal concentration for all three endpoints. The endpoints based on frond area and frond biomass were correspondent to each other. However, the endpoint based on the number of fronds differed the most compared to the other two endpoints, as already seen in figure 4 and table 5. Therefore, frond area was the endpoint that was mainly focussed on.

Combinations without U and with 2 µM U mostly yielded a negative relative growth inhibition, meaning they grew better than control plants. This is especially the case in the M0, M1, M10 and M100 metal combination. In the M1 combination without U in experiment 1, the relative growth inhibition varied between -1 and -4%, while the growth inhibition for the same metal combination but combined with 2 µM, ranged between -11 and 1%.

The growth inhibition observed at 10 µM U did show a maximum growth inhibition of approximately 24% (M1) based on frond area. This growth inhibition then increased to a final maximum growth inhibition of approximately 48% after exposure to M1000. Unfortunately, this last percentage of M1000 cannot be compared to experiment 2 because results from U10-M1000 from experiment 2 had to be removed. These plants were not exposed to the amount of 10 µM U as shown by analysis of the growth medium by ICP-MS (Appendix 4).

Table 6: Growth inhibition (relative to control) of metal mixtures based on frond area, number of fronds and frond biomass. *Lemna minor* plants were exposed for seven days to combinations of copper (Cu), lead (Pb), nickel (Ni), zinc (Zn) and/or uranium (U). The M0 combination refers to modified K-medium without additional metals. The M0 without U is used as control. The M1 combination exists of environmentally relevant metal concentrations, and M10, M100 and M1000 are a tenfold, hundredfold and thousandfold higher concentrations, respectively. Each metal mixture was combined with 0, 2 or 10 µM U. Data represent the mean ±SE of at least three biological independent replicates for each metal concentration and the control. Values in bold are significantly different from the corresponding controls. Significant differences between different U concentrations, within the same metal combination, are represented for frond area (small letter), number of fronds (capital letter) and frond biomass (small letter with apostrophes) (one-way ANOVA, p-value <0.05).

Concentration Beaverlodge	Endpoint	EXPERIMENT 1			EXPERIMENT 2		
		0 µM U	2 µM U	10 µM U	0 µM U	2 µM U	10 µM U
M0	Frond area	0.00% ±0.62%^{a}	-15.6% ±0.18%^{a}	**22.3% ±6.6%**^{b}	0.00% ±2.01%^{a}	-8.7% ±0.43%^{a}	**18.7% ±2.27%**^{b}
	No. of fronds	0.00% ±1.18%^{A}	-4.26% ±4.00%^{A}	**17.8% ±2.38%**^{B}	0.00% ±1.69%^{A}	-1.88% ±1.27%^{A}	**18.6% ±3.19%**^{B}
	Frond biomass	0.00% ±1.19%$^{a'}$	-9.5% ±0.69%$^{a'}$	**25.4% ±5.9%**$^{b'}$	0.00% ±3.31%$^{a'}$	-9.7% ±1.81%$^{a'}$	16.7% ±6.25%$^{a'}$
M1	Frond area	-3.43% ±1.28%^{ab}	-11.05% ±2.65%^{a}	12.5% ±0.67%^{b}	-6.50% ±2.07%^{a}	**-37.8% ±16.5%**^{b}	**24.8% ±2.18%**^{c}
	No. of fronds	-4.3% ±1.94%^{A}	1.65% ±2.10%^{A}	**18.9% ±3.7%**^{B}	-9.05% ±3.13%^{A}	-4.00% ±0.48%^{A}	**20.4% ±3.80%**^{B}
	Frond biomass	-1.42% ±3.17%$^{a'}$	-5.88% ±2.06%$^{a'}$	**20.6% ±1.10%**$^{b'}$	-12.06% ±1.62%$^{a'}$	**-34.3% ±15.5%**$^{a'}$	**28.3% ±2.60%**$^{b'}$
M10	Frond area	**-17.7% ±6.73%**^{a}	-16.1% ±3.31%^{a}	15.5% ±4.33%^{b}	-12.7% ±3.6%^{a}	-11.3% ±2.12%^{a}	**21.5% ±5.8%**^{b}
	No. of fronds	-4.47% ±2.81%^{A}	-6.3% ±3.25%^{A}	**18.6% ±1.35%**^{B}	-6.3% ±5.2%^{A}	-5.3% ±1.52%^{A}	**22.04% ±4.3%**^{B}
	Frond biomass	**-17.1% ±5.18%**$^{a'}$	-10.9% ±1.97%$^{a'}$	**21.8% ±4.6%**$^{b'}$	-18% ±3.8%$^{a'}$	-12.7% ±1.91%$^{a'}$	18.3% ±0.81%$^{b'}$
M100	Frond area	-6.5% ±3.69%^{a}	-12.3% ±6.64%^{a}	**19.6% ±5.3%**^{b}	-5.93% ±1.45%^{a}	-12.2% ±4.29%^{a}	**21.8% ±4.66%**^{b}
	No. of fronds	-8.1% ±3.52%^{A}	-6.5% ±3.57%^{A}	**19.4% ±1.62%**^{B}	-3.81% ±5.28%^{A}	-4.27% ±1.34%^{A}	**20.2% ±0.96%**^{B}
	Frond biomass	-5.9% ±1.39%$^{a'}$	-7.2% ±5.11%$^{a'}$	**25.1% ±5.7%**$^{b'}$	-13.3% ±3.37%$^{a'}$	-11.7% ±3.23%$^{a'}$	**31.5% ±7.52%**$^{b'}$
M1000	Frond area	**27.5% ±0.72%**^{a}	**29.3% ±4.39%**^{ab}	**48% ±2.19%**^{b}	**34.3% ±2.72%**^{a}	**22.4% ±1.80%**^{a}	
	No. of fronds	2.08% ±1.45%^{A}	**11.7% ±0.85%**^{AB}	**23.4% ±2.33%**^{B}	3.01% ±0.70%^{A}	10.5% ±0.84%^{A}	
	Frond biomass	**25.6% ±1.78%**$^{a'}$	**28.2% ±3.72%**$^{a'}$	**49.3% ±2.81%**$^{b'}$	20% ±2.11%$^{a'}$	**24.8% ±2.79%**$^{a'}$	

To analyse if there are interactions between the different components in the mixture and whether the mixture acts as CA or IA, the mixtures were plotted using MixTox. MixTox is an excel application described by Jonker *et al.*[105], which generates a predicted CA or IA curve that fits through as much data points as possible. It then checks by means of a chi-square test whether a new plot including a parameter for synergism or antagonism (S/A) better fits the data. If this is the case, then another parameter can be added to test for dose ratio-dependent (DR) deviations giving asymmetric isoboles, and dose level-dependent (DL) deviations giving variable isoboles depending of the dose level or effect concentration. A limitation of MixTox is that it requires a univariate response. As a result, the growth inhibition was visualised relative to the M10 metal combination because MixTox could not handle the negative growth inhibition values of the controls (M0) and M1.

Table 7 provides the R-squared values of the predicted fittings, which is a measure of how close the data are to the fitted regression line. Adding a new parameter to test S/A for CA within MixTox did not decrease the residual sum of squares, which resulted in a negative Chi-value. Therefore, a Chi-test was not possible. It can be concluded that this mixture acted like both the CA or IA model, because of the p-values <0.05. Based on this limited data set, there is not enough evidence to conclude synergistic or antagonistic effects.

Table 7: Concentration Addition and Independent Action predictions using MixTox.
S/A = Synergism/Antagonism. The residual sum of squares should be as low as possible.

	Concentration Addition (CA)		Independent Action (IA)	
	CA	CA vs S/A	IA	IA vs S/A
R^2	0.958	0.954	0.910	0.911
Residual sum of squares	0.184229	0.201908	0.20166	0.19998
p-value or ChiTest	9.0199×10^{-30}	N/A	1.989×10^{-22}	0.523
Preferred model	✓		✓	

Figure 5 shows a summary of the growth inhibition results together with predicted growth inhibition following IA. The IA can be seen in figure 5: the relative growth inhibition increased with higher U concentration, and also with higher metal concentration. When U was combined with the metal mixtures, the effects were greater than the effects of the corresponding metals or U alone. The predicted growth inhibition acting as IA accurately aligned with the actual data points.

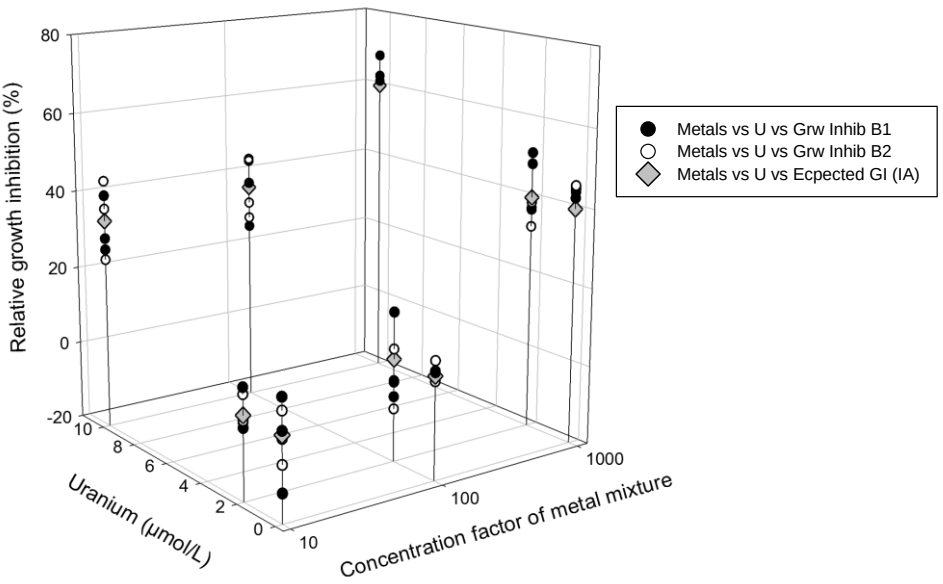


Figure 5: Growth inhibition for uranium versus the metal concentration (relative to tenfold metal mixture). *Lemna minor* plants were exposed to combinations of metal mixtures for seven days. The x-axis shows the uranium (U) concentration expressed in µM, while the y-axis shows the concentration factor of the metal mixture starting from M10. The relative growth inhibition is presented in the z-axis. B1: Batch (experiment) 1; B2: Batch (experiment) 2; GI: growth inhibition; IA: independent action.

3.4 Metal uptake in *Lemna minor* exposed to U, mixture of different metals or both

The metal content of U, Zn, Ni and Cu of the *L. minor* plants after seven days of exposure (Experiment 2; see table 6) was analysed to study whether the presence of U in a mixture changes the metal uptake by plants in that mixture (Figure 6). Because the plants from experiment 2 were used for this analysis, metal mixture M1000 combined with 10 µM U had to be removed as mentioned above. These plants were not exposed to the amount of 10 µM U as shown by analysis of the growth medium by ICP-MS (Appendix 4). Measurement of Pb was impossible as these plants were washed with $Pb(NO_3)_2$ to remove surface-bound U.

As can be observed in figure 6A, significantly more U was taken up by plants after exposure to 10 µM U than after exposure to 2 µM U, independent of the presence of the metal concentrations M1 and M10. When 10 µM U was combined with M100, significantly less U was taken up by plants compared to the lower metal concentrations (M1-M10).

Regarding the uptake of Zn (Figure 6B), a dose-dependent uptake was shown when exposed to mixtures without U, or with 2 µM U: the higher the metal concentration, the significantly more Zn taken up by the plants. This was not the case when exposed to the mixtures containing 10 µM U. When exposed to 10 µM U, Zn uptake was not significantly higher than the control, meaning the presence of U apparently diminished or counteracted Zn uptake. This was the case for exposure to the M10 or M100 mixture, in which there was a significantly lower Zn uptake with increasing U concentration. A similar trend could be seen after exposure to the M1000 mixture, but regretfully only data for control and 2 µM U and not for 10 µM U were available.

In control conditions without U added, uptake of Cu showed a significant dose-dependent increase with increasing metal concentrations. However, when U was added to the mixture, Cu

uptake diminished, which was similar to Zn uptake. This was significant within the M0, M10 and M100 mixture. The same trend was visible within the M1000 mixture, but there is no data available for 10 µM U. The Ni uptake by the plants was almost zero for all conditions and therefore negligible.

