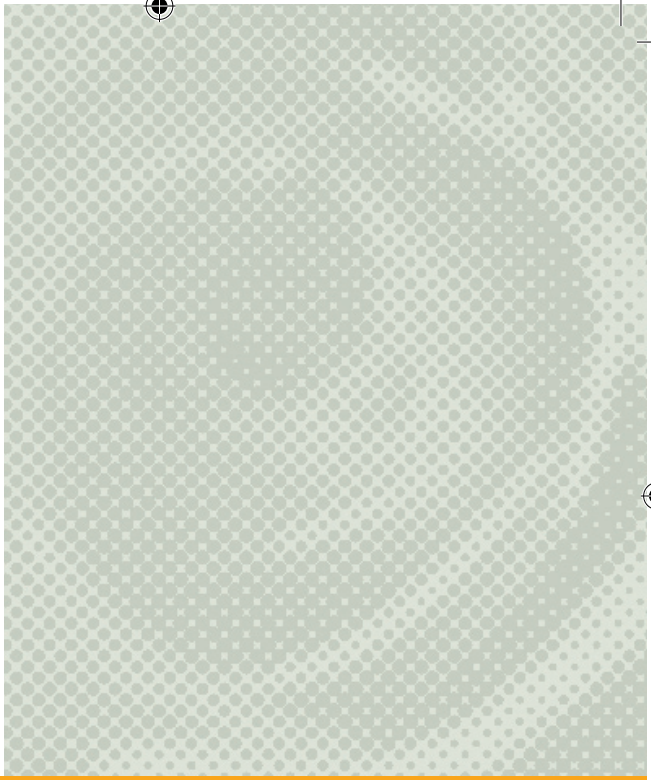
Bioaccumulation in earthworm exposed to uranium particles and anions

pabitra basnet



Norwegian University of Life Sciences

Department of Plant and Environmental Sciences

Master Thesis 2011 - 60 Credits

Preface and Acknowledgements

This master thesis has been funded by IPM with the support of the Norwegian Radiation Protection Authority and was carried out at the Isotope Laboratory, Department of Plant and Environmental Sciences (IPM) at University of Life Sciences (UMB). The thesis presents a study of the bioavailability of different uranium species in earthworms.

I am grateful to a large number of people who have been contributing to realize this master thesis. First of all, I wish to express my sincere gratitude to my main supervisor Prof. Dr. Deborah H. Oughton for giving me positive feedback, and for generously sharing time and knowledge throughout the laboratorial work and the writing process. Her encouragement was duly appreciated that I cannot express by word. I am grateful and deeply indebted to my co-supervisors professor Brit Salbu and associate professor Ole Christian Lind for their generosity in giving valuable comments and suggestions throughout the research which made the accomplishment of this work possible.

I especially want to acknowledge Claire Courtis for her efforts in planning and carrying out the uptake experiment under the advice of main supervisor. I am deeply indebted to Karl Andreas Jensen and Henrik Ness Mikkelsen, who helped me with the laboratorial work and patiently answered my questions.
Last but not least, the special thank goes to Ingrid Færgestad Bugge and Vilma Veronica Bischof together with supervisor for their legislative assistance.

Finally, I would like to express my heartiest gratitude for the moral support and encouragement that I received from my parents. My heartfelt thanks go to my wife and daughter for their love and understanding which made life more comfortable and enjoyable.

Thanks to everyone who inspired me else not mentioned by name during my time at UMB.

Pabitra Basnet

Abstract

This study contains information about the bioaccumulation of uranium (U) in earthworms following exposure of the worms exposed to different uranium species in food (horse manure). Three different uranium species were used: synthesized uranium nano-micrometer particles (UO_2 and U_3O_8) and uranyl ions at two different concentrations (50 and 500 µg/g dw manure). The study started with the culturing of worms, growing them in OECD soil and ended by performing uranium measurements by ICP-MS of four types of samples: worms, food (horse manure), soil and faeces. The analysis showed that uranyl uptake in terms of biological concentration factors (BCF, concentration in worm/concentration in food) was 10 times higher than that of particle uptake. All worms survived the treatment with no mortality during the week of uranium exposure and hence 100 % worms (50 worms) survived the experiment, although 2 worms died during the depuration period (gut emptying). However these were randomly distributed over the test groups. So, no correlation with U exposure could be observed. There were no significant effects of uranium on growth of worms, but 4 of the worms showed a reduction in weight, again randomly distributed between the groups. The test comparison of soil and manure concentration showed that the soil/manure concentration ratio for the control was much greater than for the treated soils, due to the higher concentration of natural uranium in soils with no significant difference between the different U treatments. This is the first time that UO_2 and U_3O_8 particle uptake has been studied in the earthworm, and results should provide useful information for ecological risk assessment.

Contents

Preface and acknowledgements i

Abstract ii

Contents iii

List of tables and figures v

Abbreviations vi

Chapter 1: Introduction 1

1.1 General introduction 1

1.2 Earthworm and their importance 4

1.3 Nanoparticles and toxicity 5

1.4 Uranium concentration, uptake and effects 7

Chapter 2: Materials and Methods 9

2.1 Test Materials 9

2.2 Instrumentation 11

2.2.1 UltraCLAVE microwave digestion 11

2.2.2 Inductively coupled plasma mass spectroscopy 13

2.3 Experimental Methods 14

2.3.1 Preparation of OECD soil 14

2.3.2 Culturing of worms 14

2.3.3 Acclimation 15

2.3.4 Exposure to uranium spiked horse manure 16

2.3.5 Depuration Period 18

2.3.6 ICP-MS measurements 19

2.3.6.1 Worm 19

2.3.6.2 Soil 19

2.3.6.3 Manure 19

2.3.6.4 Feces 19

Chapter 3: Results and Discussions 20

3.1 Concentration of Uranium in Soil and Manure 20

3.2 Worm Growth 21
3.3 Uranium Uptake by worms and concentration in faeces 23
3.4 Conclusions 26

References 28

Tables and Figures

Table 1: Concentration of U in environmental media 1

Table 2: Naturally occurring uranium radioisotopes 3

Table 3: Uranium concentration in manure and soil 20

Table 4: Worm growth after one week 21

Table 5: Uranium uptake by worms and comparison of concentrations 23

Figure 1: Uranium decay chains which contains a number of shorter half-life radionuclides including radium and polonium 2

Figure 2: Scaning electron microscopy picture of uranium particles in soils from Kosovo contaminated by munitions explosions 7

Figure 3: TEM of UO_2 nanoparticles 11

Figure 4: TEM of U_3O_8 nanoparticles 11

Figure 5: Schematic of digesting samples in UltraCLAVE 12

Figure 6: Schematic of ICP-MS main processes 13

Figure 7: OECD soil prepared at Isotope Laboratory 14

Figure 8: Culturing of worms using clay and horse manure as food 15

Figure 9: OECD soil prepared for acclimation of worms before putting manure 15

Figure 10: 50 boxes of 5 treatments filled with OECD soil containing one worm per box 16

Figure 11: Solutions of NP and uranyl prepared at Isotope Laboratory 17

Figure 12: Worms taken out of treatment boxes after 1 week exposure and prepared for depuration period 18

Figure 13: Worm growth removing outliers and labeling uncertainties 22

Figure 14: Bioconcentration factor of uranium particles and uranyl in worm and manure 24

Figure 15: Schematic drawing of an earth worm cross section (top) and confocal micro X-ray mapping cross sections of the reproductive organ segment and intestine segment 25

Figure 16: Ratio of U concentration in worm and U concentration in faeces 26

Abbreviations

U	- uranium
nm	- nanometer
NP	- nanoparticles
ww	- wet weight
fw	- fresh weight
dw	- dry weight
BCF	- biological concentration factor
DNA	- de-oxyribo nuclic acid
OECD	- Organisation for Economic Co-operation and Development
TEM	- Transmission electron microscopy
ICPMS	- Inductively coupled plasma mass spectrometry
CAS nr	- Chemical abstracts service number
w.r.t	- with respect to

CHAPTER 1: INTRODUCTION

1.1 General introduction

Uranium metal is a silver-white, lustrous, dense material and is found in rocks, soils, water, air, plants, animals and in all human beings (Table 1).

Table 1: Concentration of U in environmental media (Bowen, H.J.M., 1979)

Media		Concentration (mg/kg)
Geosphere	Granite	4.4
	Basalt	0.43
	soil	2
	Shale	3.7
	Limestone	2.2
	Sandstone	0.45
	Sea Water	0.0032
	Fresh Water	0.0004
Biosphere	Land Plants	0.005 – 0.04
	Edible Vegetables	0.01 – 0.06
	Mammal Muscles	0.0009 – 0.003
	Mammal Bone	0.00016 – 0.07
	Marine Algae	0.4 – 0.9
	Marine Fish	0.04 – 0.08

It has atomic number 92 and atomic mass 238.02891 and is one of the heaviest long-lived naturally occurring radionuclides. U includes three isotopes: U-234, U-238 and U-235 in nature and gives rise to the uranium decay chains which contain a number of shorter half-life radionuclides including radium and polonium which are key dose contributors for man and environment (Figure 1).

