Synthesis, characterization and uptake study of uranium nanoparticles (UO_2 and U_3O_8) in eggs of Atlantic salmon (Salmo Salar)

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Preface and acknowledgements

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Abstract

The use of nanomaterial, nanotechnology and the awareness of nanoparticles (NPs) in the environment have escalated in recent years. Uranium (U) is an element frequently used and released to the environment, being the major fuel material used in nuclear energy power plants. The study of U NPs is, however, rather scarce in literature, although studies of micro sized particles have been carried out. Since U NPs occur both naturally and can be synthesized as catalysts, this was the focus of the present thesis. Work included the synthesis of U NP, the characterization of these particles and the use of these U NPs, as well as U ions, in an uptake study in fertilized eggs of Atlantic salmon (*Salmo salar*).

Two different kinds of U oxide NPs were successfully synthesized from uranyl acetate dihydrate with depleted uranium (DU). These particles were synthesized by modifying a method previously used by Wang et al. (2008), using organic amines as reducing agents under hydrothermal conditions in a high pressure microwave (UltraClave). The product was washed with ethanol, half was freeze-dried whereas the other half was subjected to dialysis and subsequently kept in suspension. Observations in transmission electron microscopy (TEM) showed that purification of synthesized products by means of dialysis (mean diameter 85 nm for the U_3O_8 suspension and 198 nm for the UO_2 suspension) yielded smaller sized NPs than subsequent freeze-drying (mean diameter 530 nm for the U_3O_8 suspension and 220 nm for the UO_2 suspension). From these images it was also evident that the dialysis treated suspensions contained more single small particles (~<50 nm) than the freeze-dried suspensions, where almost all the U particles are in aggregates. This is possibly the result of the product being kept in suspension during all times when treated with dialysis, as opposed to freeze-drying. More than 50 % of the particles in each suspension treated with dialysis had diameters between 1-100 nm; both suspensions could hence be defined as nanomaterial according to the European Commission's definition.

In the uptake study, eggs were exposed to uranyl, UO_2 and U_3O_8 suspensions; all containing ~ 50 mg/l U. The exposure took place during 24 hours after dry fertilization, and then transferred to clean water for 1 week of depuration. Fish eggs were sampled after 3 and 24 hours exposure to U and after 3 and 7 days in clean water. In addition, samples of the eggs interior was taken, using a syringe, at each sampling point. The U concentration in eggs was determined by ICP-MS (both whole eggs, and interior), as well as in water samples taken

before and after exposure, and after 3 and 7 days of depuration in clean water. In addition to total water samples, samples collected before and after exposure, was fractionated using 0.2 µm syringe filters, high speed centrifugation (30 000 rpm; ~93 000 g) and 3 kDa membrane filters. Uranium concentration was measured in all fractions, and some were analyzed by TEM, to determine the particle distribution, and to identify whether the organic matter in the water had changed the U size distribution.

It was found that eggs exposed to U NP's contained much U on the exterior shell, while the concentration of U in the interior of eggs exposed to uranyl was higher than in those exposed to U NP. Most of the U measured in the whole eggs exposed to NP's was present on the egg shells already after 3 hours exposure, and the concentration of U did not decrease noticeably during depuration. The conclusion is therefore that U ions are more bioavailable to the fish eggs than U NPs. The U concentration did, however, decrease faster during depuration in fish eggs exposed to uranyl, than in fish eggs exposed to U NP.

From both the TEM images and the ICP-MS measurements, it was evident that more U in the water was present as HMM species after exposure than before. This led to the conclusion that either organic matter, or time, induced the formation of U particles in U suspensions. When comparing TEM images taken of total water fractions and <0.2 µm water fractions, it was evident that there was some clogging in the 0.2 µm filter, since particles with diameters lower than the filter pore size cut-off were retained by the filter. This also led to an underestimation of small sized U particles in the suspension.

Sammendrag

Bruken av nanomateriale, nanoteknologi og bevisstheten rundt nanopartikler (NP) i miljøet har eskalert de siste årene. Uran (U) er et element som er mye benyttet og som slippes ut i miljøet, fordi U er det viktigste brenselsmaterialet i atomkraftverk. Studier av U NP i litteraturen er likevel begrenset, selv om studier av partikler på mikrometer størrelse har blitt utført. Fordi U NPer forekommer både naturlig og som et produkt av katalyse, er dette fokuset i denne oppgaven. Arbeidet inkluderte syntese av U NPer, karakterisering av disse partiklene og bruk av disse U NPene, samt U ioner, i et opptakstudie med befruktede fiskeegg fra atlanterhavslaks (*Salmo salar*).

To forskjellige typer U oksid NPer ble syntetisert fra uranyl acetat dihydrat med utarmert uran (DU). Disse partiklene ble syntetisert ved hjelp av en modifisert metode tidligere benyttet av Wang et al. (2008), ved å bruke organiske aminer som reduseringsmiddel under hydrotermale forhold i en høytrykksmikrobølgeovn (UltraClave). Det syntetiserte produktet ble vasket med etanol, halvparten av produktet ble frystørket, mens resten av produktet ble behandlet med dialyse, og holdt i suspensjon gjennom hele forsøket. Observasjon ved hjelp av transmisjon elektronmikroskopi (TEM), viste at dialysebehandling av produktene (gjennomsnittlig diameter 85 nm for U_3O_8 suspensjonen og 198 nm UO_2 suspensjonen) gav NPer med mindre størrelse enn ved frysetørking (gjennomsnittlig diameter 530 nm for U_3O_8 og 220 nm for UO_2). Behandling med dialyse gav også flere single og små partikler (<50 nm) enn partikler behandlet med frysetørking, hvor det meste av materiale var i form av aggregater. Dette kan være et resultat av at produktet holdes kontinuerlig i suspensjon når den behandles med dialyse, dette er ikke tilfellet ved frysetørking. Over 50 % av partiklene i hver suspensjon, behandle med dialyse, ble funnet til å være mellom 1-100 nm i diameter; begge suspensjonene kunne derfor defineres som nanomateriale i henhold til Europa Kommisjonens definisjon.

I opptaksstudiet ble befruktede lakse-egg eksponert for uranyl, UO_2 og U_3O_8 suspensjoner med U konsentrasjon på ~50 mg/l. Eggene ble eksponert i 24 timer etter befruktning og deretter overført til rent vann, der de ble oppholdt i en uke. Det ble tatt fiskeegg prøver etter 3 og 24 timers U eksponering, samt 3 og 7 dager etter at eggene var overført til rent vann (utskillelse). I tillegg ble det tatt prøver av innholdet i egg ved hver prøvetaking ved hjelp av sprøyte. Induktivt koblet plasma emisjon masse spektrometer (ICP-MS) ble benyttet for å måle uraninnhold i egg (både hele og innhold uten sall). Uraninnhold i vannprøver både før

og etter eksponering, samt etter 3 og 7 dager utskillelse, ble analysert. I tillegg til totale vannprøver, ble det tatt vannprøver før og etter eksponering til fraksjonering. Disse ble fraksjonert med hhv. 0.2 µm sprøyte filter, høyhastighets sentrifugering (30 000 rpm; ~93 000 g) og 3 kDa membran filter. Urankonsentrasjon i alle fraksjoner ble også analysert og noen ble også analysert vha. TEM, for å bestemme fordelingen av partikkelstørrelser og for å identifisere om det organiske materialet i vannet hadde endret uran spesieringen.