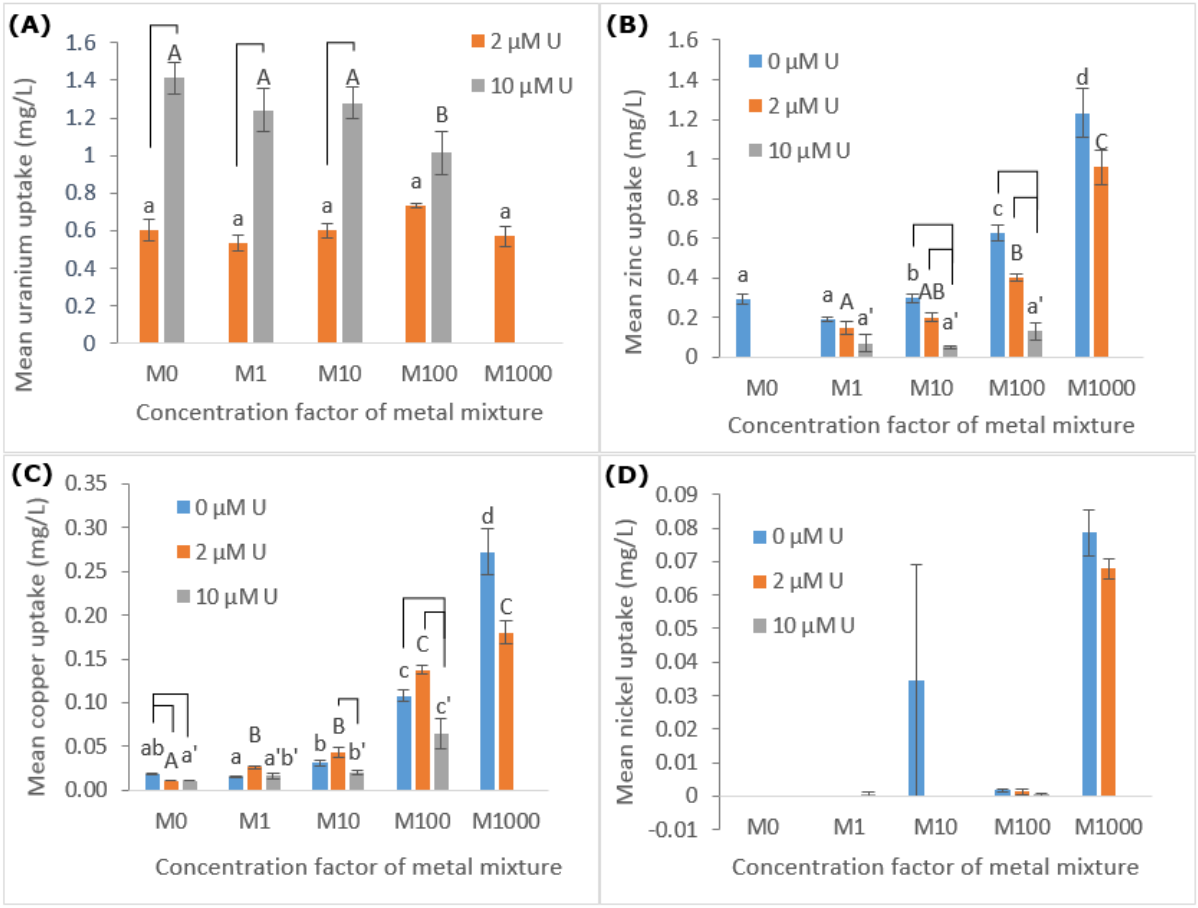


Figure 6: Metal uptake of (A) uranium, (B) zinc, (C) copper and (D) nickel in *Lemna minor* plants after exposure for seven days to metal mixtures. The M0 combination refers to modified K-medium without additional metals. The M1 combination exists of environmentally relevant metal concentrations, and M10, M100 and M1000 are a tenfold, hundredfold and thousandfold higher concentrations, respectively. Each combination was combined with 0, 2 or 10 µM uranium (U). Data represent the mean ±SE of at least three biological independent replicates for each metal concentration and the control. Different letters mean significant differences within the same U concentration. Brackets indicate significant differences within the same metal concentration, but with different U concentrations (one-way ANOVA, p-value <0.05). Statistical analysis for nickel was impossible since most values equaled zero.

3.5 Primer optimisation

Only recently a draft *L. minor* genome became available[100] and an RNAseq experiment was conducted in the BIS group on plants exposed to U, beta or gamma radiation (Van Hoeck *et al.*, personal communication). This enabled the search for genes that only reacted after exposure to U and thus potentially can be used as biomarkers, which is the goal of this study. For the first time *L. minor* genes could be studied by means of a real-time PCR analysis. From the RNAseq experiment (Appendix 1), 14 potential housekeeping genes were selected that showed a high but similar response under all tested conditions, and 10 potential U biomarkers that only responded in a dose-dependent manner to uranium exposure and not after beta or gamma radiation. Genes with the lowest overall standard deviation and a high overall expression for all exposure concentrations were taken into account. Primers were designed as described in paragraph 2.5. As such, 22 primer pairs

(primer numbers 1 through 22) designed for the 14 selected housekeeping genes, and 18 primer pairs (primer numbers 23 through 40) designed for 10 U biomarkers were tested by means of a gel electrophoresis and real-time PCR (Table 9 and 10).

Primers that were selected for further use showed in the gel electrophoresis a single amplicon, indicating a single PCR product, and a clear diminishing of the expression level proportionally to the decreasing amount of cDNA added. Additionally, only primers of which the cDNA dilution series showed a proportionate amplification during real-time PCR, or in other words showing the same interval between the dilutions, were selected. The melt curve was of less importance, because the presence of a double peak does not always indicate a non-specific amplification[122]. The complete overview of all tested potential housekeeping genes and U biomarkers can be found in Appendix 2 and 3 respectively. Primer numbers 4, 9, 15, 18, 20 and 22 were selected as housekeeping genes, and primer numbers 24, 33, 34, 36, 39 and 40 were selected as U biomarker primers. The predicted function of these genes can be found in table 8.

Table 8: Predicted function and primer numbers of selected housekeeping genes and uranium markers.

Housekeeping genes		
Gene	**Primer number**	**Function**
Lminor_003273	Primer 4	Serine/threonine-protein phosphatase
Lminor_014887	Primer 9	Subtilisin-like protease
Lminor_009513	Primer 15	Mitogen-activated protein kinase kinase
Lminor_004034	Primer 18	Tubulin beta-5 chain
Lminor_012610	Primer 20	Cytochrome P450
Lminor_009166	Primer 22	Gamma-tubulin complex component
Uranium markers		
Gene	**Primer number**	**Function**
Lminor_020037	Primer 24	Protein of unkown function
Lminor_010222	Primer 33	Cytochrome P450 family protein
Lminor_020596	Primer 34	Proliferating cell nuclear antigen
Lminor_016305	Primer 36	Peroxidase
Lminor_009276	Primer 39	Auxin-responsive protein
Lminor_014505	Primer 40	Phenylalanine ammonia-lyase

Table 9: Final selection of housekeeping genes for *Lemna minor*. Primers were verified using real-time PCR and gel electrophoresis.

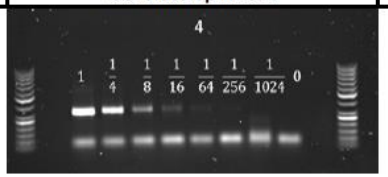
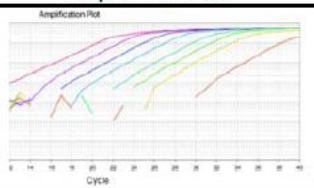
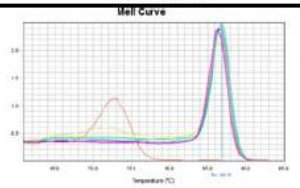
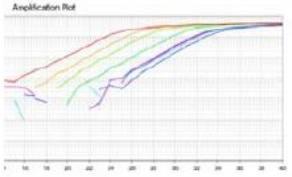
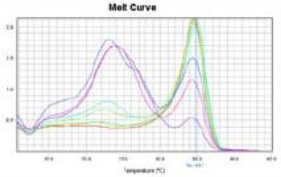
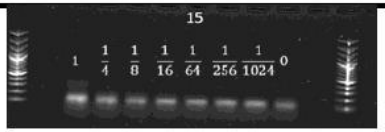
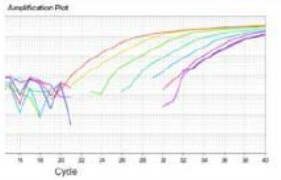
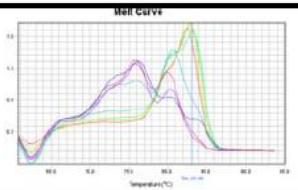
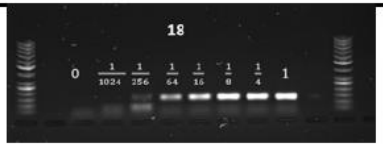
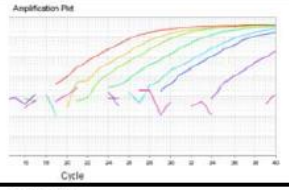
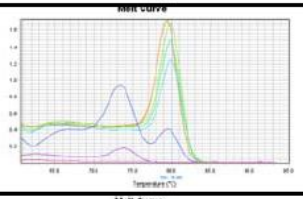
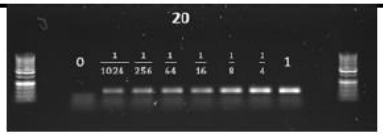
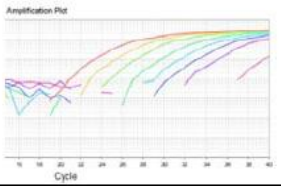
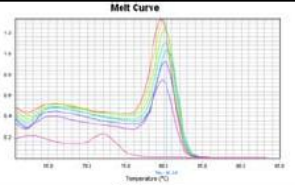
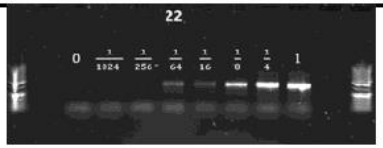
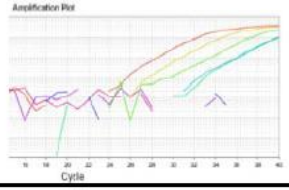
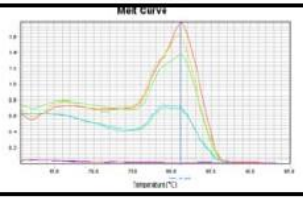
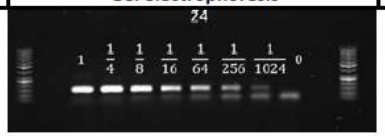
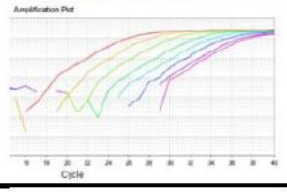
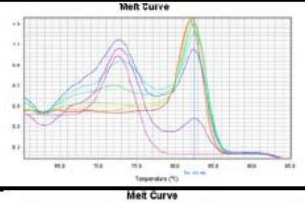
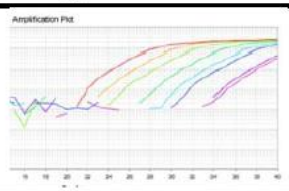
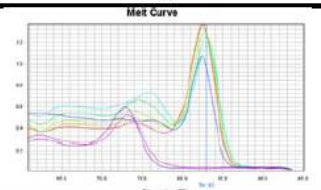
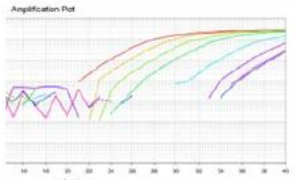
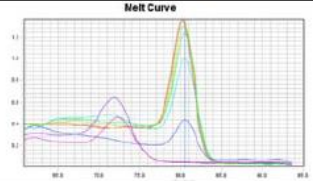
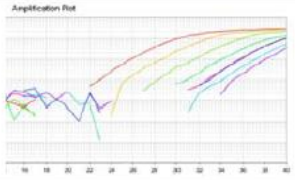
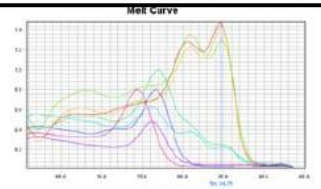
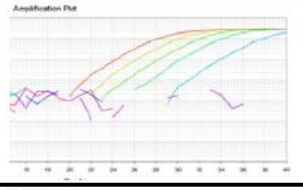
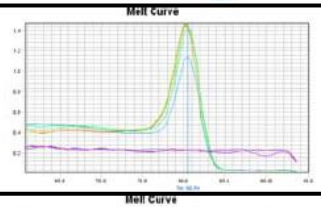
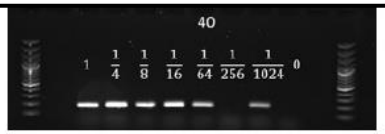
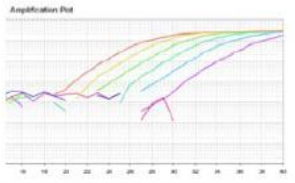
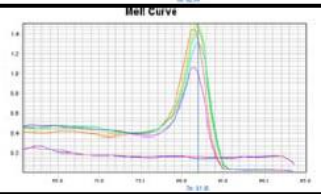
Primer No.	Primer pairs	Sequence	Gel electrophoresis	Amplification Plot	Melt Curve
4	Lminor_003273_1_F Lminor_003273_1_R	GCGCCGATCCTAGATTTGAA CAGGCCCATCCTCCTTCTTC			
9	Lminor_014887_2_F Lminor_014887_2_R	GAGACGGGGCAAGAGTTCAA CATCCACCGGCTAACCCATT			
15	Lminor_009513_1_F Lminor_009513_1_R	TTGGATCATGAGGGGAAGGAG CGTCCTGAAAGACGCCACAA			
18	Lminor_004034_2_F Lminor_004034_2_R	TTCGTCCGGATAGCTTCGTG GCCATTTTCGGATTCCTCGC			
20	Lminor_012610_1_F Lminor_012610_1_R	TTGAGCTTGCTCTTGCTGGT TCCTGCGAATGGTAAAGCCC			
22	Lminor_009166_1_F Lminor_009166_1_R	TCGTCAGCCAGCCAGTAAAG AGTCTCCCTGTCCGAGAAGA			