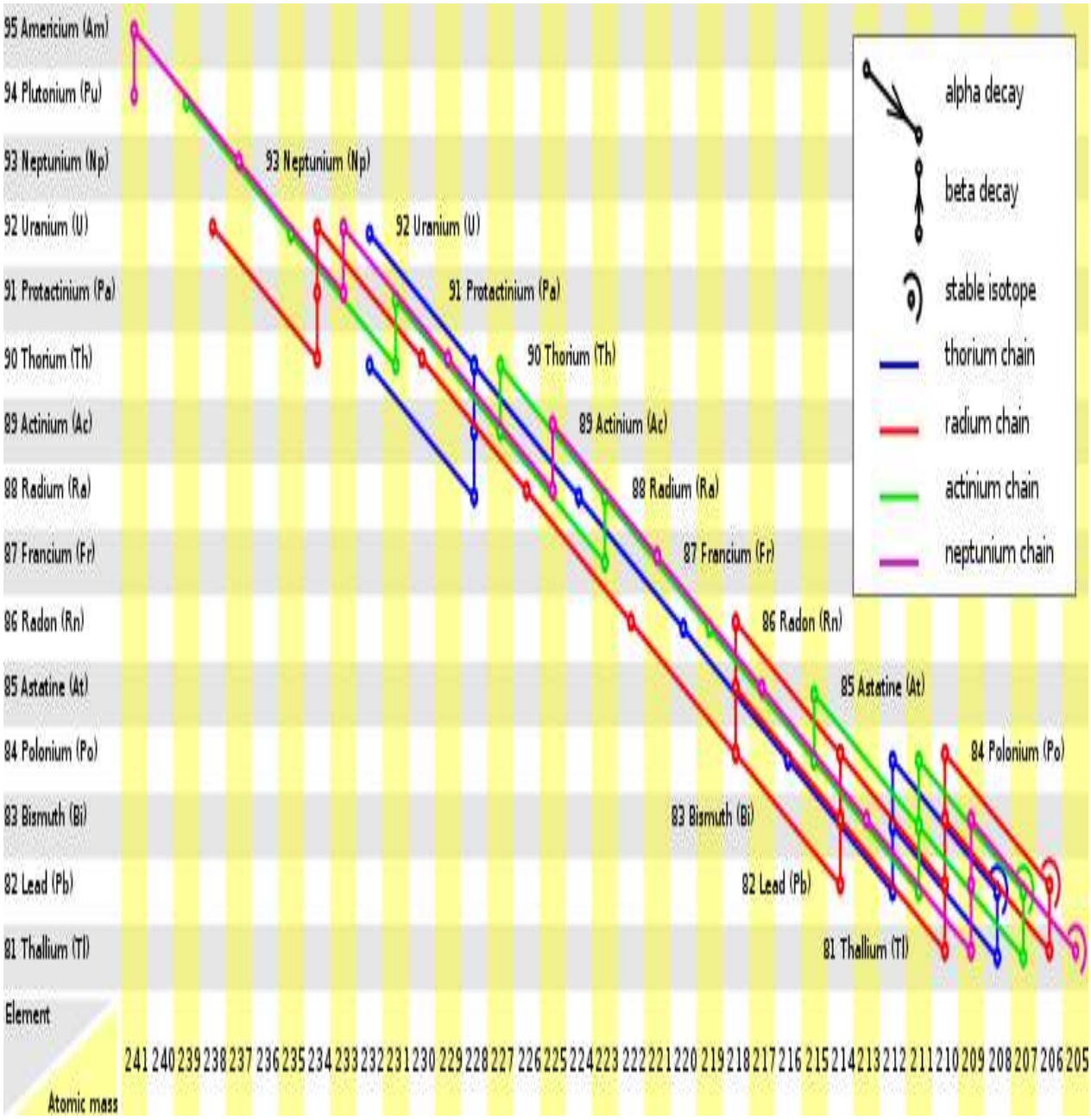


Figure 1: **Uranium decay chains which contains a number of shorter half-life radionuclides including radium and polonium** (http://en.wikipedia.org)

The chemistry of uranium is rather complex and U can be present in different physico-chemical forms (CCME, 2007). In aqueous solution, uranium can exist in five different oxidation states (+2, +3, +4, +5, and +6) (ATSDR 2011), but tetravalent (+4) and hexavalent (+6) uranium are the most

common and useful for practical importance in environmental conditions (Giammar, D. 2001). In soil, the U^{4+} valence state of uranium occurs in strongly-reducing environments and is formed due to redox processes of organic matter or iron in the soil (CCME, 2007, Angell, P., 1996). Tetravalent uranium forms hydroxides, hydrated fluorides, and phosphates and these are strongly adsorbed and very immobile in soils. The U^{6+} valence state occurs in oxidizing environments (UO_2^{2+}) and can be adsorbed by soils, or forming stable mobile complexes with many ligands notably carbonates and organic complexant agents (CCME, 2007). High ligand concentrations can result in a lower positive or negative charge and increase mobility of the complexed uranium (Sheppard and Evenden 1987).
As a heavy metal uranium can be chemically toxic as well as radiotoxic. Long half-life and slow decay of uranium isotopes facilitates their radiotoxicity to be relatively low and hence the adverse effects about uranium toxicity can be assumed due to chemical toxicity, rather than radiation effects (CCME, 2007). Uranium compounds differ substantially in their chemical properties, physiological properties and toxicological effects they exert. For example, compounds such as uranium trioxide (UO_3), uranyl chloride (UO_2Cl_2), uranyl nitrate (($UO_2(NO_3)_2$) and uranyl ethanoate ($UO_2(CH_3COO)_2$) are relatively soluble, whereas uranium dioxide (UO_2) and triuranium octaoxide (U_3O_8) are forming particles and are considered to be relatively insoluble. In the environment, uranium can be present as naturally occurring colloidal, or various types of particles ranging from nanometer to micrometer sizes, as a result of human activities, including depleted uranium from weapons and U-fuel particles from nuclear reactors (Salbu et al., 2003).

Table 2: Naturally occurring uranium radioisotopes (Bowen, H.J.M., 1979; EFSA, 2009; WHO, 2001)

Isotopes	Natural abundance (%)	Specific activity (pCi/g)	Half life (yr)
U-238	99.2745	0.336	4.47×10^9
U-235	0.7200	2.17	7.038×10^8
U-234	0.0056	6208.2	2.446×10^5

In recent years, there has been a lot of focus on the environmental and health importance of engineered nanoparticles, which are defined as particulate dispersions or solid particles having size

ranging from 10 nm to 1000 nm (Mohanraj, V.J. and Chen, Y, 2006). Due to their small size, nanoparticles are able to enter cells and interact with various cell components and hence cause adverse biological and toxic effects. (Buzea, C. et al, 2007). Uranium can also exist as nanoparticles and the aim of this study is to investigate the environmental behavior and possible chemical toxicity of uranium nanoparticles and ions. The main focus has been on studies of bioaccumulation of various chemical forms of U in earthworms. Specifically, two different types of uranium approximately nano-scaled particles: uranium dioxide (UO_2) and triuranium octaoxide (U_3O_8) and two different concentrations of uranyl ions have been fed to the earthworm (*Eisenia fetida*).

The main objectives of this study are listed as below.

- To compare the uptake of uranyl ions and U particles by the earthworm
- To calculate the bioconcentration factors of uranium to earthworms from soil and manure
- To investigate the effect of uranium on worm growth and development

1.2 Earthworm and their importance

There are about 3000 species of earthworm worldwide with different sites and ecosystems having their own indigenous species of worms. Earthworms' bodies are soft and long with a cylindrical shape. They breathe through their skin and must stay moist. Generally, earthworms live in the soil, but some species live in the mud along the shores of fresh or salty bodies of water, some in the upper leaf litter layer, topsoil, or in deeper layers in the soil and others live high above the forest floor in soils that accumulate among the branches of tree canopies in tropical rainforests. Earthworm feeding behavior depends on the type and quality of food sources. Some species prefer fresh organic matter on the soil surface, but others require partially decomposed material incorporated into the soil profile. (Syers, J.K. and Springett, J.R. 1984). Earthworms in agricultural ecosystems generally prefer protein-rich legumes. Commonly, earthworms eat dead and decomposing leaves, decaying roots, freshly dead plant material, or detritus in the soil etc.

Earthworms are one of the most important biotic components in soil and terrestrial ecosystems (Connell, D.W. and Markwell, R.D., 1990; Edwards, C.A. and Bohlen, P.J, 1996). They live in soil, feed at the soil surface level, converting dead litter to organic material, and aerating the soil by

burrowing. From and ecological perspective, earthworms are also important as they are prey to many amphibian, reptile bird and mammalian species, and due to a high uptake of metals, can transfer environmental contaminants from soil to organisms, as well as a variety of different food chains (Haimi, J.R. *et al.*, 1992). Because various earthworms can be easily cultured in a laboratory, and extensive databases are available, they have been considered one of the most suitable indicators for testing soil health and chemical toxicity in soil (OECD 1984, Giovanetti, A.,et al. 2010). The compost worm *Eisenia Fetida* is a widely used indicator in acute and chronic toxicological testing for determining the ecological risk of heavy metals, pesticides and other organic pollutants in soil (OECD, 1984), using a variety of endpoints, including morality, growth as well as molecular and cellular biomarkers. Since they breed well in captivity they can also be used in reproduction studies, and considering their body size and ease of handling, they also are ideal organisms for assessing metal bioavailability in the terrestrial environment (Norr C and Riepert F, 2007). *Eisenia Fetida* was chosen as the test reference organism in this thesis.

1.3 Nanoparticles and toxicity

Nanoparticles are defined as particulate dispersions or solid particles having size ranging from 10 nm to 1000 nm. (Mohanraj, V.J. and Chen, Y, 2006). Because of their very small size, their physical and chemical properties are unpredictable because the surface and interfacial properties may be modified in the presence of chemical agents (surfactants) (SCENIHR, 2005). The main parameters of nanoparticles are their shape, size, and the morphological sub-structure of the substance (SCENIHR, 2005). They form as amorphous or crystalline shape and their surfaces can act as carriers for liquid droplets or gases. To some extent, nanoparticles are considered a distinct state of matter, in addition to the solid, liquid, gaseous, and plasma states, due to their distinct properties i.e. large surface area and quantum size effects (Buzea, C et al, 2007). They act as an aerosol (mostly solid or liquid phase in air), a suspension (mostly solid in liquids) or an emulsion (two liquid phases) (SCENIHR, 2005). On the other hand, nanoparticles constitute interactions between other chemicals, other particles, themselves etc depending on the attractive or repulsive interaction forces between them which are very difficult to characterize (SCENIHR, 2006). Also, the chemical processes taking place on the surfaces of nanoparticles are very complicated and remain largely unknown (SCENIHR, 2006).