Det ble funnet at eggene som ble eksponert for U NP suspensjoner hadde mye U på skallet, mens konsentrasjonen av U inne i eggene var høyere i egg eksponert til uranyl i forhold til egg eksponert til U NP. Mesteparten av U målt i egg eksponert til U NP var til stede på eggenes overflate allerede etter 3 timers eksponering, i tillegg avtok ikke denne U verdien merkverdig etter overføring til rent vann (utskillelse/desorpsjon). Konklusjonen er derfor at U ioner er mer biotilgjengelig for fiskeegg enn U NP. Uraninnholdet i eggene som ble eksponert for U ioner avtok imidlertid raskere enn uranet i eggene eksponert for U NP.

Både fra TEM bildene og fra ICP-MS målingene fremgår det at konsentrasjonen av U som forelå som HMM spesier var høyere etter eksponering av egg enn før. Dette ledet til en konklusjon om at enten organisk materiale, eller tid, endrer spesieringen av U i suspensjon. Fra TEM bilder tatt av totale partikkel fraksjoner og av 0,22 µm filtrerte fraksjoner, kunne det også konkluderes med at filtreringen medførte at filtrene ble tettet (clogging). Dette førte til at partikler som hadde mindre diameter enn membranfilteret ble holdt tilbake under filtrering. Dette medfører også en underestimering av små U i suspensjon.

Abbreviations

BCF	Bioconcentration factor
CI	Confidence interval
DLS	Dynamic light scattering
DU	Depleted uranium
FESEM	Field emission scanning electron microscopy
FFF	Field flow fractionation
HMM	High molecular mass
HRTEM	High resolution transmission electron microscopy
ICP-MS	Inductively coupled mass spectrometry
IS	Internal standard
Kd	Soil/sediment-water distribution coefficient
LMM	Low molecular mass
ND	No detection
NP	Nanoparticles
SEM	Scanning electron microscopy
SSA	Specific surface area
Std	Standard deviation
TEM	Transmission electron microscopy
U	Uranium
U.P.	Ultrapure
XRD	X-ray diffraction

Contents

1 Introduction

1.1 Nanoparticles

Research on nanoparticles (NP) and nanotechnology has escalated during the last decades and hundreds of commercial products from medicines to clothes contain NPs (Christian et al. 2008; Ju-Nam & Lead 2008). In addition to the release of anthropogenic NPs, there are a lot of naturally occurring NPs in the environment. Inorganic forms of naturally occurring NPs, often called colloids, result from chemical alterations or physical erosion of minerals, or from the direct precipitation from surface or groundwater containing ions of NP elements (Geckeis et. al., 2011).

Because NPs are small compared to bulk particles, the surface area to volume ratio of the particle is larger than for its bulk counterpart. This leads to different chemical and physical properties in NPs, e.g. the reactivity tends to be greater per weight unit (Aitken et al. 2006). Nanoparticles also tend to aggregate or agglomerate into larger clusters of several micrometers, in these aggregates and agglomerates each primary particle can still be in the nanometer scale. Because of this, the larger agglomerates/aggregates can also have NP behavior, since the particles can change rapidly between solution and agglomerated/aggregated state (Handy et al., 2008). The tendency of the NPs to aggregate/agglomerate in water and hence also the stability of the particles in water is controlled by several factors. These include both the properties of the particle surface and the composition of the solution (e.g. pH, pE) (Geckeis et al., 2011). Because of their small size, NPs can also cross biological membranes and cause toxicological effects in humans, animals and plants (Lövestam et al., 2010).

The definition of NPs is still somewhat unclear, and there is more than one view of what can be defined as NPs. The definition most commonly used is that a particle can be defined as a NP if it has at least one dimension less than 100 nm (Christian et al. 2008; Ju-Nam & Lead 2008). A definition like this, that only takes the size into account when defining NPs, might be insufficient. All NPs cannot be defined by one upper and one lower limit. A purely size-based definition does not take size range, size distribution and nanoscale properties into account, which can lead to particles beyond these size limits being ignored even though they still act as NPs. With a lower limit of 1 nm some molecules might also fall under the NP definition

(SCENHIR, 2010). Another definition used by Geckeis et al., (2011), is that NPs are particles between ~1 nm to ~1 µm in size that remain suspended in solution. With this definition the density of the particles largely determines whether or not it is a NP. In the inorganic chemistry book written by Atkins et al. in 2006, a definition taking the properties of the particles into account is also used:

"A nanomaterial is any material that has critical dimension on the scale of 1 to 100 nm; a more exclusive definition is that a nanomaterial is a substance that exhibits properties absent in both the molecular and bulk solid state on account of it having a critical dimension in this range." (Atkins et al. 2006)

Although the definition also covers the aspect of NP behavior it is however so wide that the determination of what can be called NPs can be difficult. The European commission recommended a new definition of NPs and nanomaterial in 2011, this definition takes both size and size distribution into consideration:

"*Nanomaterial means a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm."* (European Commission, 2011)

In addition to this definition the European Commission recognizes that aggregates and agglomerates consisting of primary particles between 1-100 nm should be termed NP, even if the size of the total aggregate or agglomerate is above 100 nm. These agglomerates/aggregates often have close to the same surface area to volume ratio as the primary particles; this means that they can possess many of the NP properties. Primary NPs in the aggregate/agglomerate might also become unbound as a function of time. To acknowledge the primary particles in agglomerates and aggregates as NPs, the Commission recommends that material containing particles (aggregates and agglomerates) that have a specific surface area (SSA) of >60 m^2/cm^3 should be defined as a nanomaterial (European Commission, 2011).

1.2 Uranium

1.2.1 Basic chemistry and radiochemistry of uranium

Uranium (U) is part of the f block in the periodic table, more specifically the 5f actinides. Because of the high atomic number, actinides have large atomic and ionic radii. Due to the radioactive nature of the actinide elements, only a few are found naturally, U is one of these elements (Atkins et al. 2006). Uranium is highly electropositive and oxidizes readily in air; U oxide is therefore the form found most often in nature. Uranium has five natural oxidation states, these are: +2,+3,+4,+5 and +6, where +4 and +6 are most stable. In water, most U is in the hexavalent form (Craft et al., 2004; Bleise et al., 2002). According to thermodynamic calculations, U will commonly occur as uranyl carbonate (anions) in fresh water with pH~7 (Choppin et al., 2002). The toxicity of U in aquifers is dependent on the chemical and physical conditions of the water, for example, U tend to be more bioavailable in soft water with low alkalinity (Sheppard et al., 2004). The U oxide, UO_2, is one of the most common solid forms of U. This has a low point of zero charge at 5.2, which means that the particles will be positively charged in most natural aquifers (Geckeis et al., 2011), UO_2^+ is the most stable species of U in aquifers (Choppin et al., 2002).