Table 10: Final selection of uranium biomarker genes for *Lemna minor*. Primers were verified using real-time PCR and gel electrophoresis.

Primer No.	Primer pairs	Sequence	Gel electrophoresis	Amplification Plot	Melt Curve
24	Lminor_020037_1_F Lminor_020037_1_R	ACTACAACAATGGCTGGCCC ATCGGTCTGCTTGATGCTCG			
33	Lminor_010222_2_F Lminor_010222_2_R	CGACACTCTCATGTCCCTGC ATATCGTGATCCAACGCCCG			
34	Lminor_020596_2_F Lminor_020596_2_R	TGATGGACATCGACAGCGAG ATCGTTCGTACCTGTGTCGC			
36	Lminor_016305_2_F Lminor_016305_2_R	AGACAGTGGCTGGCTTCATC CGACCAGTCTCCACTCTCCA			
39	Lminor_009276_2_F Lminor_009276_2_R	CCTCGAACACCCTTCCTTCC ACGAAGTAGTCCTCTCCGCA			
40	Lminor_014505_2_F Lminor_014505_2_R	GCAGCACAACCAAGATGTGA CTGACTCACCGCGTTCTTGA			

3.6 Gene expression

Using real-time PCR, six potential biomarker primers were for the first time selected and tested for their U specificity. To do this, *L. minor* plants were exposed to the individual metals Cu, Ni, Pb, U or Zn and to a combination of metals with or without U.

Individual metals

In general, primers 24, 33 and 34 showed a dose-dependent upregulation for the treated compared to control plants, while primers 36, 39 and 40 showed a downregulation (Figure 7). For most primers a similar response was measured for all metals except for primer 33. The gene expressed by primer 33 is the only one that showed a very strong reaction only after exposure to U. For most primers (exceptions see below) the observations regarding the expression levels of U exposure were in accordance with the expression levels after exposure to U in the previously mentioned RNAseq experiment (Appendix 1). This RNAseq experiment revealed a number of genes that were upregulated and a number of genes that were downregulated only after U exposure and not after exposure to beta or gamma radiation.

Looking at the individual gene response, it could be noted that primer 24 showed a significant upregulation after exposure to 4 and 6.5 µM U toward the control, but not after exposure to 0.5 µM U. However, the response of 6.5 µM U was significantly higher than the response after 0.5 µM U. Primer 24 showed a significant upregulation after exposure to almost all metal concentrations, except the lowest concentrations of 5 and 10 µM Cu, 10 µM Ni and 10 µM Zn.

Primer 33 showed a strong significant response compared to the control after exposure to all U concentrations, and gene expression after exposure to 4 and 6.5 µM U was significantly different from exposure to 0.5 µM U. However, also a significant response toward the control could be observed after exposure to other high metal concentrations of Cu (15 and 20 µM) and Pb (100 µM). Exposure to the other metal concentrations did not elicit a significant response from primer 33.

The same trend could be observed after using primer 34. Exposure to the high concentrations of U (4 and 6.5 µM) caused a gene expression significantly higher than the control. Concerning the other metals, only a significantly higher gene expression than the control was observed after exposure to Cu (10, 15 and 20 µM).

In contrast to expectations, primer 36 did not show significant differences relative to the control in U-exposed plants. However, exposure to the other metals did elicit a significant down regulation in gene expression using primer 36. Gene expression of all metal concentrations showed a significant response, except 20 µM Cu and 10 µM Pb. Gene expression and Zn concentration were inversely related: the higher the Zn concentration, the lesser the gene expression.

The same trend was observed after using primer 39. Exposure to U only elicited a significantly lower gene expression level relative to the control after exposure to 4 µM U. All other metals showed a significantly lower gene expression toward the control except exposure to 10 and 25 µM Zn. In addition, gene expression and metal concentration were inversely related: lesser gene expression with increasing metal concentration. However, only exposure to 100 µM Zn generated a significantly lower gene expression level in comparison to exposure to 10 µM Zn.

Primer 40 yielded a gene expression response significantly different from the control after exposure to 4 and 6.5 µM U, but not after exposure to 0.5 µM U. All other responses were significantly lower relative to the control, except for the response after exposure to 10 and 25 µM Ni, and 10 µM

Zn. Again, gene expression levels and all metal concentrations seemed to be inversely related. This could in particular be deduced from the significantly lower gene expression level after exposure to 100 µM Pb compared to 10 µM Pb.

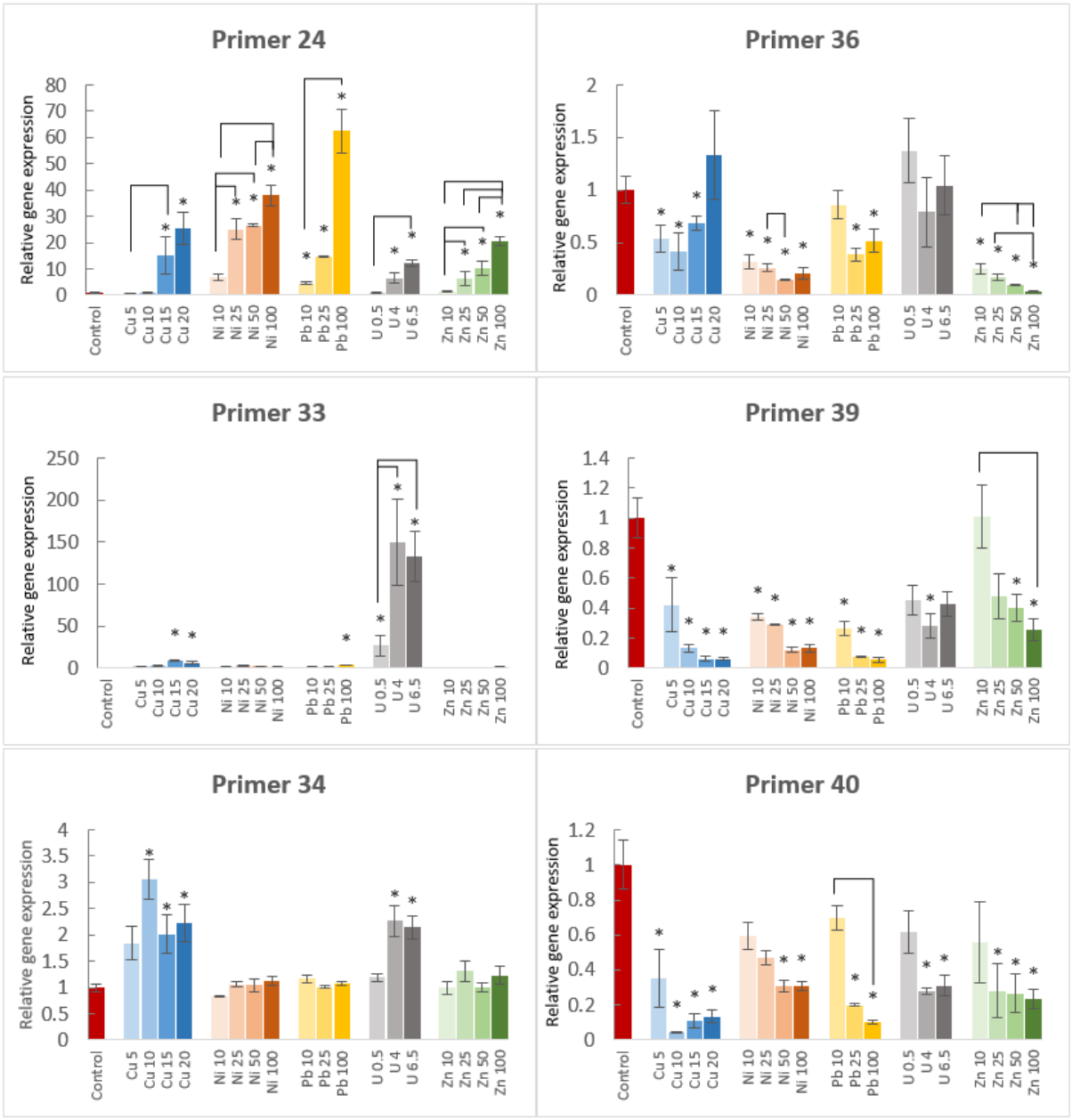


Figure 7: Gene expression levels of *Lemna minor* relative to control after exposure to single metals. *Lemna minor* plants were exposed for seven days to a range of single metal concentrations of 5, 10, 15 or 20 µM copper (Cu), 10, 25, 50 or 100 µM nickel (Ni), 10, 25 or 100 µM lead (Pb), 0.5, 4 or 6.5 µM uranium (U) or 10, 25, 50 or 100 µM zinc (Zn). Data represent the mean ±SE of at least three biological independent replicates for each metal concentration and the control. Brackets indicate significant differences within a single metal group, asterisks (*) indicate significant differences relative to the control (one-way ANOVA, p-value <0.05).

Metal mixtures

After the single metals, *L. minor* plants were also exposed to metal mixtures (Figure 8). These metal mixtures were selected based on samples taken from Beaverlodge Lake. As described in paragraph 2.2, the original (M1) sample of Beaverlodge Lake consisted of 0.0157 µM Cu, 0.0145 µM Pb, 0.017 µM Ni and 0.0765 µM Zn. This combination was multiplied by 10, 100 and 1000 to obtain the M10, M100 and M1000 combinations respectively. These combinations were then combined with 0 µM, 2

μM or 10 μM U after which all 14 combinations were tested to study the influence of U on the metal mixtures and possible interacting effects between U and metal toxicity. Here the expression of the 6 potential biomarker genes was also tested in plants exposed to U, a mixture of U and metals or the metal mixture alone.