Nanoparticle toxicity can arise if the materials are accumulated in organisms at harmful levels. Due to their small size, nanoparticles have the ability to enter and can translocate from entry portals of organism's body into the body tissues and organs and concentrate in different cells (Buzea, C.,2007). Then, they produce adverse biological and toxic effects and damage living organisms (Buzea, C.,2007). Toxicity of nanoparticles not only depends on their size, but also other many factors such as shape, origin, material, surface area, electric charge, physicochemical peculiarities of the structure, dosage, administration route, concentration in the target organ, duration of action etc (Buzea, C.,2007, Mohanraj, V.J. and Chen, Y, 2006).The chemical composition may have a direct influence on the toxicity potential of the material. For example, some NPs consist of metals that are very toxic even at low concentration of their dissolved states (e.g. Ag). The surface charge of the NPs is important for two reasons: for determining the particle dispersion characteristics, and the absorption of ions and biomolecules to the particle surface. Classically the zeta potential is often used, which is a function of the surface charge of the particle, adsorbed species on its surface, and the composition and the ionic strength of the surrounding medium (Powers et al. 2006). Again, the toxicity of any nanoparticle to an organism is also determined by the individual's genetic complement that provides the biochemical toolbox by which it can adapt to and fight against toxic substances (Buzea, C.,2007). In truth, every organism on the Earth continuously encounters nanometer-sized system in the forms of colloids and other biological entities. Even those that parasitically exploit cellular processes to replicate themselves etc., or interfering with biological systems, often causes little ill effect and harm to the organism. However, new forms of engineered nanoparticles may have the potential for adverse biological effects. As stated above uranium can exist as both natural (colloids) and human produced nanoparticles in the environment (e.g. from depleted uranium ammunitions explosions (Figure 2), and work is ongoing for production of uranium nanoparticles for catalysts and remediation. Hence, the environmental behavior of uranium nanoparticles is of interest for many reasons.

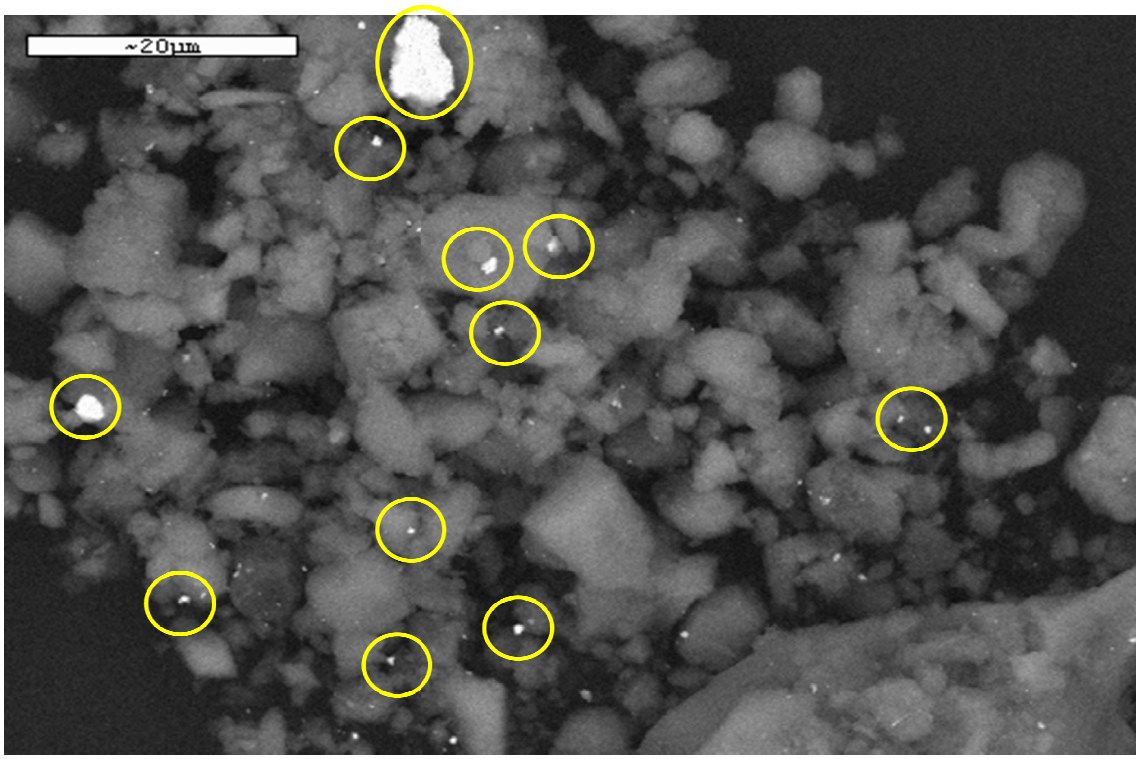


Figure 2: Scanning electron microscopy picture of uranium particles in soils from Kosovo contaminated by munitions explosions (Courtesy of Ole Christian Lind)

1.4 Uranium concentration, uptake and effects

Bioavailability is often a key indicator of potential risk of a toxic substance, radioactive or otherwise, ingested by a consumer that chemicals pose to environment and human health (Desmet et al., 1991). Since bioavailability is a combination of dynamic process (Peijnenburg et al., 1999), a combination of chemical and biological methods could be used to determine ‘‘environmental availability” and ‘‘bioavailability” of metal (M) in earthworms. (Díez-Ortiz, M. et.al., 2010). These could include direct uptake studies or soil extraction investigations. The bioavailability of many metals has been investigated, including some studies on uranium, and these are summarized below.

In a study on uranium bioavailability in soils, the concentrations of natural U in *Eisenia Fetida* exposed to U for 7 days increased from 8.0×10^{-2} $mgkg^{-1}$ (fw) at the U concentration in the soil of 1.86 $mgkg^{-1}$(dw), to 3.5×10^{1} $mgkg^{-1}$ at the concentration of U in soil of 600 $mgkg^{-1}$ (dw), resulting in a mean biological concentration factor (BCF) of $(7.4 \pm 3.3) \times 10^{-2}$ **(** Giovanetti, A. et al., 2010). However, the study also showed that the bioavailability of uranium is concentration dependent, with

higher soil to worm transfer being shown for soils with low U concentration. Other studies have suggested that for several soils, the bioavailability of U is at a minimum in the range of 10 to 100 mg U/kg dry soil. Sublethal toxicity does not occur until the ability of the organisms to restrict U uptake becomes impaired (Sheppard, S. C. and Evenden, W. G., 1992). This means there is no effect of uranium in earthworm at concentrations below 100 mg/kg soil.

In general, uranium compounds such as UF_6 are extremely soluble in organic fluids and these are highly dangerous, whereas the UO_2 and U_3O_8 particles are thought to be not so bioavailable or toxic as the solubility is low. But, the observed effects will also depend on the exposure, physiochemical properties and exposure route. In a recent study by Giovanetti, A. et al. (2010), it appeared that dry soil U concentrations up to 600 mg kg^{-1} had no detrimental effect on earthworm weight. However, DNA damage and adverse effects on lysosomal membrane stability were identified at quite low (5 - 15 mg kg^{-1}) U concentrations in the soil.

In order to maximize the uptake of the various U species in the present study, and to reduce contamination of soil, the uranium was added to the earthworm's food rather than soil. However, since the main aim of this study was to investigate bioavailability of the various U species, concentration ranges were selected at between 50-500 mg/kg food to maximize detection of uptake during one week, and to avoid confounding failure influencing on biological uptake due to toxicity effects.

CHAPTER 2: MATERIAL and METHODS

2.1 Test Materials

Animals:

Earthworms (*Esenia Fetida*). About 170 matured yellow-colored earthworm cocoons were collected from BIOFORSK earthworm farm and from the Isotope laboratory earthworm farm of Norwegian University of Life Sciences. They were cultured and acclimatized and those with weight approximately 0.3 – 0.5 g were chosen for experimental purpose.

Chemicals:

A solution of 4.5 g/1000 g of $CaCO_3$ powder was used to adjust the pH of OECD soil, pH was checked many times using $CaCl_2$ solution and KCl solution, NaCl solution (8.5 g/l) was used to make filter paper wet and is referred as saline solution, 7M HNO_3 solution was mixed with the sample before setting the Teflon tubes in UltraCLAVE and 3.5 ml was used for soil sample and 2.5 ml for other solution, 250 µl internal standard (In + Tl + Te + Rh + HNO_3) solution was mixed with the sample before setting the Teflon tubes in UltraCLAVE.

Manure:

The horse manure was collected from a domestic horse of one of the UMB staff, not commercially available and non-contaminated. It was finely sieved horse manure with 0.63 mm diameter sieve, and is a suitable food for *Esenia Fetida*. The horse from which manure was obtained was healthy during the duration of the experiment, not subjected to medication or treatment with substances such as growth promoters, nematicides or similar veterinary products that could adversely affect the worms during the test.

Soil:

The exposure experiment was carried out in OECD soil. This is a standardized mixture of soil from sand, clay and peat in set proportions (explained below). The materials used to prepare the soil are 7 kg sand, 2 kg clay and 1 kg peats.

Sand:

7 kg Askania sand (Baskarp 28) with size ranging 0.09 – 0.5 mm.

Clay:

Kaolinite clay (trade name as PURAFLO and general name Quality clay produced by WBB minerals ltd, United Kingdom)

Peats:

It is an accumulation of partially decayed vegetation matter or histosol (soil consisting primarily of organic materials). Peat forms when plant material, usually in marshy areas, is inhibited from decaying fully by acidic and anaerobic conditions. It is composed mainly of marshland vegetation: trees, grasses, fungi, as well as other types of organic remains, such as insects, and animal remains. Under certain conditions, the decomposition of the latter (in the absence of oxygen) is inhibited. Under the proper conditions, peat is the earliest stage in the formation of coal. The archeologists find it buried in earth's crust. The peat for the experiment was PLANTEJORD, distributed by Felleskjøpet and produced by Nordic Garden AS, Borgeskogen, 3160 Stokke) and it was taken 1 kg for making OECD soil.