Uranium has atomic number 92, and an atomic mass of 238.03 g/mol. There exists 19 isotopes of U and three of these are natural. The three naturally occurring U isotopes are: ^{238}U, ^{235}U and ^{234}U. These isotopes disintegrate through a series of radioactive elements, by emitting α-, γ- and β-radiation (Figure 1). The decay series end in ^{206}Pb and ^{207}Pb, which both are stable elements (Craft et. al 2004). ^{238}Uranium is the most abundant isotope and accounts for 99.3 % of all natural U (mass percentage), it is the isotope with the longest half-life (4.47×10^9 y) and consequently also the lowest specific activity (12 455 Bq/g). ^{234}Uranium is the least abundant of the natural U isotopes (0.006%), but has a shorter half-life (2.46×10^5 y) and therefore also higher specific activity (231×10^6 Bq/g) than ^{238}U. ^{235}Uranium is the isotope that is the second most abundant in nature (0.72 %), it has a half-life of 7.04×10^8 years and has a specific activity of 80 011 Bq/g (Bleise et al., 2002). ^{235}Uranium is the material used in nuclear fuel, due to its fissile properties (Chang 2008).

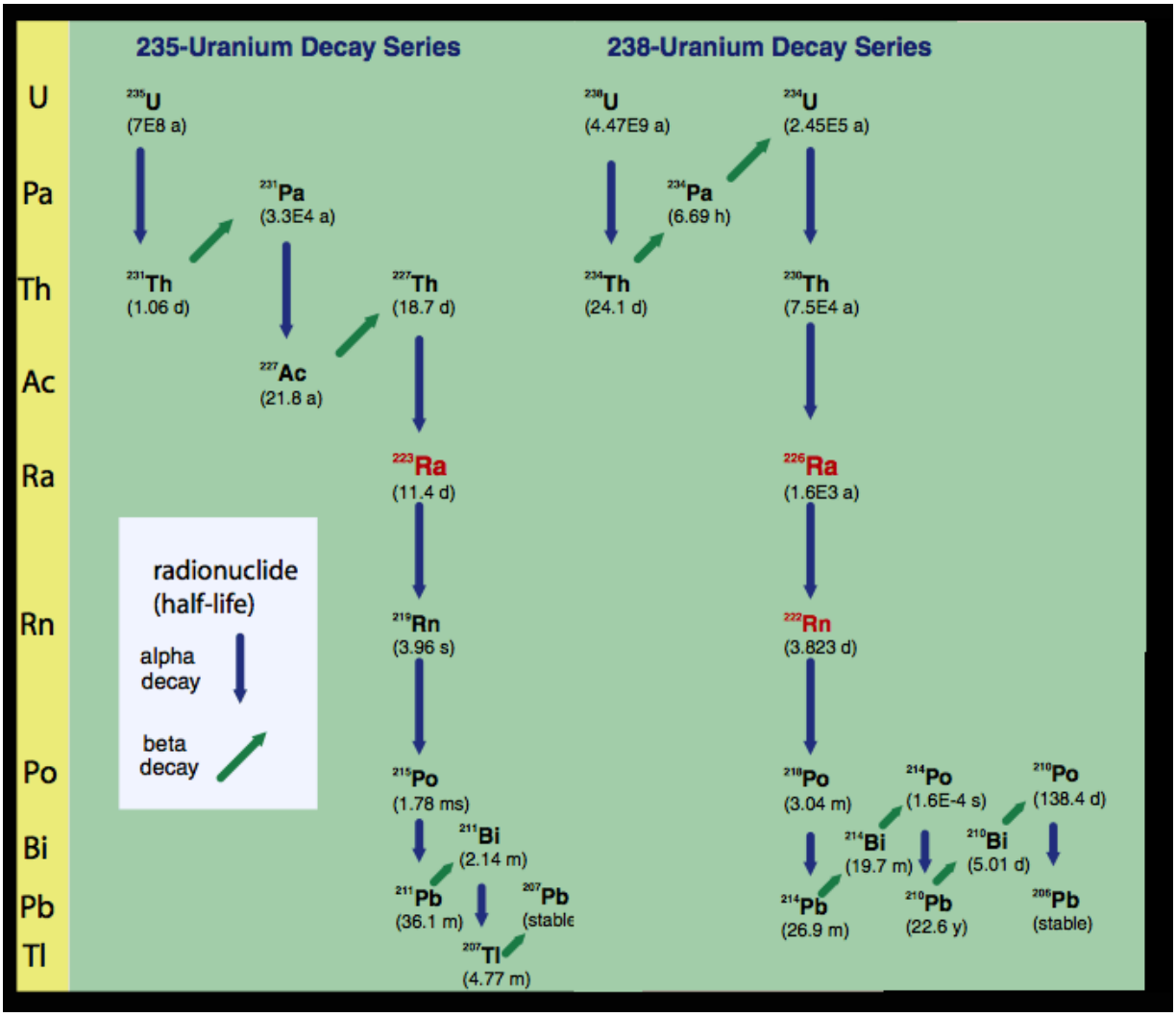

Figure 1: Decay chains of 235-U and 238-U, by alpha and beta decay. Under each element the half-life of the radionuclide is displayed. (Picture altered from USGS)

1.2.2 Depleted uranium and uranium as nuclear fuel.

The dividing of a nucleus into two new isotopes is called fission (Figure 2). Some of the mass in the original nucleus is in this process converted to energy, and this energy is used in both power plants, and nuclear bombs. Fission of ^{235}U can be triggered with high probability following absorption of a neutron and is therefore the isotope commonly used as fuel in nuclear power plants and nuclear bombs. There needs to be a certain relative amount of ^{235}U in comparison to the other U isotopes, to keep a fission chain reaction going in power plants. To get an explosion in nuclear bombs the amount of ^{235}U needed is substantially higher. Natural U is therefore processed to enrich the fuel in ^{235}U before use, commonly to 2-4 % in power plants, and to >90% in nuclear bombs. Because of this, there is a huge amount of U waste enriched in ^{234}U and ^{238}U, called depleted uranium (DU). Depleted U can also refer to spent nuclear fuel. Depleted U is ~40 % less radioactive than natural U.

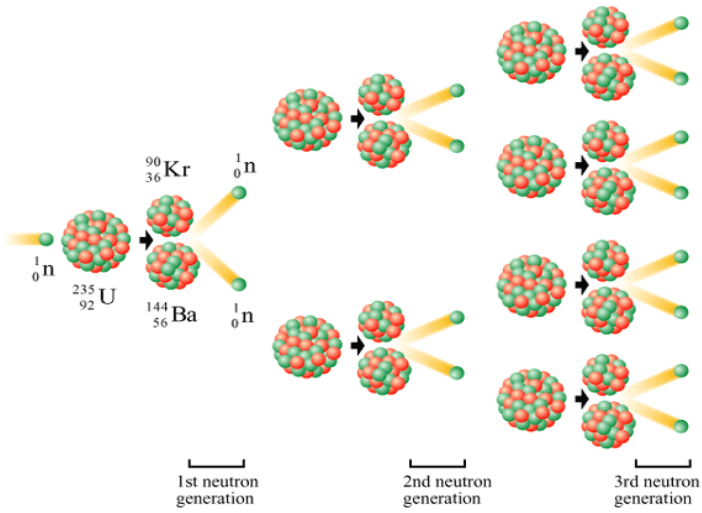

Figure 2: Illustrates the fission of 235-U into 90-Kr and 144-Ba, creating neutrons, resulting in more fissions and a chain reaction. (Picture taken from CUHK)