In accordance to exposure to single metals (Figure 7), primers 24, 33 and 34 also showed an upregulation after exposure to metal mixtures compared to the control (Figure 8). Primers 36, 39 and 40 showed a downregulation relative to the control after exposure to the metal mixtures. No significant differences were found when comparing the gene expression between U-M0, U-M1 and U-M10 for all three U concentrations. Primer 33 showed a very high increase of gene expression after exposure to 2 and 10 μM U. This is in accordance to figure 7, which also showed a very high upregulation after U exposure using primer 33. Both primer 34 and 40 only showed a significant difference in gene expression after exposure to mixtures with 10 μM U, which could be expected as figure 7 showed only a significantly different gene expression after exposure to 4 and 6.5 μM U. Accordingly, primer 36 did not elicit significant responses for after 0.5, 4 and 6.5 μM U in figure 7, and only showed significant responses after exposure to 10 μM U in figure 8. Primer 39 showed in figure 7 only a significant difference after exposure to 4 μM U and not after 6.5 μM, but in figure 8 a significant response after 10 μM U is seen.

Primer 24 showed a clear dose-dependent response to U exposure. Compared to the control, the M1000 concentrations combined with 0 and 2 μM U showed significantly higher gene expression levels, while the same U concentrations combined with the lower metal concentrations M0, M1, M10 and M100 did not elicit significant responses compared to the control. The M1, M10 and M100 combinations with 10 μM U had significantly more gene expression than the corresponding metal concentrations combined with 0 and 2 μM U. Notably, a significant response could be observed after exposure to the M1000 metal mixture without U.

Primer 33 on the other hand did not show a significantly different response relative to the control after exposure to combinations without U. When exposed to combinations containing 2 or 10 μM U, the gene expression levels for all metal concentrations (M1, M10, M100 and M1000) and U exposure alone (M0) were significantly higher than the control. The response between 0, 2 and 10 μM U combined with M10 were all significantly different from each other. This was also the case for all three U concentrations combined with M100 and M1000. It is noticeable that gene expression with primer 33 completely diminished after exposure to the highest metal combination (M1000) with 2 and 10 μM U.

Gene expression with primer 34 showed the highest significant response after exposure to 10 μM U combined with M1. The higher metal combinations M10, M100 and M1000 combined with 10 μM U showed a decreasing trend of the expression level compared to the expression after U10-M1. Gene expression levels after exposure to the combinations with 2 μM U did not elicit significantly different responses toward the control. However, exposure to U10-M1 caused a significantly higher gene expression level than the same metal concentration (M1) combined with 0 and 2 μM U.

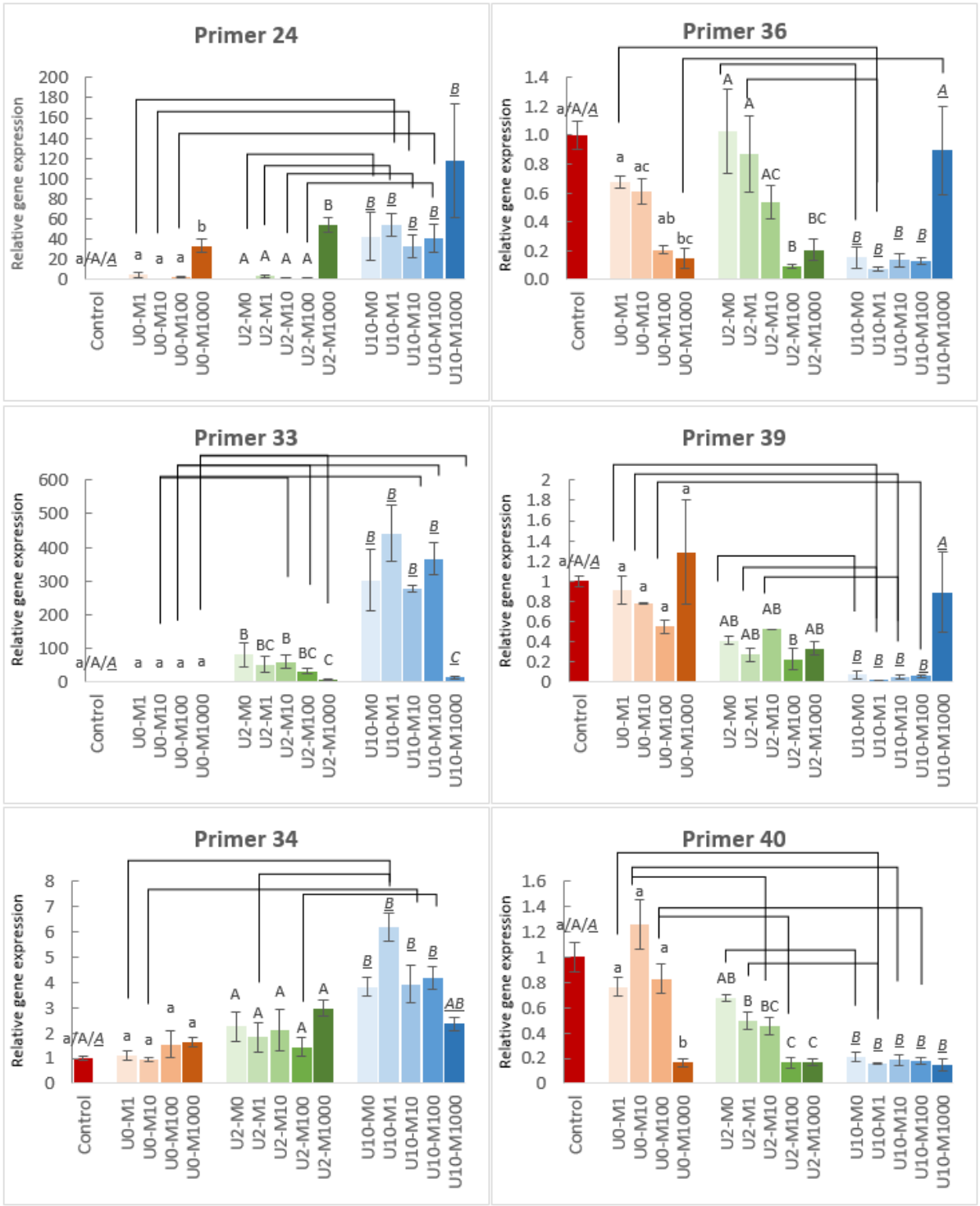


Figure 8: Gene expression levels of *Lemna minor* relative to control after exposure to metal mixtures. *Lemna minor* plants were exposed for seven days to metal mixtures combined with or without 2 µM or 10 µM uranium (U2 and U10 respectively). Metal mixture (M) 1 consists of 0.0157 µM Cu, 0.0145 µM Pb, 0.017 µM Ni and 0.0765 µM Zn. These concentrations were multiplied by 10, 100 and 1000 (M10, M100, M1000 respectively). To test effects of U alone, U was also combined with only modified K-medium without metals (M0). Data represent the mean ±SE of at least four biological independent replicates for each metal concentration and the control. Different letters within the same U concentration represent significant differences between metal concentrations, brackets indicate significant differences between different U concentrations with the same metal concentration (two-way ANOVA, p-value <0.05).

After using primer 36, the M1000 combination combined with 0 or 2 µM U had a significantly decreased gene expression level compared to the control, while the M1000 combination with 10 µM U had not. Nevertheless, this latter combination yielded a significantly higher gene expression level than the corresponding metal combination without U. The other combinations with 10 µM were significantly decreased in comparison to the control, while the corresponding metal concentrations without U or with 2 µM U were not significantly different from the control. The gene expression level after exposure to the M1 mixture combined with 10 µM U was significantly decreased compared to the M1 mixture without U or compared to the environmentally occurring M1 mixture combined with 2 µM U.

The same trend could be observed after using primer 39. The M1, M10 and M100 metal concentrations combined with 10 µM U had a significantly lower gene expression level than the corresponding metal concentrations without U. The M1 combinations with 10 µM U also caused a significant decrease of gene expression toward the M1 combination with 2 µM U. In accordance to primer 36, all metal combinations with 10 µM U were significantly lower expressed than the control except the M1000 mixture.

Primer 40 also showed dose-dependent responses. Exposure to M1000 without U caused a significantly lower gene expression toward the control. Exposure to 2 µM U caused a significantly lower gene expression level than the control for all metal concentrations except the M0. Combined with 2 µM U, the M100 and M1000 combinations also yielded a significantly lower gene expression than the M0 or M1 combination with 2 µM U. All combinations with 10 µM U had a significantly lower expression of primer 40 compared to the control, but there were no significant differences among these U10 combinations.

In conclusion a few curiosities were observed. For all genes, no significant differences were found in gene expression between one to tenfold concentrations (M1-M10) of metals as present in Beaverlodge. In addition, several M1000 mixtures generated a different reaction than the other metal concentrations. Its gene expression level fell back to the level of the control, which was the case for primer 33, 34, 36 and 39 for both 2 and 10 µM U.

4. Discussion

The aim of this study was twofold. First, we aimed at investigating possible interactions of U with other contaminants under environmentally relevant conditions. Secondly, a limited set of genes was tested for the first time as potential biomarker genes for U by measuring their response to U, single metals or a combination of U and metals. To obtain environmentally relevant conditions, the experimental design was based on samples taken from mining sites polluted with U. We focussed on samples taken from Beaverlodge Lake (northern Saskatchewas, Canada)[98]. Table 1 shows an overview of the contaminants present in this sample. A literature search was done to decide which components were the most interesting. Based on this literature search, five metals were selected, namely Ni, Pb, Zn, Cu and Fe to use in this study.

4.1 Exposure of *Lemna minor* to metals and metal mixtures

A range finding experiment was performed for these five selected metals to determine appropriate concentrations for a complete DRC, and to verify their toxicity in the modified growth medium used at BIS. A K-medium with a low KH_2PO_4 concentration is used within BIS, because the growth medium recommended by the OECD reduces U bioavailability to plants as described by Horemans *et al.*[102]. In spite of the low nutrients within this K-medium, the control conditions still met the requirements by the OECD (guideline 221)[96]. A range finding experiment was necessary, because it was uncertain if the metals in this modified growth medium would have the same degree of toxicity as described in literature. In this range finding experiment, the growth inhibition was calculated on three different endpoints: frond area, number of fronds and the frond biomass. All curves showed a dose-dependent growth inhibition, as one would expect. However, none of the curves reached a growth inhibition of 100%, which means higher exposure concentrations had to be taken into account when establishing the DRCs. The toxicity of Cu in the range finding experiment was much less than expected based on the literature search, which could be because of a different set-up, or because the modified growth medium was used instead of the suggested growth medium by the OECD[96]. Additionally, at low concentrations of Cu and Zn a negative growth inhibition could be observed, meaning a better growth at these concentrations than control plants. This can be the result of a hormetic-like response, which was also investigated using *L. minor* by Cedergreen *et al.*[123, 124]. They described hormesis as a general phenomenon that could occur among different chemicals. The underpinning mechanism(s) for this hormetic-like responses have not been resolved. It is e.g. conceivable that Cu and Zn, being two essential microelements of plant growth are rather limited in the normal K-medium. Therefore, a slightly higher Cu/Zn concentration might be beneficial for growth. Alternatively, e.g. for U a higher photosynthesis efficiency has been reported in *Arabidopsis thaliana* exposed for 3 days to low U concentrations. The excess of energy that this would generate could, if not be used in defence response, be directed to growth[125].

After exposure to single metals, the DRCs of three different growth-based endpoints, namely frond area, frond number and frond biomass were compared for each metal (Figure 4). In the ideal case, these three DRCs should be parallel. However, this is not the case, which could mean that these three endpoints do not have the same sensitivity. The DRCs representing the number of fronds always showed less growth inhibition than the DRCs of frond area and frond biomass for all metals.

This indicates that the number of fronds formed during exposure was not as affected as total frond area, and as such average frond size is smaller. Accordingly, the ECx values for all metals estimated from the number of fronds were generally higher than the ECx values estimated from the frond area or frond biomass (table 5). The ECx values based on frond area and frond biomass were more similar to each other. These findings confirm the conclusion of Horemans *et al*. that after exposure to U, growth inhibition based on number of fronds is the least sensitive endpoint for evaluation of *L. minor* growth, and thus the least reliable[101]. Dalton *et al.* concluded that the endpoint based on frond biomass was the most sensitive[126]. However, they did not include frond area while this is an easy and reliable endpoint. Frond area is non-destructive, which makes biomonitoring possible during the exposure. The fact that the number of fronds is not directly related to total biomass of the plants, implies that the frond size is not fixed, which has already been proven for U and cadmium[18]. This is the first time this was shown for other metals (Zn, Cu, Pb and Ni). In contrast, this is not the case for exposure to beta or gamma radiation, where frond area and frond number gave a similar response[127, 128]. As such, this seems to be a feature specific for *L. minor* exposed to metals.