Reference material:

China soil (GBW 07405) was used as soil reference material for determination of U concentrations.

Uranium treatments:

(i) U-ions: prepared from the salt uranyl acetate dihydrate: chemical formula ($UO_2(CH_3COO)_2 \cdot 2H_2O$) and molar mass 424.146 g/mol;

(ii) UO_2 and U_3O_8 particles: The particles were laboratory synthesized at IPM from natural uranium acetate, $UO_2(OAc)_2$, through a facile hydrothermal condition using amines as both reducing and structure – directing agents and have a size distributing ranging from 20 to 1000 nm (Wang Q et al., 2008, M Sc thesis of Tesfaye Girma Wurgie, to be submitted)

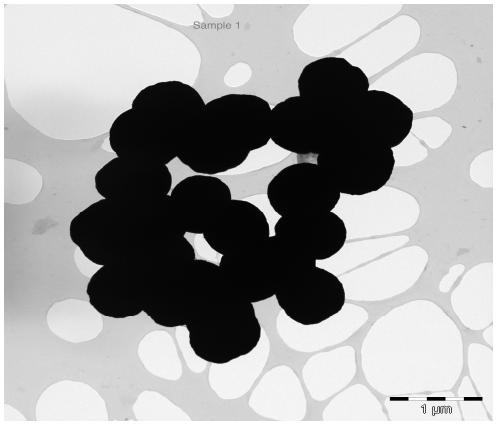


Figure 3: TEM of UO_2 nanoparticles

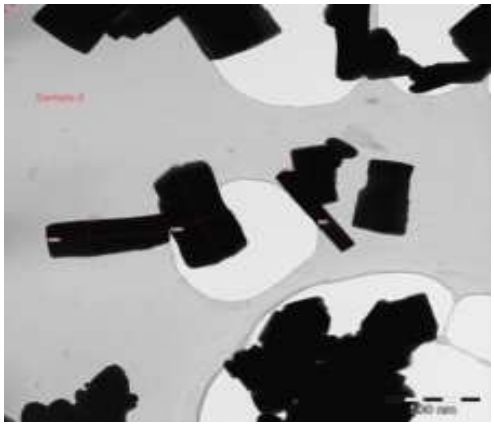


Figure 4: TEM of U_3O_8 nanoparticles

Figure 3 and 4 show a TEM image of the two U-NP treatments. The uranium oxidation state and crystalline structure was verified by Ole Christian Lind and Tesfaye Girma Wurgie (M Sc thesis of Tesfaye Girma Wurgie, to be submitted)

Tween 20:

It was mixed with U particles and ions while making U solutions (15 ml of 14%). Molecular formula: $C_{58}H_{114}O_{26,}$ CAS nr: 9005-64-5, density: 1.1 g/cm^3, Hydroxyl number 90 - 110. This is a non-toxic detergent/food additive widely used for ensuring a good dispersion of particles in aquatic solutions. It is known to be non-toxic to earthworms at the concentrations used in the experiment.

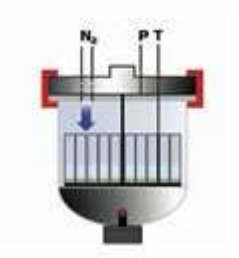
2.2 Instrumentation

2.2.1 UltraCLAVE microwave digestion

A simple sample rack is loaded with samples weighed into glass vials. The vials are capped with loose fitting caps to prevent condensation from the top of the reaction chamber dripping into the vials. The rack is fitted to the chamber top which is lowered automatically into the chamber. The sample vials sit in liquid that provides a consistent "load" for the delivered microwave energy. This insures even heating and consistent conditions from run to run. The chamber clamp is secured automatically with the UltraCLAVE, and the chamber pressurized with N_2 (40 bar). This prevents boiling of samples as the run starts and essentially acts as a "cover" over the samples, eliminating any possibility of cross contamination. The microwave program starts and all samples are digested under the same conditions. When the heating cycle stops, water cooling rapidly cools the chamber

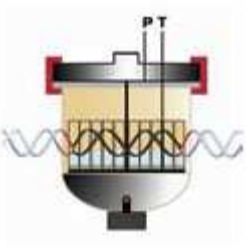
to ambient temperature. The pressure is released, the chamber is opened and acid fumes are extracted - away from the operator. The rack is then simply removed, the samples are made up to volume, and are ready for analysis.

Sample rack is lowered automatically into microwave chamber



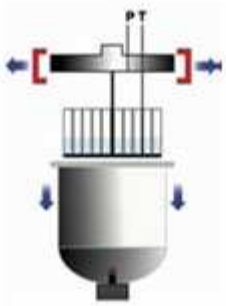
Chamber clamp is secured

Chamber is pressurized with N_2 prior to start of run – prevents any boiling of samples – cross contamination is eliminated



Microwave energy is applied. All samples under same temperature and pressure conditions

Fast cooling step due to water cooling of chamber. Chamber is vented and acid vapors extracted



Clamp is released and sample rack automatically rises from chamber

Figure 5: Schematic of digesting samples in UltraCLAVE

(http://milestonesci.com/index.php/product-menu/digestion/ultraclave)

2.2.2 Inductively coupled plasma mass spectrometry (ICP-MS)

For nearly 30 years, inductively coupled plasma mass spectrometry (ICP-MS) has been gaining favor with laboratories around the world as the instrument of choice for performing trace metal analysis. Most commercial ICP-MS systems is a quadruple mass spectrometer which rapidly scans the mass range. Samples are introduced into argon plasma as aerosol droplets. The plasma dries the aerosol, dissociates the molecules, and then removes an electron from the components, thereby forming singly-charged ions, which are directed into a mass filtering device known as the mass spectrometer. At a time, only one mass/charge ratio will be allowed to pass through the mass spectrometer from the entrance to the exit. (www.perkinelmer.com, 03/12/2011) Upon exiting the mass spectrometer, ions strike the first dynode of an electron multiplier, which serves as a detector. The impact of the ions releases a cascade of electrons, which are amplified until they become a measureable pulse. The software compares the intensities of the measured pulses to those from standards, which make up the calibration curve, to determine the concentration of the element. For each element measured, it is typical to measure just one isotope, since the ratio of the isotopes, or natural abundance is fixed in nature.

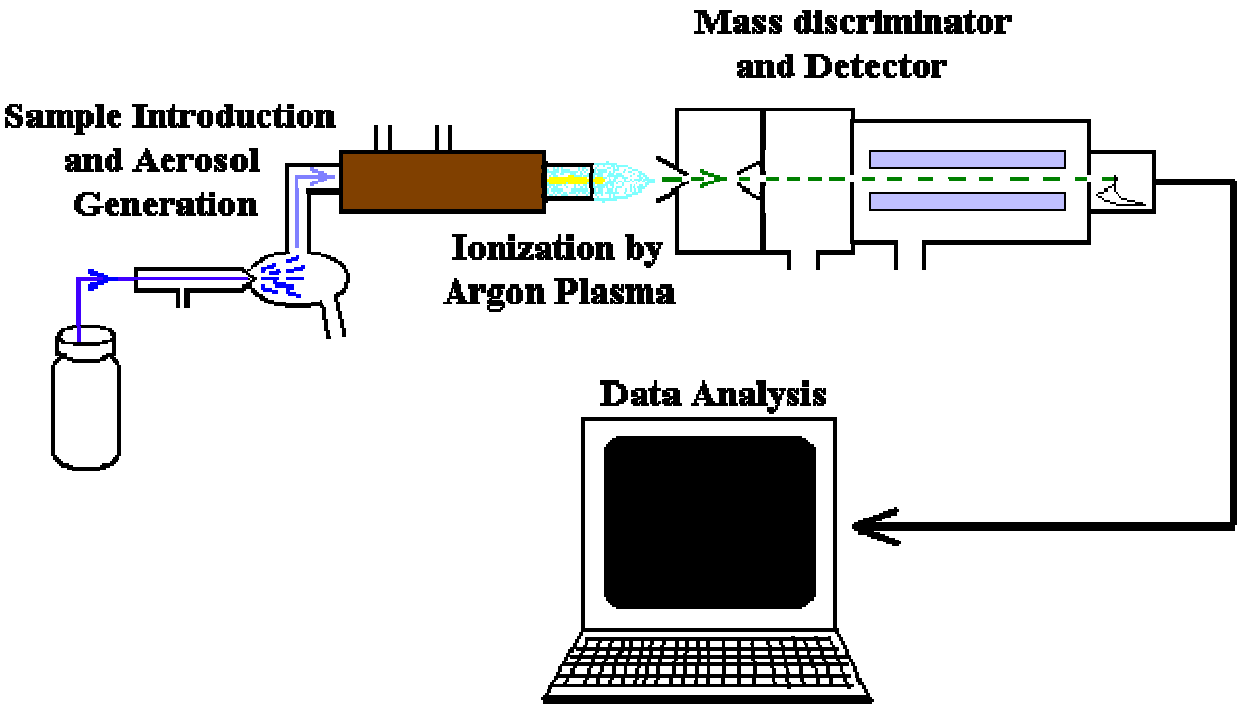


Figure 6 : Schematic of ICP-MS main processes (Steve Kvech, http://www.cee.vt.edu/ewr/environmental)

2.3 Experimental methods

2.3.1 Preparation of OECD Soil

First of all, 7 kg Askania sand, 2 kg kaolinite clay and 1 kg peats were weighed with the help of calibrated weighing machine and mixed finely after pouring in a big plastic box. This was the OECD soil prepared based on the OECD guidelines 1984. The pH of OECD soil was very low 2.8 approximately which was not adequate for growing the worms. So the pH of the soil was adjusted to 6.10 ± 0.08 by adding 4.5 g/1000 g of $CaCO_3$ powder which was within the OECD guidelines 6.0 ± 0.5.