Depleted U has currently many uses. The high density (19.07 g/cm^3 at 25°C), the low specific radioactivity and the high atomic number makes DU suitable for both civilian (e.g. radiation shielding, counterweight in planes and containers for transport of radioactive matter) and military purposes (e.g. armors and ammunition) (Craft et. al 2004; Bleise et al., 2002). Military use is currently the major source of DU particle discharge to the environment (Lind et al., 2009)

1.2.3 Sources and release scenarios of uranium and uranium nanoparticles in the environment.

The natural abundance on U in the earth's crust is about 0.0003 % (3 mg/kg); it occurs in soil minerals and dissolved in water. Because U readily reacts with air and oxidizes, it occurs mainly as oxides in solid forms. Near U rich ores, aquifers can be contaminated with U, mostly as the aqueous hexavalent form, uranyl (UO_2^{2+}), which is both a mobile and bioavailable form of U. The release is due to weathering of the natural U in the ores, but anthropogenic discharges from the U fuel cycle, can also contribute to the dissolved U in aquifers. Wherever there is dissolved U in the water, natural U NPs can also occur. These NPs are commonly of the form UO_2 (uraninite) where the U is tetravalent, which is much less soluble than the hexavalent form. These NPs are usually produced through a reduction of U(VI) to U(IV), and the reducing agent in this reaction is often microbial (Lee et al., 2010; Bargar et al., 2008). UO_2 can also occur in water through corrosion of old nuclear fuel (Kaminski et al. 2005). If the conditions in the aquifers are highly oxidizing, the UO_2 particles

can be oxidized to U_3O_8. Oxidation is also known to happen to UO_2 fuel in reactors during accidents, creating stable U_3O_8 particles (Salbu, 2000). Particles of various sizes of U_3O_8 and UO_2 also occur in nature as discharge from high temperature events, such as DU used as ammunition (Lind et al., 2009). UO_2 and U_3O_8 have low solubility in water and are often thought of as virtually insoluble (~0.1 µg/l for UO_2) (Lind et al., 2009), however in 2000, Chazel et al. found that the solubility of UO_2 and U_3O_8 increased when the SSA of the particles increased (a ~2.5 fold increase in solubility when the SSA increased ~1.7 fold). This indicates higher solubility in NP's of UO_2 and U_3O_8, due to the large SSA of NPs in comparison to bulk particles.

Several studies have been carried out on bioremediation of U (VI) to the less soluble and possibly less bioavailable U (IV). Lee, Baik, and Choi (2010) did an experiment where *Shewanella putrefaciens* CN32 was used to reduce solved U (VI) to U (IV). The product was characterized with X-ray diffraction (XRD), field emission scanning electron microscopy (FESEM), scanning electron microscopy (SEM) and high resolution transmission electron microscopy (HRTEM). The results of the characterization showed clusters ranging from 50 to100 nm of UO_2 NPs (Lee et al., 2010). Despite the consensus that such NPs exist in the environment there is a lack of knowledge regarding the toxicology and bioavailability of these U NPs. Therefore there is a lack of knowledge about the uptake, bioavailability and potential biological effects of U oxide NPs compared to U ions.

1.3 Mobility and bioavailability

To estimate the transport and mobility of elements in aquifers, the distribution coefficient (Equation 1) is usually applied.

Equation 1

$$Kd = \frac{Cs}{Cw}$$

Where Cs is concentration of the element in the sediment/soil (stationary phase), and Cw is the concentration of the element in water (mobile phase) (Salbu et al., 1998). When the element is on the form of NPs or bound to colloids, the element is not truly dissolved in water, in the sense of forming soluble ionic species, but the NPs can still be transported with the water if it is in suspension. The transport and mobility of elements that do not form soluble

ions in water can therefore be greatly underestimated. It might therefore be beneficial to consider colloidal and nanometer sized particles as a separate phase, in addition to water phase and soil phase, when considering mobility of species in water (Geckeis et al., 2011).

The bioavailability of species in nature depends both on the physico-chemical form and the mobility of the species. Species with low molecular mass (LMM) are said to be more bioavailable than high molecular mass (HMM) species, such as colloids or particles. LMM is the fraction smaller than ~1-4 nm (Figure 3) (Salbu, 2000), this illustration shows the size distribution of different species in the aquatic environment, it also shows the mobilization and growth mechanisms of the species.

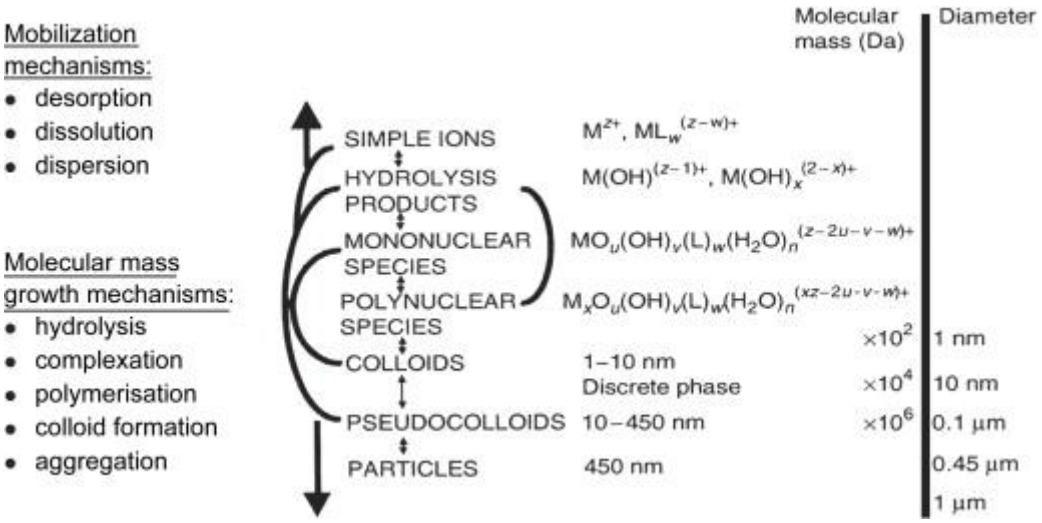

Figure 3: Size classes of elements and nuclides, the illustration also shows growth and mobilization mechanisms. (figure from Salbu (2000))

According to this classification, NPs are in the same size range as colloids and pseudocolloids. Nanoparticles are hence considered HMM, which is the fraction larger than the LMM but smaller than particles (<0.45 µm). LMM species are highly bioavailable because these have the ability to cross biological membranes. HMM species are thought to be biologically inert (Salbu et al., 2004), although recent studies on the bioavailability of NPs suggest that this might not always be the case (Handy et al., 2008). Bioavailability of a contaminant to a given organism in water can be determined by the bioconcentration factor (BCF) (Equation 2). The BCF is the concentration of a contaminant taken up by the organism, versus the concentration of the contaminant in the surrounding water. The BCF is not a constant, but will vary with both physical and chemical factors. The alteration of the BCF, can amongst others, be a result of: varying physico-chemical form of the contaminant, chemical or

physical changes in the water, time of year or life stage of the organism.