Similar to the observation during the range finding experiments, a few data points of the DRCs also showed a negative growth inhibition at low exposure concentrations, which means the plants exposed to these concentrations grew better than the control plants. As described above, this could be the result of a hormetic-like response. The DRCs of U and Zn did not reach a growth inhibition of 100%. The highest concentration used for exposure was possibly too low to reach a maximum growth inhibition of 100%. The highest U concentration in this study was 25 µM U, while the highest U concentration used by Horemans *et al.* was as high as 150 µM[18]. They did obtain a relative growth inhibition of 100% and a sigmoid curve after exposing *L. minor* for seven days using the same growth medium as used in this study. They found an EC50 of U of approximately 29.5 µM ±1.9 µM, which is close to the mean EC50 of U of approximately 22.7 µM found in this study. However, the low growth inhibition of Zn was unexpected as the literature search identified a maximum EC50 of 48 µM. In this experiment, an EC50 of approximately 1400 µM was found. For Pb, this study identified an EC50 of 38 µM, while the literature search mentioned 19 µM[110]. The EC50 values of Cu defined in this study fall in the EC50 range described by one literature search article about Cu[39]. The other two articles that defined an EC50 for Cu mentioned lower EC50 values ranging between 3 and 10 µM[7, 108]. However, they exposed the plants to $CuCl_2$ while in this study $CuSO_4$ was used. For Ni on the contrary, two articles mentioned an EC50 of approximately 6 µM[42, 108], while in this study EC50 values for Ni ranged between 140 and 252 µM. However, as mentioned in literature, the interpretation of ECx values from different studies should be done with care, because the ECx values greatly depend on the experimental set-up, such as metal species and growth medium[101, 129].

Plants were exposed to metal mixtures, with or without U, composed of Cu, Pb, Ni and Zn. These experiments were done twice, which produced marginal and non-significant differences between the two experiments indicating that both experiments were similar (Table 6). Cedergreen *et al.* found that in binary mixtures dose-dependent effects could not be repeated consistently, and results should therefore be interpreted with great care[130]. Inconsistent results increase with increasing variation in the test system, however *L. minor* is known to be a test system with a low variability and thus a high reproducibility[130]. This is because *L. minor* plants are clones from each other which are grown in aseptic controlled conditions, following strict guidelines[96, 130].

In the second experiment, the highest metal concentration (M1000) of 10 µM U could unfortunately not be used due to an error introduced during the execution of the experiment. For both experiment taken together, no antagonistic or synergistic effects could be proven, but the mixtures of U and metals followed an IA or CA model. In other words, estimating the toxicity based only on either U or the metals alone will underestimate the joint effect present. The predictions of both models were similar, therefore, either one of these two models is sufficient for a safe evaluation of the ecotoxicological risk. Furthermore, plotting the data against predicted values acting as IA generated an accurately alignment of all data points (Figure 5). It was concluded before that IA does not predict the mixture effect significantly better than CA, but CA was the most conservative model based on the EC50 values generated by MixTox[78, 131]. However, at low metal concentration as found in the Beaverlodge sample (M1), it is clear that the effect of U alone is higher than when combined with metals in the M1 combination. Although the effects of the metals in the M1 mixtures were very low and even yielded a negative growth inhibition (Table 6). Only a limited number of combinations was tested, which can add to the fact that no interacting effects were statistically proven. However, there were signals that antagonism is present, because Zn and Cu uptake were diminished with higher U concentration. In addition, a significantly decreased U uptake was also observed when the metal concentrations were higher (Figure 6). This could be the reason why plants exposed to 2 µM U in combination with the onefold Beaverlodge concentration grew better than plants exposed to 2 µM U alone. To gain more knowledge about the toxicity of mixtures containing U, other contaminants in the Beaverlodge sample, and also in samples from other U mining sites should be investigated in the future[98].

4.2 Metal uptake

To study whether the presence of U in a mixture changes the metal uptake by *L. minor* plants in that mixture, the metal content of U, Zn and Cu in the plants was analysed. As described above, metal mixture M1000 combined with 10 µM U were not tested. At the high metal combinations M100 and M1000, plants take up less U even after exposure to 10 µM U. However, the percentage growth inhibition after 10 µM U at this high metal combination was as high as growth inhibition after 2 µM U or even 0 µM U. This means the toxicity at the M100 and M1000 is mainly caused by the metals rather than the U.

The uptake of Zn significantly decreased with increasing U concentration when exposed to the M10 and M100 mixture. The same trend was seen after exposure to the M1000 mixture, but since there is no data for the M1000 mixture containing 10 µM U, this cannot be supported. This trend was also visible regarding Cu uptake, although this was not significant. The uptake of Ni by *L. minor* plants in this experiment was almost negligible. There was no bioaccumulation of Ni. This was also found in *L. gibba* by Khellaf *et al.* who concluded that the bioconcentration factor, which is an indicator of the plants ability to accumulate trace elements relative to their concentration in the external nutrient solution, was very low for Ni, but very high for Cu[107]. Contradictory, Axtell *et al.* mentioned a more rapid Ni uptake by *L. minor* than Pb uptake[132]. To obtain more conclusive results, this experiment needs to be repeated with the incorporation of the U10-M1000 combination, and analysis of metal uptake after exposure to single metals.

4.3 Gene expression

Potential primers for biomarker genes were selected and tested using real-time PCR. *Lemna minor* plants were exposed to the single metals Cu, Ni, Pb, U or Zn and mixtures of these combined metals to test the U specificity of the potential biomarkers. Six primers were tested of which the gene expression of three (primer 24, 33 and 34) showed an up- and of three others (primer 36, 39 and 40) a downregulation after exposure. Except for primer 36, this is in consistency with the expression levels of the previously mentioned RNAseq experiment (Appendix 1). This study was a pilot study in which a limited number of genes was tested. The genes for this experiment were selected in such a way that three out of six would be upregulated, and the other three downregulated after exposure to the single metals and metal mixtures (Figure 7 and 8 respectively). After exposure to single metals, only primer 33 showed a reaction that was much more pronounced or stronger after exposure to U compared to the other metals. The other primers also showed a dose-dependent response after exposure to other metals, which means they were not specific to U exposure. As these genes were selected based on the fact that they did not or barely respond to gamma and beta radiation this could indicate that the response was more specific to metal exposure in general.

After exposure to the metal mixtures, gene expression using primer 33 remained stable within the same U concentration, independent of metal concentrations. This is ideal when designing potential U biomarkers. Primer 33 will hybridise to a gene that has an annotated function as cytochrome P450 (Table 8). Cytochromes P450 are one of the largest and oldest superfamilies coding for hemoproteins present nearly ubiquitously in organisms of all biological kingdoms[133]. They execute a very wide and extremely divergent range of reactions. In general, they are final oxidase enzymes within electron transfer chains[133]. They also act as signalling molecules and have a role in the metabolism of xenobiotics, detoxification of poisons, and biosynthesis of hormones[134]. A number of cytochrome P450s have been shown to be stress-induced proteins. The high gene expression of the gene picked up by primer 33 is the first indication of a role of cytochrome P450 in the response of *L. minor* to low U concentrations. Therefore, it could be useful to investigate this in more detail.

However, gene expression with primer 33 completely decreased after exposure to the highest metal combination (M1000) in combination with both 2 and 10 µM U. This was exactly the same for primer 39, of which gene expression remained stable within the same U concentration, independent of the metal concentrations, except for the U10-M1000 mixture. On the one hand this could be due to the high toxicity caused at these extremely high metal concentrations, which is a thousandfold higher than environmentally relevant concentrations. Therefore, it is possible there was less gene expression at these high concentrations due to the high mortality of the plants. This has been described by Saenen *et al.* who found that after exposure to extremely high U concentrations a DNA/RNA degradation occurred[135]. As a result of the degraded DNA/RNA there will be no *de novo* protein synthesis, and therefore no gene expression. On the other hand, it was seen that there is a lesser U uptake by the plants at this high M1000 metal combination, while the amount of growth inhibition remained high. Therefore, the degree of toxicity is caused by the metals rather than the presence of U. The potential biomarker genes are chosen to react after U exposure, which means if there is less U in the plants at the M1000 level, the primers might not react. It was in this study only

possible to study a small selection of genes due to time restrictions. Since this is the first time the *L. minor* genome was used to design biomarkers. Considerable effort was put into testing of the different primer sets including in finding good primers for housekeeping genes. Therefore, to gain a clearer conclusion, this experiment should be repeated in the future and more *L. minor* biomarkers should be designed and tested.

5. Conclusion and synthesis

This study mainly focussed on studying the toxic effect of U on growth in an environmental relevant mixture of different metals, and on the design of potential biomarker genes specific for U exposure in the floating macrophyt and exotoxicological species *L. minor*. The presence of U is an environmental problem, but toxicity as such is at the moment mainly studied at umbrella endpoints like growth, which are not specific. Therefore, this study aimed to find more U-specific and sensitive endpoints that could have the potential to be a biomarker. The specificity of selected genes was tested by measuring their response to U, single metals or a combination of U and metals. In addition, mixed exposure conditions were used to investigate possible interactions of U with other metals at growth related endpoints. To obtain environmentally relevant exposure conditions, the experimental design was based on samples taken from mining sites polluted with U. We focussed in particular on samples taken from Beaverlodge Lake (northern Saskatchewas, Canada). To determine the most important toxic components in this sample, a literature search was done, based on which the metals Ni, Pb, Zn, Cu and Fe were selected.

To confirm toxicity of these five metals in the modified growth medium used at BIS, a range finding experiment was performed. The toxicity of the metals was indicated with the percentage growth inhibition. The growth inhibition was calculated on three different growth-related endpoints: frond area, number of fronds and the frond biomass. All growth inhibition curves of the range finding exhibited a dose-dependent growth inhibition for all growth-related endpoints tested, but none reached a 100% growth inhibition. In addition, at low concentrations of Cu and Zn a negative growth inhibition was seen, which means the plants grew better at these concentrations than control plants. This could be the result of a hormetic-like response or the fact that Cu and Zn are essential micronutrients, but are limited in the growth medium the control plants grew in.

Exposure of *L. minor* plants to different concentrations of single metals (Cu, Zn, Ni, Pb and U) also resulted in a dose-dependent growth inhibition based on the three different endpoints frond area, frond number and frond biomass. These three DRCs were not completely parallel for each metal, because the different endpoints probably did not have the same sensitivity for metal toxicity. This effect was observed on the entire curve as demonstrated by a list of EC10, EC30 and EC50 values for the three endpoints separately. Frond area was the most sensitive endpoint, while number of fronds was the least.

Exposure to metal mixtures based on the Beaverlodge sample was done twice, which provided similar results that were not significantly different from each other. The metal mixtures were combined with or without U to investigate the contribution of U to the mixture toxicity. As metal concentrations in the original Beaverlodge sample (M1) were low, and to ascertain toxicity induced by the metals alone, the original metal were also added as a multitude of the original M1 concentration, namely multiplied by 10 (M10), 100 (M100) and 1000 (M1000). Using the program MixTox, it was predicted that these mixtures followed both IA or CA. This means the joint effect of U and metals is underestimated when

the toxicity is based on either U or the metals alone. Either one of these two models is sufficient for a safe evaluation of the ecotoxicological risk, but CA is the most conservative model.
It was concluded that the effect of U alone (M0) is higher than when combined with metals in the M1 combination. This U uptake was decreased when the concentration of the mixture of other metals in the medium increased. In addition, the uptake of Zn and Cu significantly decreased with increasing U concentration. The uptake of Ni was almost zero when combined with other metals, which could mean that Ni has a low bioconcentration factor. However, the plant uptake when exposed to Ni alone is unknown so far, which makes it difficult to make a conclusion. Taken together the decreased uptake of U and other metals into the plants could lead to apparent lower toxic effects visible as an antagonistic interaction, when growth inhibition is expressed on external metal concentrations.

As a pilot study, the expression of six genes were tested as potential biomarkers for U exposure of which three showed an up- and three a downregulation after exposure. One gene (primer 33) in particular showed an extremely strong response after exposure to U alone and also to U in the metal mixtures. The other genes were not specific to U exposure, since they also showed a dose-dependent response after exposure to other metals as well. Hence, the change in expression of these genes was more specific to metal exposure in general. Primer 33 will test for the expression of a gene encoding for a cytochrome P450 superfamily. Cytochrome P450 performs a wide and divergent range of reactions and is known to react under stress conditions. The induction of cytochrome P450 under U stress has not been documented before and might be a focus of future research.