Figure 7: OECD soil prepared at Isotope Laboratory

2.3.2 Culturing of worms

About 170 matured yellow-colored earthworm cocoons were collected from BIOFORSK earthworm farm and from the Isotope laboratory earthworm farm of Norwegian University of Life Sciences. About 85 worm cocoons per box were placed in two plastic boxes half-filled with non-contaminated kaolinite clay for culturing for about three weeks. Two boxes were used in order to give sufficient space for the hatched earthworms to grow. During the three week culturing period, non-contaminated horse manure from the same horse was given as food once a week, at the rate of 0.5 g/worm at regular time intervals starting from the first day. Each time when horse manure was added, it was moistened by adding double the weight of Milli Q-water to ensure that the earthworm had an adequate environment. This culturing process produced enough and similar typed worms (weight, health etc) grown up under the same environmental conditions.

Figure 8: Culturing of earthworms using clay and horse manure as food

2.3.3 Acclimation

Prior to U treatment, all worms were acclimatized in artificial OECD soil for 2 weeks. First of all, a plastic box (acclimation box) was filled with 3 kg artificial OECD soil of pH adjusted to 6.10 ± 0.08 prepared according to OECD guidelines (OECD 1984). The soil was moistened by adding 3000 g × 0.354 = 1062 g of MQ-water. Then, 80 matured worms with well-developed clitella were taken out from the culturing box and placed on the top of soil in acclimation box, and acclimatized for 2 weeks. In this period, 40 g dw (dry weight) non-contaminated horse manure from the same horse moistened with 80 g MQ-water was given as food once a week at regular time intervals.

Figure 9 : OECD soil prepared for acclimation of worms before putting manure

2.3.4 Exposure to Uranium spiked horse manure

After the 2 week acclimation period, 50 worms with well-developed clitellum and weight ranging 0.2 g – 0.5 g were taken out from acclimation box and put in 50 small plastic boxes each filled with approximately 67.7 g wet OECD soil such that there was one worm per box (Figure 10).

Figure 10: 50 boxes of 5 treatments filled with OECD soil containing one worm per box

These worms were randomly distributed between the 5 treatments, taking 10 worms per treatment. The 5 treatments were control, UO_2 exposed, U_3O_8 exposed, uranyl-low exposed and uranyl-high exposed. The different uranium solutions were added directly to horse manure, and then mixed thoroughly. For all the treatments both the uranyl solutions and the nano-microparticles were dispersed in 15 ml of 14% (by weight) TWEEN (Figure 11), then 14 ml was added to 7 g dry manure in 1 ml aliquots to ensure even distribution, and the manure mixed well mechanically using a spoon. The uranyl salt solutions were added to give 0.16 and 1.6 mg uranium per gram wet weight manure (50 and 500 ug/g dw), and taken from a stock solution of 100 mg/L natural uranium as the uranyl salt. The nano-micrometer sized particles (UO_2 and U_3O_8 shown in Figure 3 and 4) were also suspended in 14 % TWEEN, and then allowed to settle for 10 minutes to allow sedimentation of the larger particles. Aliquots of both the added suspensions and the mixed horse manure was retained for TEM and total uranium analysis.

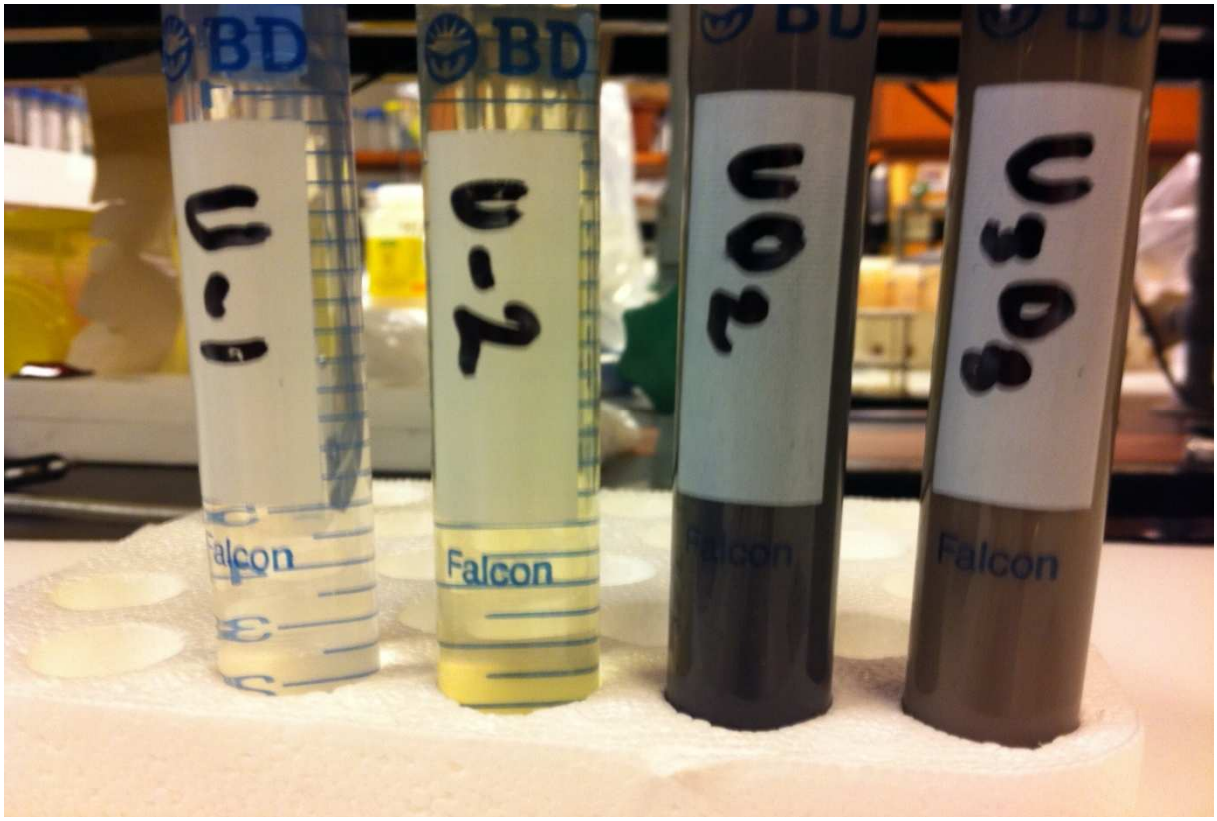


Figure 11: Solutions of NP and uranyl prepared at Isotope Laboratory (from left uranyl – low, uranyl – high, UO_2 and U_3O_8 solutions)

For the control, 1.5 g non-contaminated horse manure was used. All these small boxes were kept in climate chamber maintained at 20□ C for the period of one week.

2.3.5 Depuration Period

After one week, the worms were sorted out from the soil, washed in NaCl solution (0.85%), weighed individually and were put into a corresponding test boxes containing wet filter-paper (Figure 12). These were kept for depuration period for 2 days changing the filterpaper after one day keeping worms as usual. The used filterpapers and Faeces of first day were transferred to 50 corresponding plastic vail. After 2 days, each worm was weighed and put in separate labelled new plastic vails. The filter papers were transferred to the same corresponding vails as previous day's filterpaper were kept. At the end of depuration, the worms were transferred to and placed in the freezer at temp – 1 8 □ C to kill the worms.

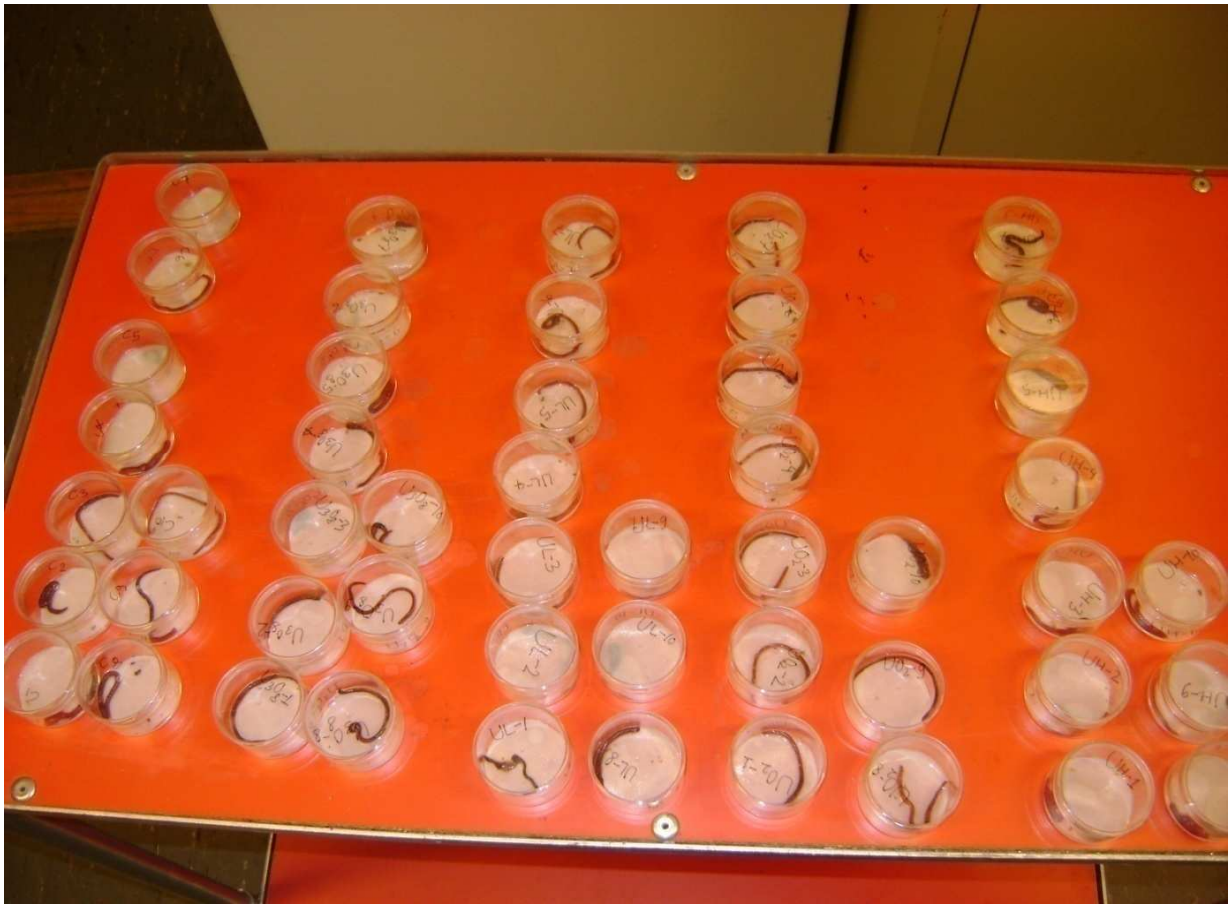


Figure 12: Worms taken out of treatment boxes after 1 week exposure and prepared for depuration period