Equation 2

$$BCF = \frac{\mathrm{Co}}{\mathrm{Cw}}$$

Where Co is the concentration of contaminant in the organism, and Cw is the concentration of the contaminant in the surrounding water (Salbu, 2000).

1.4 Characterization methods

Information on the physical-chemical speciation of a contaminant is important to predict the mobility (Kd) and the bioavailability (BCF) of a potential contaminant (Salbu et al., 1998). Nanoparticles are in the lower size area of what is classically termed colloids. They are therefore defined as HMM, and should have both low mobility and low bioavailability, but as discussed in the previous section, this might not be the case (Salbu, 2000). The need to characterize NP's, and fractionate them from LMM, particulate and greater (>100 nm) colloidal matter, is therefore important. Several characterization and fractionation methods can be used for this purpose, characterization methods are also valuable for quantitative and qualitative analysis (Table 1).

Table 1: Shows some analytical methods for characterization of NPs (Compiled from Hasselöv et al., 2008 and Handy et al. 2008)

Analytical methods		
Method	**Information**	**Comments**
ICP-MS	Quantitative information. Can also be used for qualitative information coupled with fractionation methods	Samples must be in solution, decomposition can therefore be required. Some NPs will however vaporize in the high temperature plasma. Very low detection limit for most elements.
ICP-OES	Quantitative information. Can also be used for qualitative information coupled with fractionation methods	Samples must be in solution, decomposition can therefore be required. Low detection limit for most elements.
FFF	Diameter, hydrodynamic diameter, size distribution, amount of particles within specific size ranges	Must be coupled with quantitative methods.
TEM	Particle size distribution and shape.	Visualization of particles, often a need for manual measuring of particle size. Good resolution, particles down too ~1 nm can be seen. Unless coupled with EDX techniques, not element/species specific.
Centrifugation	Size distribution (LMM, HMM, colloidal, particulate)	Must be coupled to quantitative methods
DLS	Hydrodynamic radius, particle size distribution and aggregation state.	Particles must be in suspension. The signals can be dominated by the largest particles, and hence measurement of heterogeneous suspensions can be challenging.
Filtration	Size distribution (LMM, HMM, colloidal, particulate)	Must be coupled to quantitative methods

1.4.1 Fractionation techniques

When fractionating environmental samples, the volumes can potentially be large, and many fractionation techniques are available for such purposes. Cross flow ultrafiltration such as hollow-fibers is the preferred method for fractionating particle sizes when the volume is large, and the particle concentrations low, since the techniques reduces clogging. However, all filtration methods can to some degree suffer from clogging; especially when the filters used is on the nanometer scale. Simple syringe filters with higher cut-offs can, on the other hand, be used to filter out the larger particles and aggregates, from small volume suspensions, without too much clogging.

A method of fractionating samples containing NPs, without the need for large water volumes, is centrifugation. Centrifugation separates fractions according to size, shape and density. The separation is accomplished by applying additional g forces to the suspension, causing heavy and large particles to sediment. The cut-off sizes and/or centrifugation time can be calculated using Equation 3 and Equation 4.

Equation 3

$$t = \frac{9}{2} * \frac{\eta}{\omega^2 * rp^2 * (\rho p - \rho v)} * \ln\left(\frac{Rb}{Rt}\right)$$

Equation 4

$$\omega = \frac{2 * \pi * \text{rpm}}{60}$$

Where:

rpm = revolutions per minute

η = viscosity of solution, in g/s*cm

rp = particle radius, in cm

ρp = particle density, in g/cm^3

ρv = solution density, in g/cm^3

Rt = distance from the center of centrifuge rotor to top of solution in centrifuge tube (cm)

Rb = distance from the center of centrifuge rotor to bottom in centrifuge tube (cm)

(OECD, 2000)

By selecting centrifugation time and speed, different fractions can be separated and analyzed individually. Several fractions of one sample can be divided by sequential centrifugation, where large particles are extracted first by centrifugation at low speed. Smaller sized particle fractions can be extracted by centrifuging at higher speeds. Centrifugation is however a somewhat inaccurate fractionation method, as there are many variables to consider. The particles have to be uniform in density and shape to get a clear cut-off. Information about chemical and physical properties of the solvent as well as the properties of both the centrifuge and the rotor is also required. When using centrifugation as a fractionation technique the particle concentration in the solution cannot be too great, as this will induce aggregation. Larger particles and aggregates can also induce co-precipitation of smaller particles as they sediment.

Another mean of fractionating small volume samples is membrane ultracentrifugation. In this method, the sample to be fractionated is added to a centrifuge tube, containing a membrane filter. This membrane filter can have several cut-offs, depending on the fraction to be analyzed. The tube, containing the sample, is centrifuged. The fractions smaller than the cut-off size of the filter will cross the membrane along with the solvent. The fractions larger than the cut-off size will however be retained by the filter, and this is separated from the

suspension.

1.4.2 Quantitative and qualitative analysis

Information about particle size and shape can mainly be found by microscopy or light scattering techniques. Size distribution can also be found indirectly by fractionating the sample according to size, and analyzing the fractions by quantitative methods. Because microscopes must have very good resolution to be applied to NP samples, the selection of microscopes that can be utilized is limited. Transmission electron microscopy (TEM) is, however, a microscopy method with satisfying resolution to characterize NPs. In a TEM microscope, 5-100 nm of sample must first be prepared on a sample holding grid. In the microscope, the sample grid is bombarded with an electron beam and the electrons transmitted through the sample are the basis for the image. The electrons that are not scattered or absorbed by the particles in the sample are focused on an image detector beneath the sample, usually a fluorescent plate. The resulting image is a function of both the particle distribution and the sample thickness (Reimer & Kohl 2008). Previous studies on synthesis of U NPs and comparison of TEM and DLS (diffractive light scattering) analysis showed that TEM gave far more reliable particle size estimates (Wurgie, unpublished results), hence a combination of TEM and filtration methods were chosen for this study.

Inductively coupled plasma mass spectrometry (ICP-MS) is a quantitative multi element detection method well suited for accurate quantification of heavy elements. The ICP-MS measures the mass over charge (m/z) ratio of an element, and the total content of any given element (ion) in a solution. The solution is decomposed, atomized and ionized in argon plasma, before it is separated according to m/z in the mass spectrometer. The ions are detected by an electron multiplier (Agilent technologies, 2005).

Another ICP method for quantifying elements is optic emission spectrometry (ICP-OES). As in the ICP-MS the solution is decomposed and atomized in the plasma, the elements will be present as single atoms or ions[1]. Because of the extreme heat (up to 10 000 K) in the plasma some ions and/or atoms will excite, when the ions returns to ground state, the atom/ion will send out energy in the form of a photon. The wavelength of this photon is unique for the

[1] This can be regulated by regulating the heat in the plasma, depending on what is to be measured on.

atom/ion in question and by detecting the amount of photons with the desired wavelength, the concentration of the atom/ion in question can be determined (Perkin-Elmer, 1997).