When exposed to the M1000 mixtures combined with 2 or 10 µM U, gene expression of the six tested genes completely diminished. This is caused by either the high toxicity and thus high individual cell mortality of this M1000 mixture, which is a thousandfold higher than environmentally relevant concentrations, or, in the case of primer 33, because there is less U taken up at these high metal concentrations. If there is no U taken up, the potential biomarkers specific for U exposure do not react.

This study concluded two elements. First, the toxic effect of U on plant growth in an environmental relevant mixture of different metals seem to follow both CA and IA. Second, a gene encoding for a cytochrome P450 might be a potential candidate as potential biomarker gene specific for U exposure. To gain a more thorough knowledge of the toxicity of mixtures containing U, other contaminants in the Beaverlodge sample, and also in samples from other U mining sites should be investigated in the future. Since Ni uptake was negligible after exposure to a mixture, analysis of metal uptake after exposure to single metals could be interesting.
As a result of the high response of primer 33 after U exposure, it could be useful to investigate if there is a role of cytochrome P450 in U toxicity.
It was in this study only possible to study a small selection of genes due to time restrictions and due to the fact that primer optimisation and finding good housekeeping genes was quit labour and time intensive. Therefore, to gain a clearer conclusion, this experiment should be repeated in the future and more potential *L. minor* biomarker genes should be designed and tested. Also potential biomarkers on other biological levels such as protein levels of cytochrome P450 would be interested to investigate.

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Supplemental information

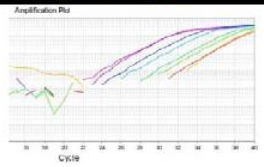
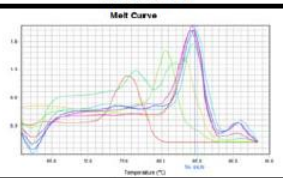
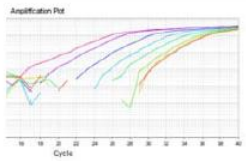
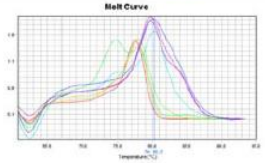
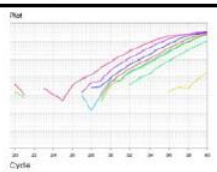
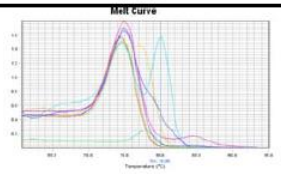
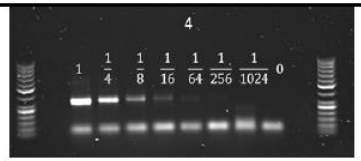
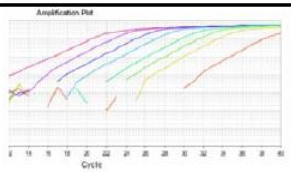
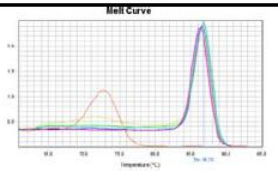
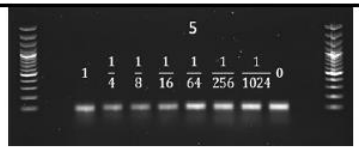
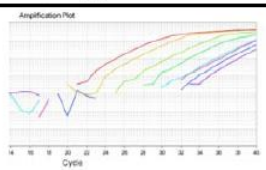
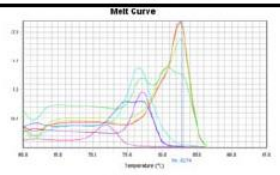
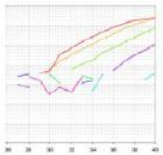
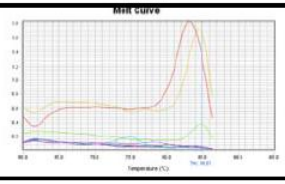
Appendix 1: Expression levels of *Lemna minor* genes after exposure to uranium, strontium-90 and gamma radiation

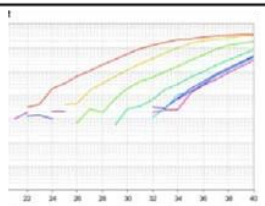
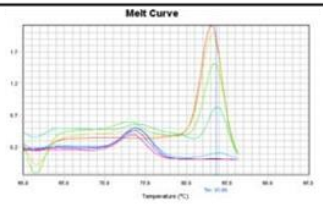
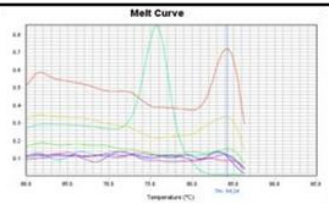
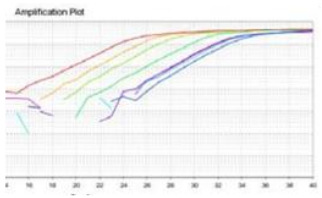
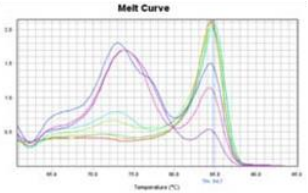
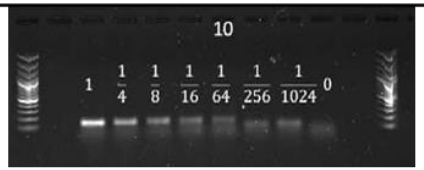
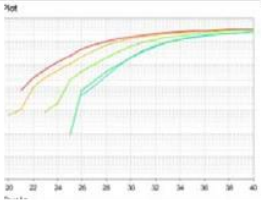
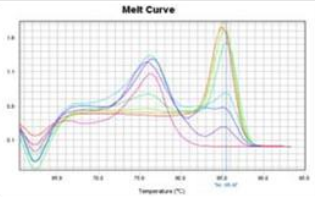
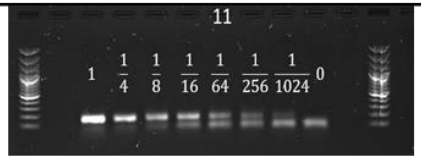
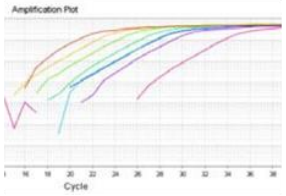
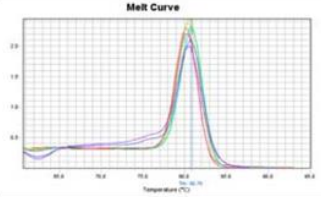
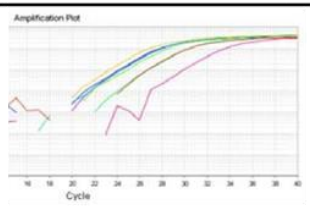
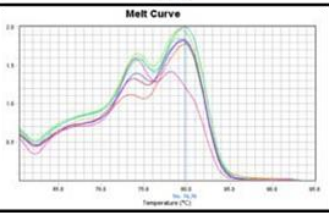
Table A1: Expression levels of *Lemna minor* genes after exposure to uranium, strontium-90 and gamma radiation
Sr = strontium; Green = upregulation; Red = downregulation; Yellow = neutral. Primer numbers are given together with their possible function.

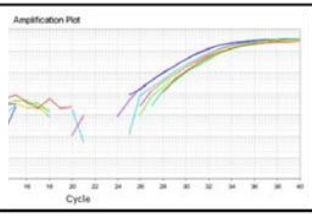
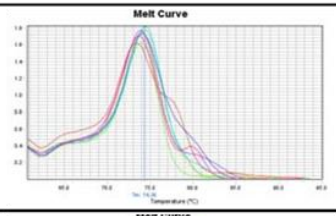
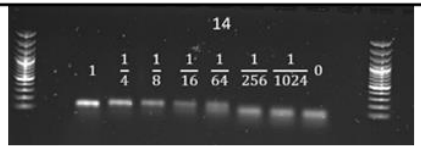
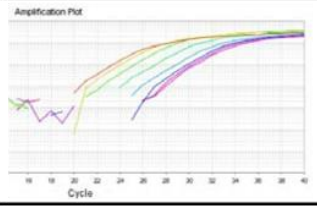
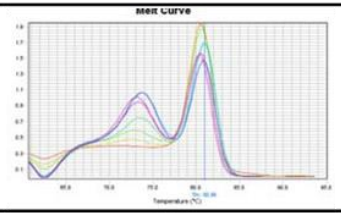
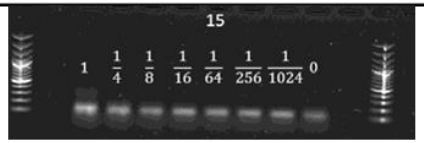
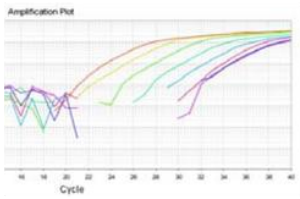
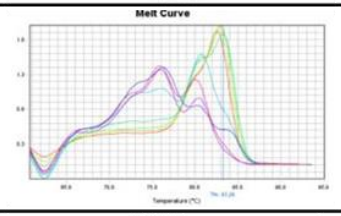
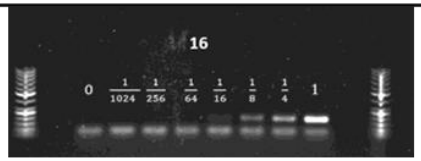
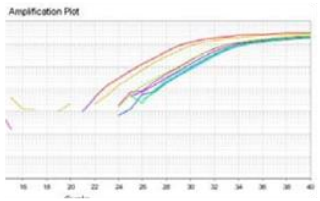
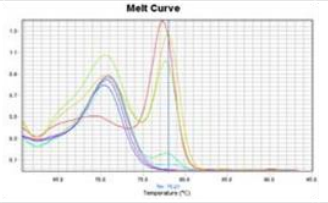
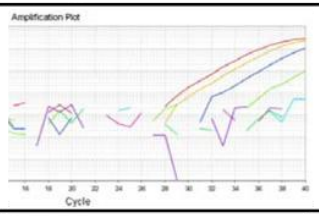
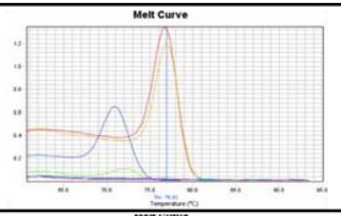
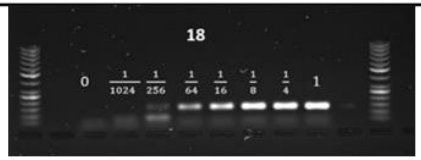
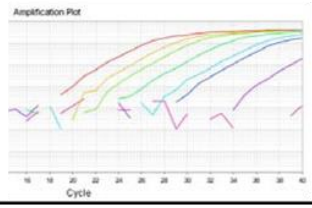
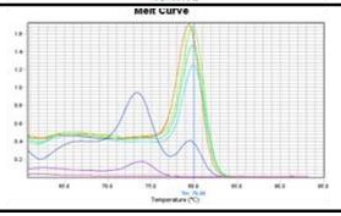
Function	Name	Primer number	Expression levels											
			Uranium (µM)				Bèta radiation (Sr) (mGy/h)				Gamma radiation (mGy/h)			
			0.5	4.5	6	10	0.08	0.9	9	97	53	120	232	423
Cytochrome	Lminor_010222	Primer 33	3.457	5.688	5.971	5.687	NA	NA	NA	NA	NA	NA	NA	NA
Defensin	Lminor_020037	Primer 24	1.87	7.958	6.018	5.173	NA	NA	NA	NA	-0.172	-0.728	-0.902	3.473
Glabra	Lminor_006454		0.237	5.315	5.038	4.735	NA	NA	NA	NA	0.13	0.393	0.922	2.674
Response to stress	Lminor_004343		0.714	3.13	4.33	4.505	NA	NA	NA	NA	NA	NA	NA	NA
Response to abscisic acid	Lminor_004343		0	3.13	4.33	4.505	NA	NA	NA	NA	NA	NA	NA	NA
Response to stress	Lminor_016959		-0.193	2.713	4.15	4.35	0.414	0.292	0.281	0.69	0.873	0.619	1.142	2.575
Response to abscisic acid	Lminor_016959		0	2.713	4.15	4.35	0.414	0.292	0.281	0.69	0.873	0.619	1.142	2.575
Cytochrome	Lminor_017373		0.242	3.27	3.453	3.348	0.319	0.458	0.68	1.253	0.081	-0.364	0.213	1.395
Response to oxidative stress	Lminor_003255		0.854	4.138	3.365	3.147	0.023	0.398	0.754	0.153	0.136	0.403	0.207	1.894
Regulation of DNA replication	Lminor_020596	Primer 34	2.326	3.931	3.213	3.082	-0.248	0.017	0.571	0.082	0.575	0.908	1.183	1.698
Response to oxidative stress	Lminor_017426		2.284	4.505	3.455	3.062	NA	NA	NA	NA	NA	NA	NA	NA
Response to stress	Lminor_007686		-0.363	1.752	2.547	2.747	0.06	-0.033	-0.301	-0.188	0.55	0.548	0.606	1.701
Response to oxidative stress	Lminor_015761		0.035	1.117	1.999	2.063	-0.161	-0.281	-0.48	-0.23	0.23	0.195	0.085	0.456
Oxidative stress	Lminor_015761		0.035	1.117	1.999	2.063	-0.161	-0.281	-0.48	-0.23	0.23	0.195	0.085	0.456
Microtubule-based process	Lminor_009053		-0.301	1.067	1.451	1.807	NA	NA	NA	NA	NA	NA	NA	NA
Base-excision repair	Lminor_003219		0.237	0.74	0.951	1.025	0.058	0.027	-0.069	-0.032	-0.147	-0.108	-0.28	-0.412
Anthocyanidin	Lminor_010814		1.261	1.848	0.631	0.14	0.649	-0.074	0.194	0.181	-0.577	-0.847	-1.39	-2.354
Metal ion transport	Lminor_016210		9E-04	0.178	-1.373	-1.534	-0.087	0.532	0.6	0.141	0.232	0.928	0.165	0.351
Metal ion transport	Lminor_014830		-0.138	-1.023	-1.745	-1.777	-0.401	0.057	-0.048	-0.566	-0.32	-0.198	-0.015	2.427
Oxidative stress	Lminor_016305	Primer 36	0.154	0.726	-2.265	-1.833	-0.122	0.087	1.262	-0.079	0.272	NA	-0.367	2.015
Oxidative stress	Lminor_015401		-0.378	-1.999	-1.018	-1.978	-0.053	0.195	0.155	0.828	0.364	0.833	1.823	4.899
Flavin adenine dinucleotide	Lminor_004705		0.229	-0.469	-1.421	-1.983	-0.946	-0.667	-0.685	-1.352	1.041	0.609	-0.092	-0.079
Flavin adenine dinucleotide	Lminor_002530		-0.074	-2.529	-2.444	-2.348	-0.45	-0.229	0.42	0.19	-0.977	-0.601	-0.103	-1.602
PAL	Lminor_014505	Primer 40	0.421	0.231	-2.042	-3.593	-0.276	0.154	1.177	-1.018	0.991	0.905	0.359	0.943
Flavin adenine dinucleotide	Lminor_003343		-1.129	-2.906	-4.013	-4.162	-0.489	-0.601	-0.946	-1.527	0.38	0.631	-0.142	-1.571
Cytochrome	Lminor_012582		-0.363	-2.363	-4.171	-4.37	-0.467	-0.452	-0.202	-0.841	-0.268	-0.297	-1.192	-2.733
Fatty acid biosynthetic	Lminor_002943		0.32	-1.41	-4.422	-4.499	1.41	1.429	0.969	0.473	0.044	-0.479	-0.545	-0.892
Response to auxin	Lminor_009276	Primer 39	0	-1.266	-3.747	-4.591	NA	NA	NA	NA	0.159	0.5	-0.075	-0.664
Oxidative stress	Lminor_010161		-0.297	-3.258	-6.907	-7.891	-0.015	-0.466	-1.962	-2.899	0.836	-0.37	-3.616	-3.75
Fatty acid biosynthetic	Lminor_013027		-2.017	-4.999	-6.813	-8.948	0.86	0.418	-0.603	-2.465	-0.003	-1.535	-1.994	-4.337