2.3.6 ICP-MS Measurements

2.3.6.1 Worm

For uranium analysis in worm, 6 worms out of 10 worms from each treatment were chosen for ICP-MS analysis. Each worm was transferred to a corresponding Teflon tube and 5 blank samples were also taken to check the results. About 3.5 ml HNO_3 and 250 µl internal standard (In + Tl + Te + Rh + HNO_3) were added into all tubes. These samples were then digested for two and half hour in UltraCLAVE. Then, these samples were transferred to 50 ml plastic vails and diluted to 50 ml by adding MQ water. Finally, all samples were analysed by ICP-MS.

2.3.6.2 Soil

To calculate the concentration of uranium in the soil in which earthworm were grown up, approximately 0.25 g of soil in each of three vials out of 10 for each treatment together with 4 blank samples and 2 reference material sample were taken. The same process for digesting and preparing samples as done for worm was repeated by taking 5 ml HNO_3 instead of 3.5 ml HNO_3 and also 1 ml hydrogen-fluoride was added to improve digestion of the sample. To avoid toxicity of fume of hydrogen fluoride, some boric acid was also added in each sample. Lastly, samples were analyzed with respect to U by ICP-MS machine. Even though, all samples were digested, some yellow types of residue appeared on the bottom of the tube.

2.3.6.3 Manure

To determine the concentration of U in the manure, approximately 0.25 g of horse manure with replicates for each treatment was taken from the remaining content of manure that was used for earthworms together with 4 blank samples. The same procedure for digesting and preparing samples for worm was carried out and samples were analyzed by ICP-MS machine.

2.3.6.4 Faeces

To determine the concentration of U in the faeces, four vials of faeces samples containing filter-paper per treatment were chosen together with 2 blank samples. The same procedure for digesting and preparing samples for worm was used plus 3 ml MQ water was also added to the Teflon tubes to dilute the sample. Lastly, samples were tested by ICP-MS.

CHAPTER 3: RESULTS and DISCUSSIONS

3.1 Concentration of Uranium in Manure and Soil

The horse manure was contaminated by the solutions of uranium particles and uranyl and the samples were prepared from that contaminated manure. This contaminated manure was kept on the surface of soil and the soil was contaminated by the remaining concentration that the earthworms have eaten. So, clearly the soil contains less uranium than the manure (Table 3).

Table 3: Uranium concentration in manure and soil

Exposure Gr	U Conc Manure (mg/g ww) n = 3	U Conc in soil (μg/g ww)* n = 3	Conc in soil/Conc in manure
Control	$(2.37 \pm 0.48) \times 10^{-4}$	1.76 ± 0.09	7.4 ± 1.6
UO_2	1.32 ± 0.02	12 ± 6	$(7.6 \pm 3.8) \times 10^{-3}$
U_3O_8	2.53 ± 0.35	37 ± 7	$(1.4 \pm 0.3) \times 10^{-2}$
Uranyl-Low	0.16 ± 0.01	2.92 ± 0.35	$(7.41 \pm 1.04) \times 10^{-3}$
Uranyl-High	1.55 ± 0.14	11.3 ± 1.5	$(6.16 \pm 0.97) \times 10^{-3}$

* After 1 week feeding.

There is a clear difference between the U concentration in control treatment and in samples with added U. The concentration of uranium in both soil and manure was significantly higher than that in the control. For manure, the concentrations ranged from 0.16 to 2.53 mg/g as compared to 0.24 μg/g for the control; and for soil from 2.9 – 12 μg/g as compared to 37 μg/g for control. The total concentrations of U in particles and uranyl- high ion spiked manure were a similar order of magnitude by design. The small variations were due to the fact that it was difficult to transfer an exact mass of U particle suspensions, because of settling out of the larger particles. But the variability in concentration between subsamples of manure was acceptable and mostly below 10 - 15 %. The concentration ratio between uranyl-low and uranyl-high manure confirmed the 1:10 ratio selected for these treatments.

The bigger difference between treatment and control for manure as compared to soil simply reflects that the U was added directly to manure, while the soil had been contaminated as a result of the

worms eating manure and excreting into the soil. Background concentrations of U in soil are 7 times greater than those in manure: 1.76 as compared to 0.24 μg/g. For the U treated boxes, the resulting levels in the soil follow the concentrations in manure, with the U-low soil being only slightly above the background control level (Table 3). As expected the soil/manure concentration ratios for the control was much greater than for the treated soils: 7.3 as compared to 6.16×10^{-3} - 1.4×10^{-2}, with no significant difference between the different U treatments. The variability between soil replicates was greater for the particle contaminated (20 - 50 % error) as compared to uranyl contaminated treatments, possibly reflecting the inhomogenity of particles treatments.

3.2 Worm growth

All worms survived the treatment, and there was no mortality observed in any of the boxes during the period of one week while measuring growth. Of course, the earthworms have a high potential for adaptation and survival in highly contaminated environments (Spurgeon and Hopkin, 1996; Corp and Morgan, 1991). The results in Table 4 and Figure 8 are of worm growth in terms of increased weight taken in gram.

Table 4: Worm growth after one week

Exposure group	Starting weight (g)	Worm growth (in terms of increased wt. (g)) n = 10	Worm growth removing outliers* (g) n = 8 - 10
Control	0.30	0.08 ± 0.05	0.08 ± 0.05
UO2	0.32	0.05 ± 0.04	0.05 ± 0.04
U3O8	0.28	0.06 ± 0.07	0.08 ± 0.06
Uranyl-Low	0.29	0.07 ± 0.04	0.07 ± 0.04
Uranyl-High	0.28	0.07 ± 0.06	0.07 ± 0.05

* Outliers taken to be worms that lost weight (4 worms) during the experiment.

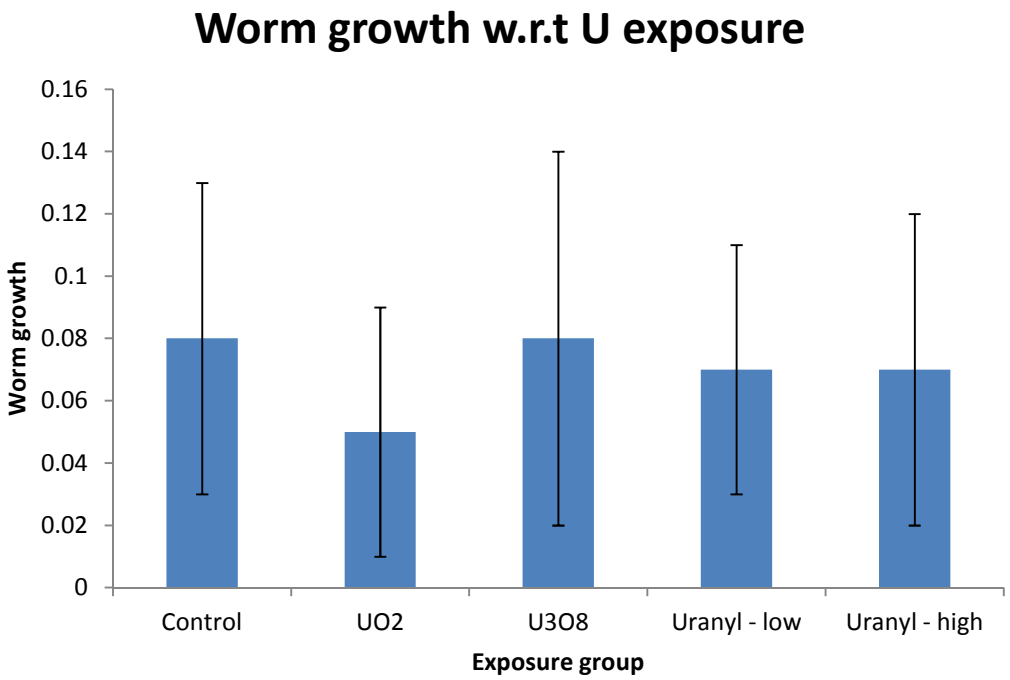


Figure 13: Worm Growth removing outliers and labeling uncertainties

The worms gained the weight ranging 0.05 – 0.08 g in the period of one week in which earthworms were fed with horse manure. But, the growth of individual worms ranged from 0.002 g to 0.156 g, and varied quite largely within each group. But, four worms lost their weights – all from different groups, hence this was not attributed to U concentration. One of the worms which had lost weight was treated with UO_2. The initial and final weights in one week period were 0.335 and 0.304 g unit respectively. It has eaten the manure and was active in both one week growth measurement and 2 day's depuration period, so this may be a metabolic difference. Other two worms whose weights decreased had not eaten much, but they were still active and these were treated with U_3O_8 and uranyl- low. But, after one day's depuration period, the worm treated with uranyl-low was found dead. The filter paper was wet with yellow liquid and no special black gut was found on the surface of filter paper. The last worm that lost weight was treated with uranyl – high, had eaten horse manure and was inactive, but it was still alive. But, it had found died by the 2nd day's depuration, leaving no faeces on the surface of filter paper similar to the previous worm. Comparing the results of different treatments, it seems that there is no significant difference in the values. Therefore, it can be concluded that there is no statistically significant effect of uranium on either mortality or on

worm growth. This is in line with what was expected from previous studies, for example, in the study of both natural and depleted U treatments involving 28 days of exposure where no significant changes were observed in terms of either weight or mortality (Giovanetti, A.et al., 2010).