When performing quantitative analysis several uncertainty factors can occur during preparation and the actual analysis. Loss of analyte during preparation, or different behavior of sample solution and standard solutions, might result in wrong quantitative measurements. To prevent this, internal standards (IS) are often added to both sample solutions, blank solutions and standard solutions in equal amounts. An internal standard is a non-analyte isotope that has a relatively similar atomic mass and ionization potential as the analyte. When an internal standard is added, the concentration of the analyte is always measured relative to the IS. If some analyte is lost during preparation, or sample introduction, the same amount of IS will also be lost, and the error will be corrected for.

1.5 Aquatic environment and fish

Run-off from domestic, anthropogenic and natural sources ultimately ends up in the aquatic environment. This run-off can contain contaminants off different sorts, including colloids and NPs (Baun et al., 2008). Aquatic environments can also be subject to direct release of contaminants. This makes the aquatic environment a significant sink for toxins and pollutants. Research on contaminant speciation, mobility, uptake and toxicity is therefore especially important (Van der Oost et al., 2002; Farré et al., 2009). In situ toxicity testing in freshwater lakes has also the advantage that the ecosystem is fairly enclosed, this makes it easier to know when and what the organisms has been exposed to. Because of this, various types of fish are used in both toxicity, behavior and uptake studies, in situ and at lab/in vitro. In Norway, one of the most relevant species to study is the Atlantic salmon (*Salmo Salar*), as this is an important species in the Norwegian aquatic environment. Atlantic salmon is also of great economic interest as Norway is the largest exporter in the world of Atlantic salmon (LaksefaktaTM, 2011).

1.5.1 Fish egg

To understand the routes and mechanisms for the uptake of contaminants in fish eggs, information on structure, construction and development of the eggs are important. Atlantic salmon eggs are enveloped by a thick membrane called chorion. Through this goes a narrow channel to the plasma membrane of the egg (micropyle) (Figure 4), which is used by the

spermatozoa during fertilization (Yanagimachi et al., 1992). Chorion pore canals also cross the two external layers of the chorion, the canals start on the outside of the egg shell (Fausto et al., 1994). When Atlantic salmon eggs are placed in freshwater after fertilization, the eggs become 'water-hardened' after some time (Figure 4). The permeability to water decreases and the eggs become almost impermeable to water solutes when the 'water-hardened'-state is reached. Water can, however, permeate the chorion during this condition (Potts & Rudy JR., 1968). It is possible to carry out ecotoxicological studies on a number of different fish species and life-history stages. Studies on eggs have advantages in terms of the higher throughput of studies, lower volumes of contaminated water and ethical issues of using live species.

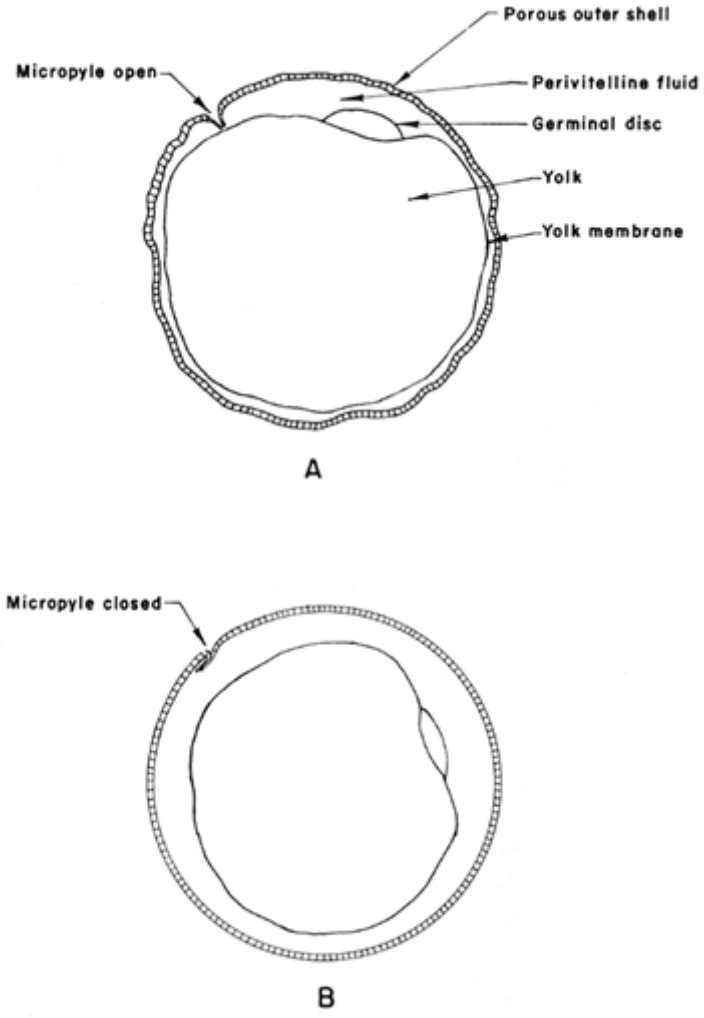

Figure 4: A- Newly-taken trout egg, outer shell not firm and mycropyle channel is open. B- Egg after water-hardening, micropyle closed. (Leitritz, 1959)

During swelling, fish eggs absorb surrounding water and are hence more permeable to substances solved or dispersed in this water (Jezierska et al., 2008). This might in turn lead to the absorption of metal ions, or metal NPs, such as uranyl, UO_2 or U_3O_8. Several experiments

have been carried out on the uptake and toxicity of metals in fish eggs. Jezierska et al., (2008) exposed *Cyprinus carpio* eggs to copper during swelling and detected up to 40 % reduction in the swelling of the eggs exposed. Another fish egg experiment conducted with eggs from *Pimephales Promelas* by Beniot and Holcombe (1978), showed a change in the egg surface after zinc exposure. Beattie and Pascoe (1978) exposed *Salmo Gairdneri* eggs to 10 mg/l of cadmium for 22 hours; they found that 98 % of the cadmium taken up by the eggs during this time was deposited in the chorion or the egg membrane. A similar result was found when Michibata (1981) exposed *Oryzias Latipes* eggs to cadmium; 94.4 % of the cadmium in these eggs was also found in the chorion. Lee et al. (2007) did an experiment exposing embryos of zebra fish to 5-46 nm sized silver NPs and found that the NPs were transported both actively through the chorion pore canals, and inactively as Brownian diffusion into the eggs.

1.6 Objectives

The overall aim of the study was to synthesize and characterize U NP and to utilize these in biotest experiments to identify if U NP was able to cross biological membranes. Two types of U NPs (UO_2 and U_3O_8) were synthesized according to a modified version of the method used by Wang et al. (2008). These synthesized NPs were characterized both quantitatively and qualitatively using various fractionation methods, spectrometric methods and microscopy. In the first part of the experiment the aim was to see the size distribution of the synthesized particles and the quantity of the synthesized material. Two different purification procedures were used; the products from both of these procedures were later characterized to find the most suitable method for obtaining particles in the nanometer range. Transmission electron microscopy (TEM) with and without an emulsifying agent (Tween 20) were used to characterize the NPs, to see whether this prevented aggregation of the particles.