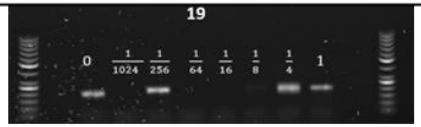
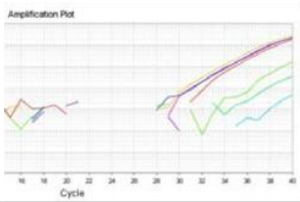
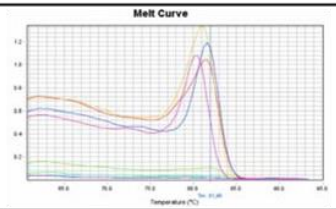
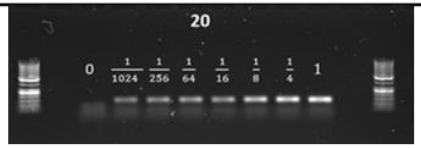
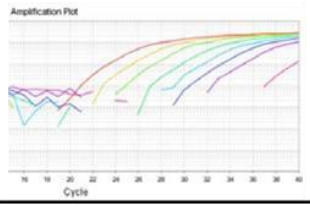
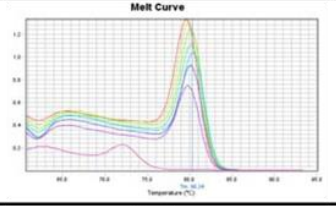
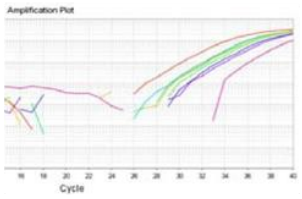
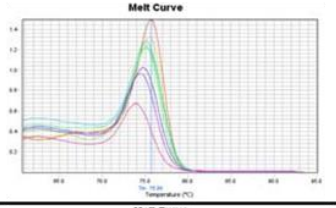
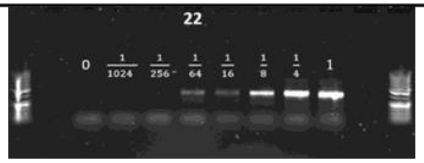
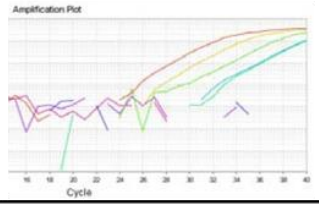
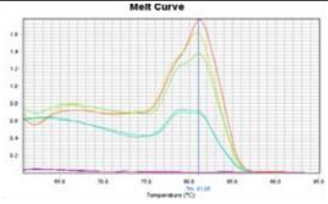
Appendix 2: Specificity testing of potential housekeeping genes for *Lemna minor*

Table A2: Potential housekeeping genes for *Lemna minor* based on real-time qPCR (amplification plot and melt curve) and gel electrophoresis.

Primer No.	Primer pairs	Sequence	Gel electrophoresis	Amplification Plot	Melt Curve
1	Lminor_012134_1_F Lminor_012134_1_R	ATCCAGCTTCTACAGTGGCG CTTCAACTTCACACGGAGCC			
2	Lminor_007986_1_F Lminor_007986_1_R	TATGGAAGCGATCTTCCCGC GCAAGAGCAAATGTGAGCCC			
3	Lminor_008384_1_F Lminor_008384_1_R	CCCCTCCCCTGTCATCTGTT GGCTCGATCAACACAGTCGC			
4	Lminor_003273_1_F Lminor_003273_1_R	GCGCCGATCCTAGATTTGAA CAGGCCCATCCTCCTTCTTC			
5	Lminor_001303_1_F Lminor_001303_1_R	ACCCACGAGCTTCAAACGAT CTGGTCTACAACACAGCCAA			
6	Lminor_011702_1_F Lminor_011702_1_R	ATACTACGGCCAGCGTTCTG GAGCCGACCTGCTATCAAGA			

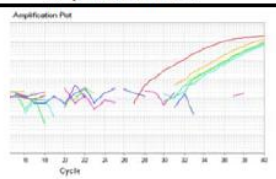
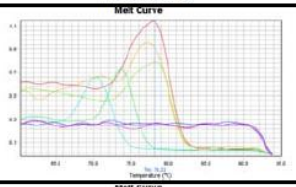
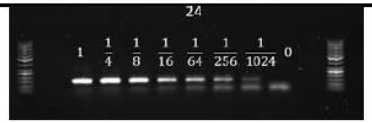
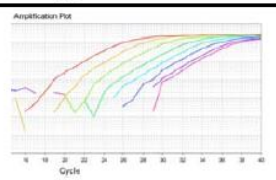
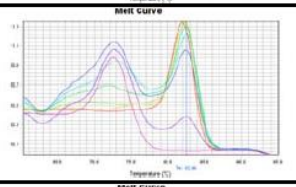
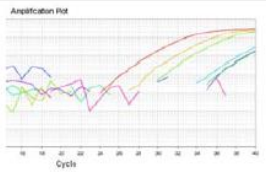
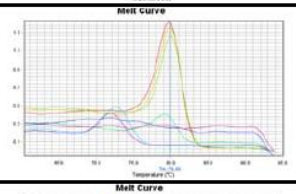
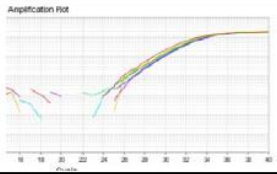
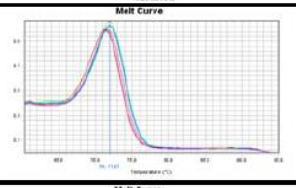
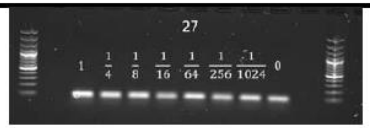
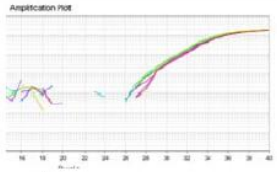
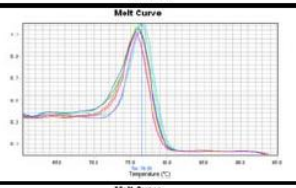
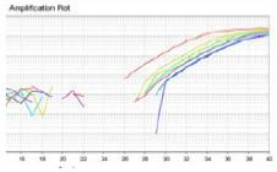
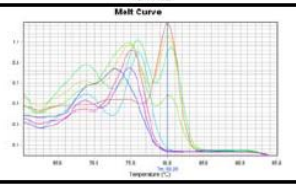
7	Lminor_007997_1_F	CTTTCCTCACCGGCCACTTC			
	Lminor_007997_1_R	CCAACCATAGGCTGGCAGAA			
8	Lminor_014887_1_F	CGTAAATGGGTTAGCCGGTG			
	Lminor_014887_1_R	CCCCGTCCACAAACACAAAC			
9	Lminor_014887_2_F	GAGACGGGGCAAGAGTTCAA			
	Lminor_014887_2_R	CATCCACCGGCTAACCCATT			
10	Lminor_007997_2_F	CTAACAGCTCTACTGCAGCG			
	Lminor_007997_2_R	TTGACGTCCCGATGGAAGTG			
11	Lminor_007986_2_F	GGGCTCACATTTGCTCTTGC			
	Lminor_007986_2_R	GGAGCGACATAGCCAGTTGT			
12	Lminor_012134_2_F	GAAAGGGCTCCGTGTGAAGT			
	Lminor_012134_2_R	TTCAAGTCCGTGAACATGCC			