3.3 Uranium uptake by worms and concentration in faeces

The experimental results in the following Table show uptake by the worm after 1 week feeding.

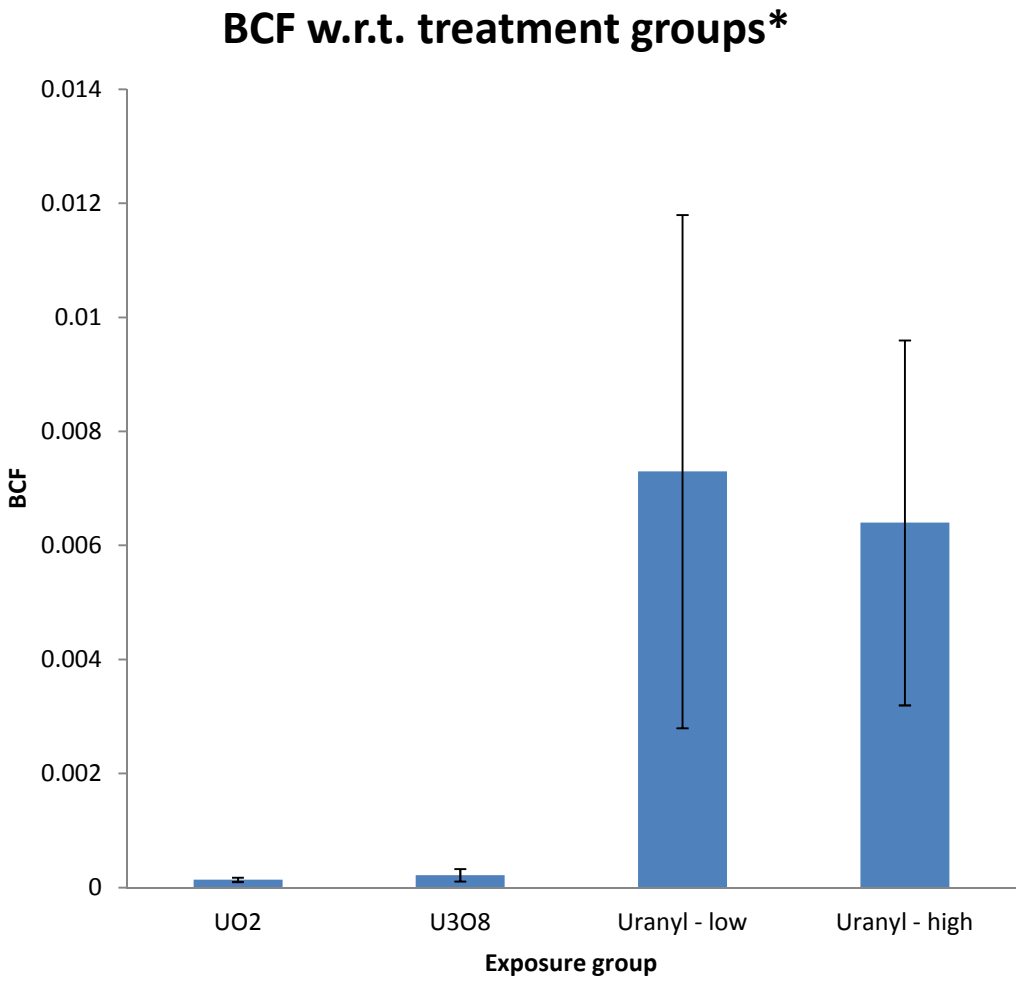
Table 5: Uranium uptake by worms and comparison of concentrations

Exposure Group	U conc in Worm (μg/g)* n = 6	U conc added Manure (μg/g) n = 3	U conc Faeces (μg/g) n = 4	Conc in worm/conc in manure	Conc in worm/Conc in faeces
Control	0.06 ± 0.01	0.24 ± 0.05	0.71 ± 0.09	0.23 ± 0.08	$(7.7 \pm 2.2) \times 10^{-2}$
UO2	0.19 ± 0.05	1320 ± 20	21.5 ± 1.1	$(1.41 \pm 0.39) \times 10^{-4}$	$(8.7 \pm 2.4) \times 10^{-3}$
U3O8	0.57 ± 0.30	2530 ± 348	110 ± 88	$(2.2 \pm 1.1) \times 10^{-4}$	$(5.4 \pm 4.9) \times 10^{-3}$
U-Low	1.15 ± 0.70	156 ± 11	NA**	$(7.3 \pm 4.5) \times 10^{-3}$	NA
U-High	9.96 ± 4.8	1550 ± 140	14.2 ± 7.1	$(6.4 \pm 3.2) \times 10^{-3}$	0.70 ± 0.50

* After 1 week exposure and 2 days depuration

** NA – not analyzed

Analyzing the result for the uptake by worm in terms of manure, i.e. the ratio of concentration of uranium in worm to the concentration of uranium in manure, or bioconcentration factor (BCF) a clear difference was observed between uranium particles and uranyl, with the uranyl treatment having higher uptake than that of particle uptake (Table 5). For particles, the ratio ranged in the order of 10^{-4} with lowest value 1.41×10^{-4} whereas for uranyl it is in the order of 10^{-3} with highest value 7.3×10^{-3}. Both values are low in comparison to the ratio 0.23 for control treatment, but this reflects the fact that control worms would be acquiring U from the soil rather than the manures, and indeed the concentration ratio of worm/soil for controls was 3.1×10^{-2}, which is in line with previous studies **(**Giovanetti, A. et al., 2010). The BCF of U_3O_8 was higher than that for UO_2, although the differences were not significant; this is in line with what would be expected from the solubility of the different U oxidation states. Higher uptake for uranyl must be due to the greater bioavailability of ions as compared to the nano-microparticles.



* Control not taken as figure distorted

Figure 14: Bioconcentration factor (BCF) of uranium particles and uranyl in worm and Manure

Moreover, in a recent pilot experiments at HASYLAB BL, involving μ-SRXRF confocal mapping, U-particles (aggregates) were also observed within earthworms following the exposure to ~100 nanometer-micrometer sized uranium particles (Figure 15). The U_3O_8 particle concentration was found in reproductive organs and in intestine being the concentration in the intestine more than that of reproductive organ even after 60 hr depuration period, but UO_2 particle was not observed (Lind O.C. et al., 2011 unpublished).

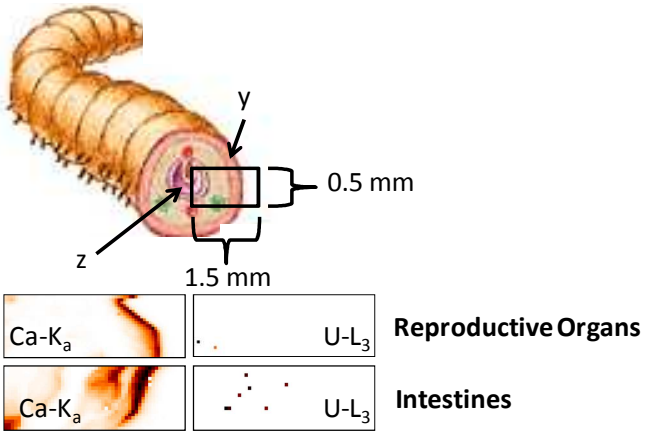


Figure 15. Schematic drawing of an earth worm cross section (top) and confocal micro X-ray mapping cross sections of the reproductive organ segment (middle) and intestine segment (bottom) showing U L_3 x-ray signals as evidence of U uptake during 1 week exposure to synthesized nm-µm U_3O_8 particles (2.5 mg/g) in horse manure. Depuration was performed on wet filter paper where earth worms were allowed to empty their intestinal contents for 60 hrs (Lind et al, 2011 unpublished).

For the ratio of U concentration of worm to the U concentration of faeces, the ratio is not consistent on going through all the treatments including control even with values ranging from 5.4×10^{-3} for particles to the value 0.70 for uranyl. The data of concentration for all the treatments is random and is probable that the levels in faeces depend on how much soil or manure the worm had in its gut at the time of depuration.