Following the characterization, the NPs with the smallest size distribution were used in a fish egg exposure experiment with the aim to compare U uptake in fish eggs exposed to U NPs (UO_2 and U_3O_8) and U ions, respectively. The main hypotheses of the study are:

- Uptake of U ions (uranyl) will differ from that of U NPs.
- Uptake of different U NPs will vary with chemical form.

To distinguish between U actually taken up in the eggs with that first deposited on the shells, samples of whole eggs as well as the eggs interior were taken. Exposures took place over 24 hours, just after fertilization, which is the period where the eggs swell, and take up much of the surrounding water. The eggs was then moved to clean water for a depuration period of 7 days, to see whether the eggs continued to take up the U deposited on the shell. The depuration also determines how much and how fast the eggs can dispose of the U taken up. Finally, the exposure solutions also underwent more thorough characterizations so as to know exactly what the eggs were exposed to.

2 Materials and methods

All water used in this experiment was purified with a Milli-Q water purification system. The water used for the exposure experiment was synthetic Maridalsvann, based on the composition of a well-characterized natural lake North of Oslo, and used in many previous studies (Table 2). The complete process of treatments to the synthesized U particles is illustrated (see Figure 5).

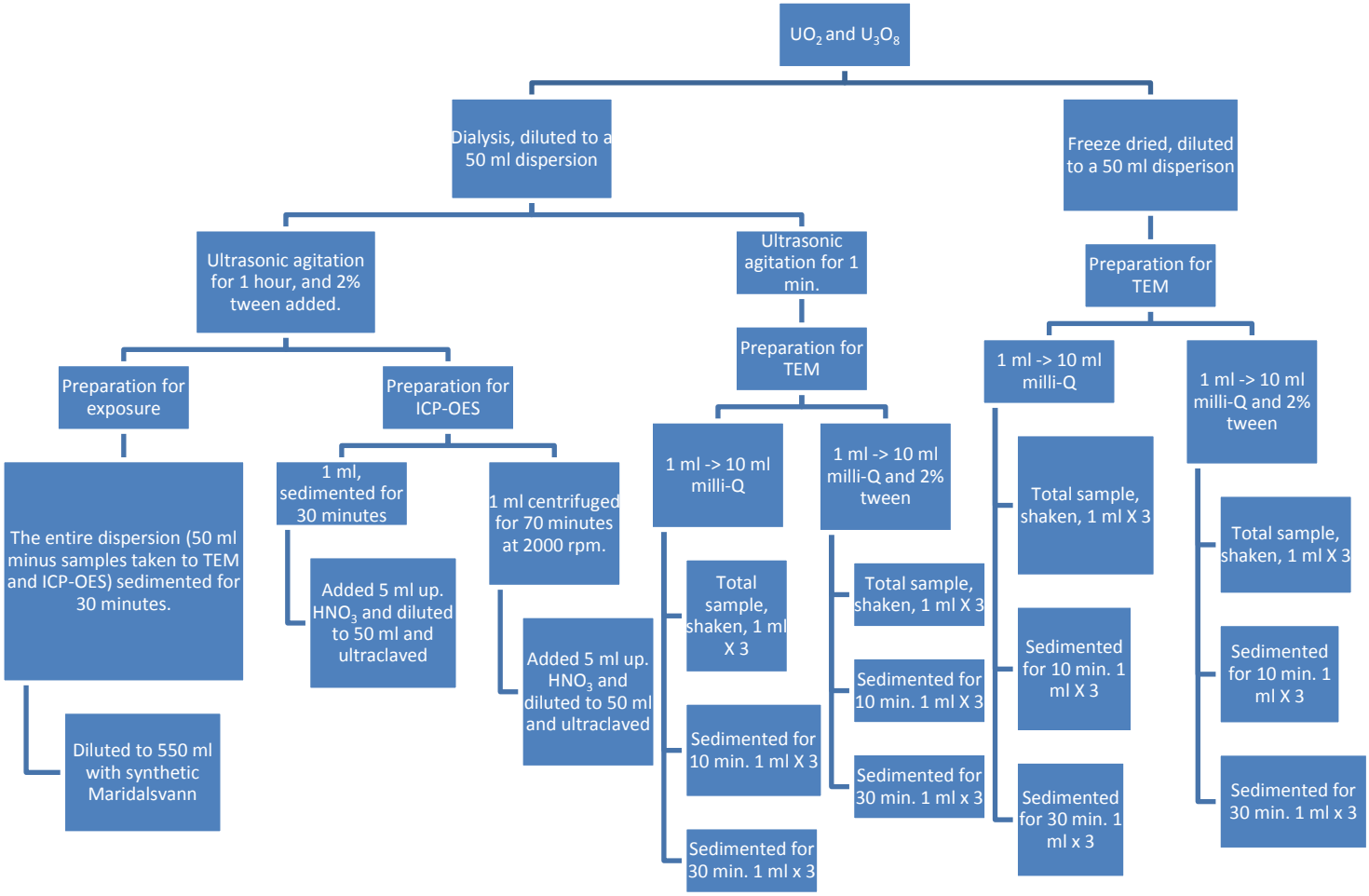

Figure 5: Illustrates the treatment and procedures performed on the UO_2 and U_3O_8 NPs after synthesis.

2.1 Synthesis of uranium oxide nanoparticles (UO2 and U3O8) and characterization of the products

For synthesis of the U oxide NPs, an UltraClave technique based on a procedure published by Wang et al., (2008) was used. The UltraClave is a microwave technique used to decompose samples, and is operated under high pressure to obtain high temperatures. The electric field made by the microwaves, causes the ions and dipole molecules to migrate and vibrate, and this induces friction heat. In the UltraClave, the samples are placed in a load, with distilled water, sometimes containing sulphuric acid and hydrogen peroxide. Acid is added to get more ions into the load, and hence increase the temperature. Hydrogen peroxide is added to reduce potential nitrous gases. It is the load that is heated by the microwaves, and the samples are again heated by the load. This ensures the same temperature and pressure in each sample container and in different runs.

The procedure used for synthesis of the U oxide NPs was adopted from Wang et al. (2008) and slightly modified. Into two Teflon-vials (35 ml sized, constructed for use in the UltraClave) 0.22 g (0.5 mol) of uranyl acetate dihydrate (UO2(CH3COO)2·2H_2O, purity ≥98.0 %, specific activity 1.459×104 Bq/g, 58.5 % of the radioactivity from natural U, Fluka, Sigma-Aldrich, Buchs, Switzerland) and 15 ml of Milli-Q water was added. The mixtures were stirred to homogeneous solutions with a magnet stirrer. To one vial, 5 ml (0.075 mol) of ethylenediamine ($C_2H_8N_2$, Fluka analytical, purris p.a. Absolute, assay spec ≥99.5 %) was added, whilst 14.27 ml (0.075 mol) tripropylamine ($C_9H_{21}N$, Sigma Aldrich, Aldrich Chemistry, assay spec ≥98 %) was added to the remaining vial. The solutions were continuously stirred while adding the amines, the stirring continued for ten minutes after the addition. The vials were then put into an UltraClave (Milestone) for 48 hours at 160°C (Appendix 1.1). After the solutions were cooled, the product from each vial was transferred to centrifuge vials, and centrifuged at 4000 rpm (~1700 g) for 1 hour. The supernatant was then removed and the precipitates was washed with ethanol (Absolutt alkohol prima, Kemetyl Norge AS) and centrifuged again for 45 minutes at 4000 rpm, this was repeated three times. Half of the products were freeze-dried, to dry off the ethanol (which was the final step of the Wang et al. (2008) procedure), while the other half was rinsed further by dialysis.