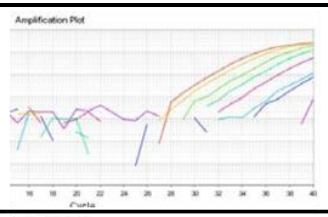
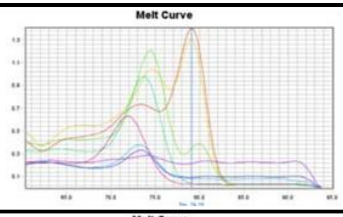
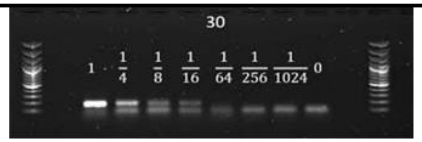
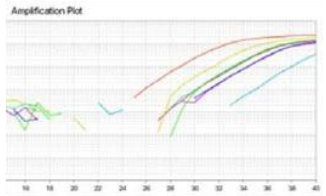
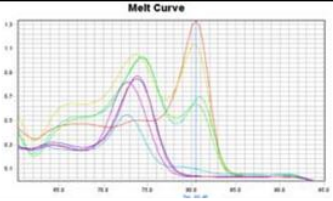
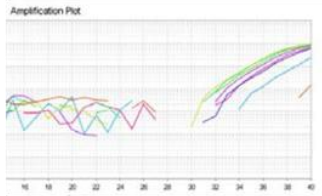
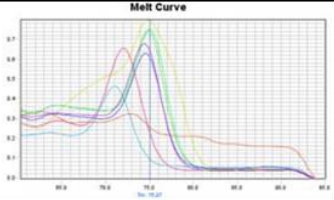
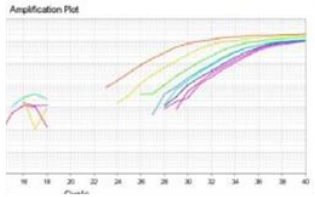
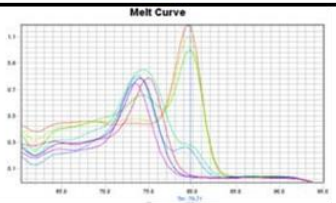
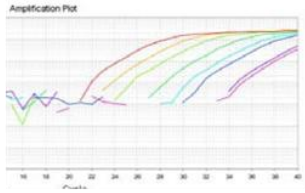
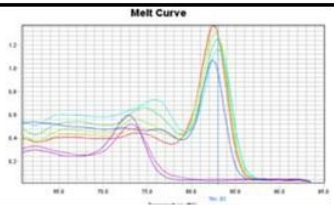
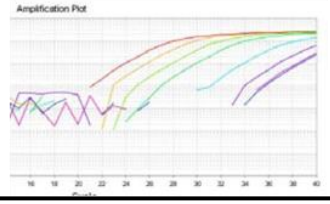
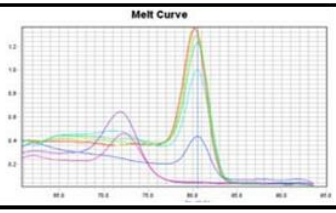
13	Lminor_004034_1_F Lminor_004034_1_R	ATGGCGACCTTCTTCAAGGG GCTCAGGCTGCCAATTTTCC			
14	Lminor_010330_1_F Lminor_010330_1_R	ACTCCTCGGAGAGCTTGGAT TTGAAGATCCGGCCAGCAAT			
15	Lminor_009513_1_F Lminor_009513_1_R	TTGGATCATGAGGGGAAGGAG CGTCCTGAAAGACGCCACAA			
16	Lminor_012134_3_F Lminor_012134_3_R	AGTACGACCGCATCATTGGG TCAACTTCACACGGAGCCC			
17	Lminor_009513_2_F Lminor_009513_2_R	AGTTCTTGGATCATGAGGGGA GACGCCACAACCTGATTGAAG			
18	Lminor_004034_2_F Lminor_004034_2_R	TTCGTCCGGATAGCTTCGTG GCCATTTTCGGATTCCTCGC			

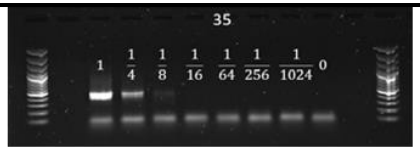
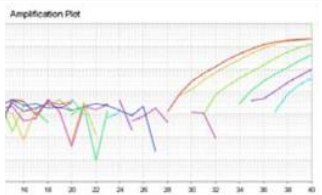
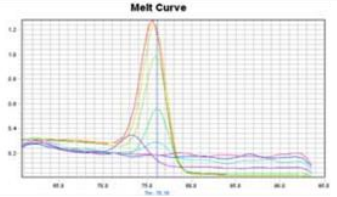
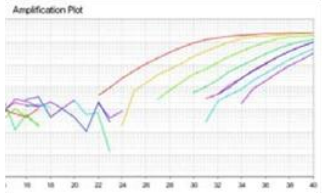
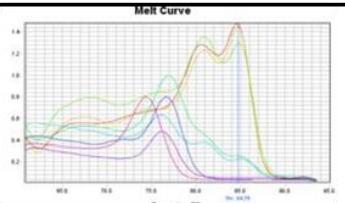
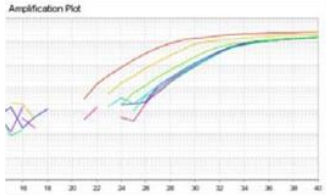
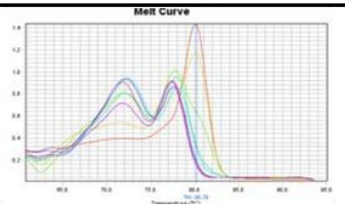
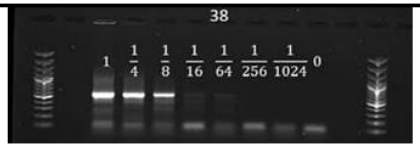
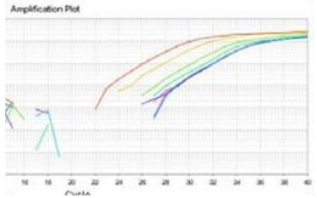
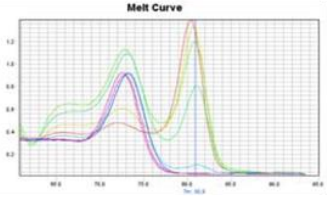
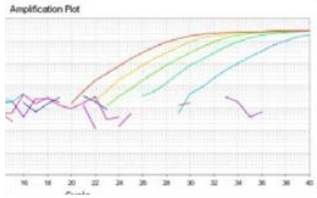
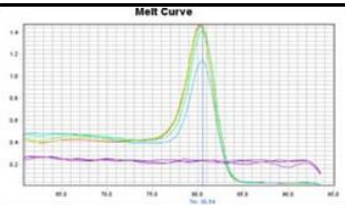
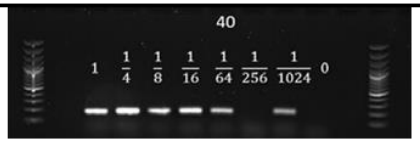
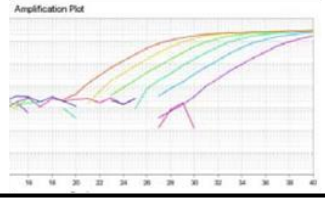
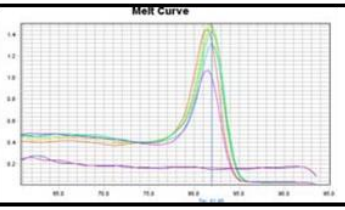
19	Lminor_014887_3_F Lminor_014887_3_R	AGTTCAACACCTCCCACTGC ATCCACCGGCTAACCCATTT			
20	Lminor_012610_1_F Lminor_012610_1_R	TTGAGCTTGCTCTTGCTGGT TCCTGCGAATGGTAAAGCCC			
21	Lminor_001303_1_F Lminor_001303_1_R	GCTTGTGGCCAAGGTTTGAG CCTGGTCTACAACACAGCCA			
22	Lminor_009166_1_F Lminor_009166_1_R	TCGTCAGCCAGCCAGTAAAG AGTCTCCCTGTCCGAGAAGA			

Appendix 3: Specificity testing of potential U biomarker genes for *Lemna minor*

Table A3: Potential U biomarker genes for *Lemna minor* based on real-time PCR (amplification plot and melt curve) and gel electrophoresis.

Primer No.	Primer pairs	Sequence	Gel electrophoresis	Amplification Plot	Melt Curve
23	Lminor_010222_1_F Lminor_010222_1_R	GGCGTTGGATCACGATATGC GCTTGGAGAAGAGGTGGGAG			
24	Lminor_020037_1_F Lminor_020037_1_R	ACTACAACAATGGCTGGCCC ATCGGTCTGCTTGATGCTCG			
25	Lminor_020596_1_F Lminor_020596_1_R	GCGACACAGGTACGAACGAT GGTGGAGAACTTGACGCCTT			
26	Lminor_017426_1_F Lminor_017426_1_R	ACGACACCAACATCAAGCCT ATCCCTAGCCAGTCCAGAGG			
27	Lminor_016305_1_F Lminor_016305_1_R	TCCACGACTGCTTCGTTAGG GACCAGTCTCCACTCTCCAG			
28	Lminor_015401_1_F Lminor_015401_1_R	GAGCAAGACGACCTTCGAGT CCTCCAATCCTAAGCCGCTC			

29	Lminor_012582_1_F Lminor_012582_1_R	GGCGCCTCTTTTCTCTGTACT CGCCATTTTTCTCCGTCCAC			
30	Lminor_002943_1_F Lminor_002943_1_R	TTGTACCACGAGTCGGCTTC AGCGTTCCTCCACCATTCAA			
31	Lminor_009276_1_F Lminor_009276_1_R	TCCGATGTGCCAAAAGGACA GGAAGGAAGGGTGTTCGAGG			
32	Lminor_014505_1_F Lminor_014505_1_R	TACCTGGTGGCTCTCTGTCA TTCTTCGCCACCTGACTCAC			
33	Lminor_010222_2_F Lminor_010222_2_R	CGACACTCTCATGTCCCTGC ATATCGTGATCCAACGCCCG			
34	Lminor_020596_2_F Lminor_020596_2_R	TGATGGACATCGACAGCGAG ATCGTTCGTACCTGTGTCGC			

35	Lminor_017426_2_F Lminor_017426_2_R	CCTCTGGACTGGCTAGGGAT ATCCGGTGCTCTGTAACGTG			
36	Lminor_016305_2_F Lminor_016305_2_R	AGACAGTGGCTGGCTTCATC CGACCAGTCTCCACTCTCCA			
37	Lminor_012582_2_F Lminor_012582_2_R	TTCTGTTCCTTCTCGTGGCG AGCTGCACTCCCTTAACGAC			
38	Lminor_002943_2_F Lminor_002943_2_R	CTACAAACTCCGGGGACGAC TGAGGTCTGGGTGCTTCTTC			
39	Lminor_009276_2_F Lminor_009276_2_R	CCTCGAACACCCTTCCTTCC ACGAAGTAGTCCTCTCCGCA			
40	Lminor_014505_2_F Lminor_014505_2_R	GCAGCACAACCAAGATGTGA CTGACTCACCGCGTTCTTGA			

Appendix 4: Exposure concentrations of uranium at the start of the experiment

Table A4: Exposure concentrations of uranium at the start of the experiment, tested with ICP-MS.

Sample ID	[U] (mg L^{-1})
U2-M0/1	550 ± 50
U2-M0/2	540 ± 50
U2-M0/3	540 ± 50
U2-M1/1	530 ± 50
U2-M1/2	530 ± 50
U2-M1/3	530 ± 50
U2-M10/1	520 ± 50
U2-M10/2	550 ± 50
U2-M10/3	510 ± 50
U2-M100/1	550 ± 50
U2-M100/2	540 ± 50
U2-M100/3	550 ± 50
U2-M1000/1	500 ± 50
U2-M1000/2	500 ± 50
U2-M1000/3	490 ± 50
U10-M0/1	1560 ± 160
U10-M0/2	1540 ± 150
U10-M0/3	1530 ± 150
U10-M1/1	1590 ± 160
U10-M1/2	1570 ± 160
U10-M1/3	1580 ± 160
U10-M10/1	1560 ± 160
U10-M10/2	1550 ± 150
U10-M10/3	1540 ± 150
U10-M100/1	1490 ± 150
U10-M100/2	1480 ± 150
U10-M100/3	1470 ± 150
U10-M1000/1	2.97 ± 0.30
U10-M1000/2	2.96 ± 0.30
U10-M1000/3	2.94 ± 0.30

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Richting: **master in de biomedische wetenschappen-milieu en gezondheid**
Jaar: **2016**

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Knaepen, Elke

Datum: **8/06/2016**