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Influence of nutrient medium composition on uranium toxicity and choice of the most sensitive growth related endpoint in *Lemna minor*

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ABSTRACT

Uranium (U) toxicity is known to be highly dependent on U speciation and bioavailability. To assess the impact of uranium on plants, a growth inhibition test was set up in the freshwater macrophyte *Lemna minor*. First growth media with different compositions were tested in order to find a medium fit for testing U toxicity in *L. minor*. Following arguments were used for medium selection: the ability to sustain *L. minor* growth, a high solubility of U in the medium and a high percentage of the more toxic U-species namely UO_2^{2+}. Based on these selection criteria a with a low phosphate concentration of 0.5 mg L^{-1} and supplemented with 5 mM MES (2-(N-morpholino)ethanesulfonic acid) to ensure pH stability was chosen. This medium also showed highest U toxicity compared to the other tested media.

Subsequently a full dose response curve for U was established by exposing *L. minor* plants to U concentrations ranging from 0.05 μM up to 150 μM for 7 days. Uranium was shown to adversely affect growth of *L. minor* in a dose dependent manner with EC10, EC30 and EC50 values ranging between 1.6 and 4.8 μM, 7.7–16.4 μM and 19.4–37.2 μM U, respectively, depending on the growth endpoint. Four different growth related endpoints were tested: frond area, frond number, fresh weight and dry weight. Although differences in relative growth rates and associated ECx-values calculated on different endpoints are small (maximal twofold difference), frond area is recommended to be used to measure U-induced growth effects as it is a sensitive growth endpoint and easy to measure *in vivo* allowing for measurements over time.

1. Introduction

Uranium is a metal and radionuclide naturally present in both aquatic and terrestrial environments. The average natural background U concentrations in surface water of freshwater typically ranges from 0.03 to 2.1 μg/L (0.00012 μM – 0.008 μM) (Bleise et al., 2003; WHO, 2001). Due to anthropogenic activities, such as uranium mining and milling, concentrations have locally risen to levels that potentially pose ecological risks. As such, in the vicinity of former U-mines elevated concentrations ranging up to 450 μg/L (~2 μM) (Czech Republic, Rapantova et al., 2013) or even 1200 μg/L (~5 μM) (Kazakhstan, Salbu et al., 2013) have been measured. These concentrations far exceed the reported predicted-no-effect-concentrations for U for freshwater plants of 5 μg/L (0.021 μM) (Sheppard et al., 2005).

Uranium is present in the environment in various oxidation states and forms. Natural U consists of a mixture of three U isotopes with U-238 (99.27%) being the most dominant one. Uranium-238 has a physical half-life of 4.5×10^9 years resulting in a low specific activity of 1.24×10^4 Bq g^{-1} U. Hence U toxicity originates more from its chemical characteristics than from its ability to release alpha particles (Sheppard et al., 2005).

In geological environments, U (IV) and U (VI) redox forms are present with U (VI) dominating in oxic conditions and as such in most freshwater. Several physicochemical factors are known to influence U speciation, bioavailability and toxicity such as water hardness, pH and complexing inorganic and organic ligands

Abbreviations: ECx, concentration of toxicant resulting in x% effect; MES, (2-(N-morpholino)ethanesulfonic acid); SFW, Synthetic Freshwater; SIS-medium, Swedish standard medium; T_d, doubling time.

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(Markich, 2002, 2013; Mkandawire et al., 2007; Saenen et al., 2013; Sheppard et al., 2005). As such, it is generally assumed that the total metal concentration is not a good predictor of its bioavailability, bioaccumulation or toxicity. Uranium toxicity is known to be highly dependent on the physicochemical form (or speciation) with reasonable evidence that aqueous uranyl (UO_2^{2+}) and UO_2OH^+ are being the most toxic species e.g. to freshwater bivalves (Markich et al., 1996, 2000), green hydra (Trenfield et al., 2011), the unicellular alga *Chlorella* sp. (Trenfield et al., 2011) and possibly also in the algae *Chlamydomonas reinhardtii* (Fortin et al., 2007). As such, Markich et al. (1996) demonstrated that about 96% of the U toxicity can be explained by the presence of UO_2^{2+} and UO_2OH^+ but that in contrast to the expectations UO_2^{2+} is about twice as toxic as UO_2OH^+. In plants it was shown that U toxicity is more pronounced in media with low phosphate concentrations and low pH (Markich et al., 1996; Saenen et al., 2013; Vanhoudt et al., 2008). Geochemical speciation modelling indicates that U has a high affinity to form precipitating phosphate complexes in normal plant growth media that contain relatively high phosphate concentrations and hence UO_2^{2+} concentrations are low at high phosphate concentrations (Mkandawire et al., 2007; Saenen et al., 2013; Vanhoudt et al., 2008).

To test U toxicity to freshwater plants, we selected *Lemna minor*, a free floating macrophyte. *L. minor* is an easy to culture and handle vascular plant that is relatively sensitive to different toxicants and hence suitable for ecotoxicological testing (Fenske et al., 2006; Moody and Miller, 2005). It is a common, relatively simple structured freshwater plant belonging to the *Lemnoideae* (duckweed subfamily). Standard guidelines to perform a growth inhibition test on *L. minor* were published e.g. by the Organisation for Economic Co-operation and Development (OECD, 2006). In natural waters of an uraniferous region in Portugal, Favas and co-workers (2014) recently studied the accumulation of natural U in different aquatic plants. From the different species tested they found that for *L. minor* plants and a bryophyte species, U uptake into the plants correlated best with the ambient U concentrations. Based on these results, the authors suggested that despite its floating and therefore mobile nature, *L. minor* could serve as an indicator species for U contamination.