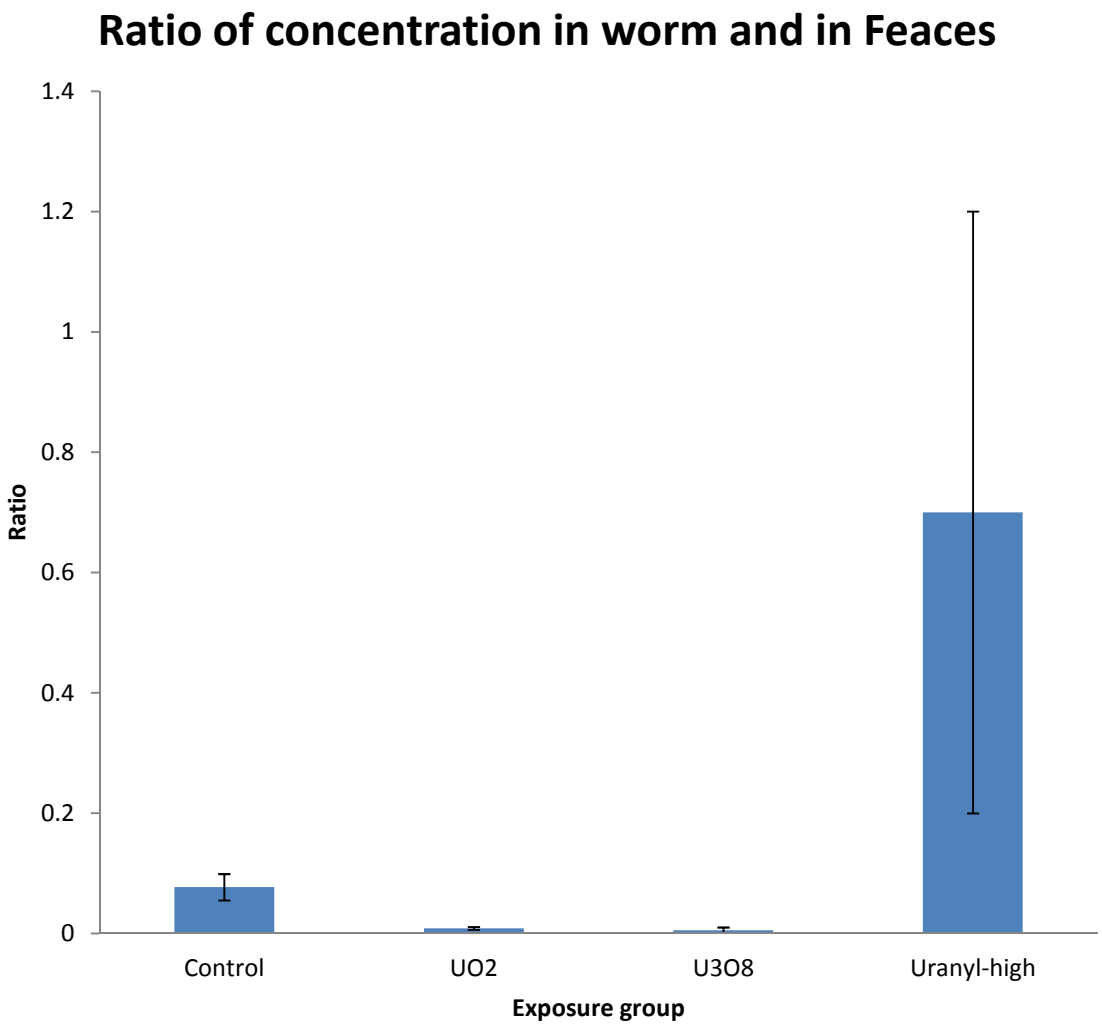


Figure 16: Ratio of U concentration in worm and to the U concentration in faeces

3.4 Conclusions

This study highlighted some important points of bioaccumulation of different U species by earthworms. There is very little data on bioconcentration factors of U in earthworms, and none comparing the bioavailability of uranium particles (UO_2 and U_3O_8) as compared to uranyl treatments. While measuring growth, it is concluded that all worms survived the treatment, and there was no mortality observed in any of the boxes during the treatment period of one week. Analyzing the result for the uptake by worm in terms of manure, a clear difference was observed between uranium particles and ions, with the uranyl treatment showing a significantly higher uptake

than that of particle uptake, with bioaccumulation of uranyl being approximately 10 times higher than that of particles, and with U_3O_8 showing a higher uptake than UO_2. This was supported by comparisons of worm/soil concentration ratio and worm/faeces concentration ratio. However, at present it is not known whether the BCF for nano-microparticles reflects the uptake of uranium as NP or whether the uptake is of U ions released from the NP. Further studies on the speciation of uranium in soil and in the earthworm would help to further understand the influence of U speciation on exposure and uptake.

REFERENCES

Angell, P., Apps, J.A., Cragnilino, G.A., Hsiung, S.M., Lichtner, P.C., Murphy, W.M., Pabalan, R.T., Pickett, D.A., Sridhar, N., Stothoff, S.A., Turner, D.R., 1996. Evolution of the near-field environment in the proposed high-level waste repository at yucca mountain-a review of hypotheses. Center for Nuclear Waste Regulatory Analyses San Antonio, Texas. 1 - 67

ATSDR (Agency for Toxic Substances and Disease Registry), May 2011. Draft toxicological profile for uranium (Public comment period ended on 29 July 2011). Agency for Toxic Substances and Disease Registry (ATSDR), Atlanta, GA. 1 - 452

Bowen, H.J.M., 1979. Environmental Chemistry of Elements. Academic Press; London. 333. ISBN: 0121204502 9780121204501

Buzea, C., Blandino P. I. I., and Robbie, K., 2007. Nanomaterials and nanoparticles: Sources and toxicity. Biointerphases vol. 2, issue 4, pages MR17 - MR172, 1−103

CCME (Canadian Council of Ministers of the Environment), 2007. Canadian soil quality guidelines for the protection of environmental and human health: Uranium (2007). Excerpt from Publication No. 1299; 14. ISBN 1-896997-34-1

Connell, D W, and Markwell, R D, 1990, Bioaccumulation in the soil to earthworm. Chemosphere, 20. Elsevier Ltd. 91–100.

Corp, N., Morgan, A.J., 1991. Accumulation of heavy metals from polluted soils by the earthworm Lumbricus rubellus: can laboratory exposure of 'control' worms reduce biomonitoring problem? Environmental Pollution, Volume 74, Issue 1, 39 - 52.

Desmet, G.M., van Loon, L.R., Howard, B.J.,1991. Chemical speciation and bioavailability of elements in the environment and their relevance to radioecology. Science of the Total Environment, volume 100, 105-24

Díez-Ortiz, M.; Giska I.; Groot, M; Borgman E. M.; Cornelis A.M.; Gestel V., 2010, Influence of soil properties on molybdenum uptake and elimination kinetics in the earthworm *Eisenia Andrei*, Chemosphere 80. 1036–1043

Edwards C A, Bohlen P J, 1996. Biology and Ecology of Earthworm (3rd ed.). London: Chapman and Hall. 426.

European Food Safety Authority(EFSA), 2009; Scientific Opinion, Uranium in foodstuffs, in particular mineral water Scientific Opinion of the Panel on Contaminants in the Food Chain; The EFSA Journal 1018, 1-59

Gaimmar, D., 2001, geochemistry of uranium at mineral-water interfaces: rates of sorption-desorption and dissolution-precipitation reactions, Ph D thesis submitted to the California Institute of Technology, USA, 1 - 277

Giovanetti, A., Fesenko, S., Cozzella, M. L., Asenci, L. D., Sansone, U., 2010, Bioaccumulation and biological effects in the earthworm *Eisenia fetida* exposed to natural and depleted uranium, Journal of Environmental Radioactivity 101, 511−516

Haimi, J. R., Salminen, J., Huhta, V., Knuutinen, J., Palm, H., 1992. Bioaccumulation of organochlorine compounds in earthworms. Soil Biology and Biochemistry, 24: 1699 – 1703.

Mohanraj, V.J. and Chen, Y, 2006, Nanoparticles – A Review, Research Article, Tropical Journal of Pharmaceutical Research, 5 (1): 560 - 562

Norr, C. and Riepert, F., 2007, Bioaccumulation studies with *Eisenia Fetida* using an established degradation test system. J Soils Sediments 7 (6) 390 – 395

OECD (Organization for Economic Co-operation and Development), 1984. OECD guidelines for testing of chemicals 207: Earthworm, Acute Toxicity Tests. 1 – 9.

Powers, K. W., Brown, S. C., Krishna, V. B., Wasdo, S. C., Moudgil, B. M. & Roberts, S. M., 2006, Research strategies for safety evaluation of nanomaterials. Part VI. Characterization of nanoscale particles for toxicological evaluation. Toxicological Sciences, 90 (2): 296 – 303.

Sheppard, S. C. and Evenden, W. G., 1992, Bioavailability Indices for Uranium: Effect of Concentration in Eleven Soils, Archives of environmental contamination and Toxicology 23, 117-124

Salbu, B., Janssens, K., Lind, O.C., Proost, K., Danesi, P.R.; 2003. Oxidation states of uranium in DU particles from kosovo. journal of environmental radioactivity 64. 167 – 173.

SCENIHR(Scientific Committee on Emerging and Newly Identified Health Risks), 2006. opinion on the appropriateness of the risk assessment methodology in accordance with the technical guidance documents for new and existing substances for assessing the risks of nanomaterials. C_7 - risk assessment, directorate C - public health and risk assessment, European Commission

SCENIHR(Scientific Committee on Emerging and Newly Identified Health Risks), 2005, the appropriateness of existing methodologies to assess the potential risks associated with engineered and adventitious products of nanotechnologies, c7 - risk assessment, directorate C - public health and risk assessment, European Commission

Sheppard, S. and W. Evenden. 1987. Review of effects of soil on radionuclide uptake by plants. Research report prepared for the Atomic Energy Control Board. Ottawa, Canada. INFO-0230.

Spurgeon, D.J., Hopkin, S.P., 1996. The effects of metal contamination on earthworm populations around a smelting works: quantifying species effects. Applied Soil Ecology 4, number 2, 147-160.

Syers, J.K., Springett, J.A.,1984, Earthworms and soil fertility, Plant and Soil 76. 93-104

WHO, 2001. Depleted uranium: sources, exposure and health effects. Department of Protection of the Human Environment. World Health Organization. Geneva. WHO/SDE/PHE/01.1. http://www.who.int/ionizing_radiation/pub_meet/ir_pub/en/

SUPLIMENTARY REFERENCES

Courtesy of Ole Christian Lind

Lind, O.C., Oughton, D., Salbu, B. (Norwegian University of Life Sciences, UMB), Vanmeert, F., Nuyts, G., Janssens, K. (University of Antwerpen), 2011, An unpublished study.

M Sc thesis of Tesfaye Girma Wurgie, to be submitted

Radioactive decay chain diagram
http://en.wikipedia.org, 20/11/2011

Schematic of ICP-MS main processes by Steve Kvech
http://www.cee.vt.edu/ewr/environmental, 02/12/2011

The 30 minute Guide to ICP – MS
http://www.perkinelmer.com/, 03/12/201, Perkinelmer Inc.

UltraCLAVE Microvave digestion
http://milestonesci.com/index.php/product-menu/digestion/ultraclave, 02/12/2011