The toxicity of U to freshwater biota has been tested on a number of species. Uranium was shown to be genotoxic and to induce oxidative stress e.g. in zebrafish (Barillet et al., 2011, 2007; Pereira et al., 2012), goldfish (Lourenco et al., 2010) and daphnia's (Biron et al., 2012; Massarin et al., 2010) leading to reduced growth, decreased reproduction and survival rates. Similar U toxicity responses have been found in photosynthetic organisms especially for algae (see e.g. Charles et al., 2002; Franklin et al., 2000; Lavoie et al., 2014) and terrestrial plants (Saenen et al., 2013; Sheppard et al., 2005; Vandenhove et al., 2006; Vanhoudt et al., 2011a, 2011b). Toxicity to freshwater macrophytes was recently studied under laboratory conditions in *Ceratophyllum demersum* (Markich, 2013), *L. minor* (Goulet et al., 2015; Horemans et al., 2015) and *Lemna aequinoctialis* (Charles et al., 2006). Some controversy still exists among these studies that can be attributed either to the complexity of U chemistry and its dependency on pH, water hardness and alkalinity or to differences in species sensitivity. For example, Markich (2013) indicated that U toxicity measured as growth inhibition varied with a factor of four in test systems with a 20-fold difference in water hardness. Markich (2013) attributed this difference to a changed cell surface binding and/or U uptake due to changed Ca-concentration rather than to a difference in U speciation. In contrast, Goulet et al. (2015) reported that it were pH and alkalinity and not water hardness that influenced U-toxicity to six different freshwater organisms including one macrophyte tested.

The tropical macrophyte *Lemna aequinoctialis* showed an EC50 value for U of 0.758 ± 0.035 mg/L (*i.e.* 3.2 ± 0.1 μM) (Charles et al., 2006). *L. minor* seems to be less sensitive with reported EC50 values of 7.0 ± 0.4 mg/L (*i.e.* 29.5 ± 1.9 μM) (Horemans et al., 2015) or 16.4 mg/L (*i.e.* 68.9 μM) (Goulet et al., 2015). However, as stated by Goulet et al. (2015) the composition of the plant nutrient medium, e.g. the higher amounts of phosphate necessary to sustain *L. minor* growth, could greatly influence U speciation. The nutrient medium described in the guidelines for a standard *L. minor* growth inhibition test (OECD, 2006) is indeed rich in phosphate and other ions and if toxicity is expressed on nominal U concentrations this potentially underestimates U toxicity to *L. minor* under more natural conditions.

The objective of the present work is firstly to select an optimal standard growth medium to use in growth tests to study toxicity of U to the freshwater macrophyte *L. minor*. Secondly, for the growth medium selected, to develop U dose response curves and compare growth rate inhibition on the basis of different growth endpoints (frond area, frond number, fresh weight and dry weight) to choose the most sensitive endpoints of toxicity based on the ECx. The total U concentration, U speciation, growth of *L. minor* and toxicity for one selected U concentration was evaluated for different growth media varying in pH, phosphate and carbonate concentration.

2. Materials and methods

2.1. Plant culture

L. minor cv. Blarney plants were obtained from Dr. M. Jansen (University College Cork, Ireland) and cultured aseptically in 250 mL glass erlenmeyers containing half-strength Hütner medium (Brain and Solomon, 2007) under continuous light (Osram 400 W HQI-BT daylight, 102 ± 0.9 μmol/m^2 s) at 24 ± 0.5 °C. Plants were sub-cultured every 10–12 days by transferring three plants to 100 mL of fresh growth medium. One week prior to an experiment five mature plants (three to four fronds) were transferred to 100 mL of fresh medium to obtain a homogenous plant population.

2.2. Different culture media used in seven days inhibition test

In order to find a suitable medium maximising *L. minor* growth, limiting U-precipitation and favouring the presence of possible key U species such as uranyl, we evaluated three different media used for *Lemna* species growth inhibition test: (i) the SIS-medium described in the OECD guideline 221 (OECD, 2006), (ii) the K-medium used in herbicide binary mixture experiments (Cedergreen et al., 2007e), and (iii) a slightly enriched Synthetic Freshwater (SFW) used to test U toxicity to a tropical *Lemna* species (Charles et al., 2006). The standard composition of these different media is presented in Table 1. Additional changes to the medium such as modifications of pH, the phosphate and/or carbonate concentrations are also indicated in Table 1. As the SIS-medium is recommended by OECD most variations were tested for this medium first.

2.3. Uranium speciation modelling

U speciation in the different growth media was predicted with Geochemist's Workbench®, version8.0 (React Software, (Bethke and Yeakel, 2010). As thermodynamic database, the Thermo Minteq database from Visual MINTEQ release 2.40 was used. Geochemist Workbench® can simulate solution, oxido–reduction and precipitation equilibrium. For all calculations temperature was fixed at 25 °C and redox simulations were enabled whereas precipitation was disabled. Precipitation was disabled since the Geochemist Workbench only makes a thermodynamic simulation.

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