2.1.1 Dialysis

Half of the product was further purified by dialysis to remove remaining ions and ethanol. The dialysis was chosen as an alternative to freeze-drying, due to concerns about being able to resuspend the freeze-dried product in the lake water for the exposure experiments. During dialysis, the product is kept in suspension through the whole procedure, and hence never dried; as is the case when freeze-drying the product. The dialysis procedure was carried out as described on the user manual for the dialysis membranes (Spectra/Por® 3 dialysis membrane, Spectrum laboratories, Inc.). The dialysis membrane tubes, with a cut-off of 3 kDa, were cut into proper pieces (~30 cm) and soaked in Milli-Q water for 30 minutes; the tubes were then rinsed through by Milli-Q water. The tubes were tied at one end, and the NP solutions were added, then the other ends were tied as well to enclose the NP suspensions, some air remained inside to keep the tube afloat. The dialysis tubes were soaked in two large beakers, containing Milli-Q water, and the solutions were stirred with a magnetic stirrer for five days (Figure 6). Water was changed five times during the dialysis cleaning and the final products were suspended in 50 ml Milli-Q water for further characterization.

Figure 6: Dialysis bags with NPs in large beakers containing Milli-Q water placed on magnetic stirrers.

2.1.2 Characterization of synthesized particles

Both quantitative and qualitative analysis was performed to characterize the particles after synthesis. The aim was here to characterize the size after different treatments, to determine which treatment would give the suspension with the desired quality. Quantitative analysis was carried out to find the U concentration of the suspensions after synthesis and purification.

Transmission electron microscopy (TEM)

The purified particles (dialysis and freeze-drying, respectively), were dispersed in 50 ml of Milli-Q water. These two suspensions were further diluted and prepared for TEM. The preparation and characterization was carried out after different settling times (0, 10 and 30 min), to see which would give the preferred cut-off size for the particles. From each fraction, 2x1 ml samples were prepared for TEM. Tween 20 was added to one of the replicates (to 2 %) and all samples were filled with Milli-Q water to 10 ml (Figure 5). Tween 20 is an emulsifying agent added to prevent aggregation of particles. With automatic pipettes 4x5 µl of each prepared suspension was dropped on separate copper grids with Formvar carbon films (100 mesh copper Formvar carbon 50ct, Chemi-Teknik as). This was allowed to dry under a lamp between each application. With the TEM (Morgagni 268), several images with different magnifications were produced. The size of the particles in the TEM images was measured manually.

Inductively coupled plasma- optic emission spectrometry (ICP-OES)

Two fractions of the dialysis cleaned suspensions were analyzed by ICP-OES (see Figure 5). The two fractions were:

1. Supernatant, after the suspension was allowed to sediment for 30 minutes
2. Supernatant, after centrifugation in eppendorf tubes for an hour, to get a cut-off of 100 nm by design, the speed was calculated with Equation 3 and Equation 4.

The fractions were decomposed by the UltraClave (Appendix 1.1) before ICP-OES analysis. Uranium was quantified using both radial and axial analysis, and with two different wavelengths (385,958 nm and 409,014 nm).

2.2 Fish egg exposure

The fish eggs (delivered by Aquagen) were fertilized by mixing the fertilizing milk (spermatozoa) and the eggs. Immediately after fertilization the eggs were moved to the exposure beakers, and the exposure started. The eggs were stored in a climate refrigerator keeping a temperature of 5-6ºC, during the whole experiment, to get the most relevant exposure in comparison to conditions in nature.

2.2.1 Preparation

The suspension cleaned with dialysis containing Tween 20 was the suspension with the preferred size distribution and therefore used for the exposure experiments. This was decided by studying the TEM images of the suspensions given the various treatments. The suspensions were subjected to ultrasonic agitation for an hour, to dissolve the aggregates. Because too much U material was lost during centrifugation (section 2.1.2 and 3.1.2), the supernatant from the 30 minutes settling was used (see Figure 5). This still contained an acceptable concentration of particles with diameters between 10-100 nm.

For the exposure solution, synthetic Maridalsvann was used. This was made by mixing six different kinds of salt (Table 2) with distilled water. The water was pH adjusted with 0.1 M HCl until pH 7.

Table 2: Contents of the synthetically made Maridalsvann, table shows: type of salt, molecular weight of the salt, amount added to the water and additional product information of the salts.

SYNTHETIC MARIDALSVANN				
Salt		Molecular weight salt (g/M)	Amount salt (mg/L)	Product information
KCl		74.56	0.629306	Pro analysis, 99.5%, Merck, Struers Kebolab
MgCl2	MaCl2* 6H2O	203.3	3.262168	99%, Riedel-de, Haën
CaCl2		147.02	1.872374	--
CaOH2		74.09	2.275479	Pro analysis, 96%, Merck, Struers kebolab.
Ca(NO3)2	Ca(NO3)2* 4H2O	236.15	3.999057	98.4%, VWR, prolab BDH
NaSO4	Na2SO4* 10 H2O	322.19	11.07145	Merck, Struers kebolab

2.2.2 Experimental setup

The exposure type and technical details of the exposure setup can be viewed in Table 3. Each experimental replicate was conducted in a 300 ml beaker containing 150 ml of the exposure media and approximately 70 fish eggs (Figure 7). The eggs were exposed during swelling, for 24 hours, before they were moved to clean water. The depuration period in clean water lasted for 7 days. Egg samples from exposure number 1 through 6 (Table 3) were taken after 3, 24, 96 and 192 hours after start of experiment. For exposure number 7 (Table 3), egg samples were only taken after 24 hours. For each sample, from each exposure beaker, 3 eggs were taken for total analysis, and 3 eggs were taken for dissection (using a syringe). Water samples for full characterization were taken before and after the exposure period, samples for total quantitative analysis were also taken after 96 and 192 hours. Units containing only the two U NP suspensions and no eggs (samples 3 and 5), were included to study the stability of the U NP in the exposure media. A control unit using only Tween 20 was included to check for any impact of the additive on fish egg development. Three eggs were also taken from each exposure beaker after 24 hours for light microscopy to control the fertilization degree and the development of the eggs.

Table 3: Table shows the different exposure media added to the exposure beakers, it shows the approximate U concentration of each beaker, the approximate amount of fish eggs in each beaker and the number of replicate beakers.

Number	Exposure type	No. of replicates	Nominal U concentrations (mg/l)	Eggs
1	Control	3	0	~70*
2	U3O8-NP	3	~50*	~70*
3	U3O8-NP	1	~50*	0
4	UO2-NP	3	~50*	~70*
5	UO2-NP	1	~50*	0
6	Uranyl (U-ions)	3	~50*	~70*
7	Control with Tween	3	0	~70*

**Accurate concentrations and numbers are given in results.*

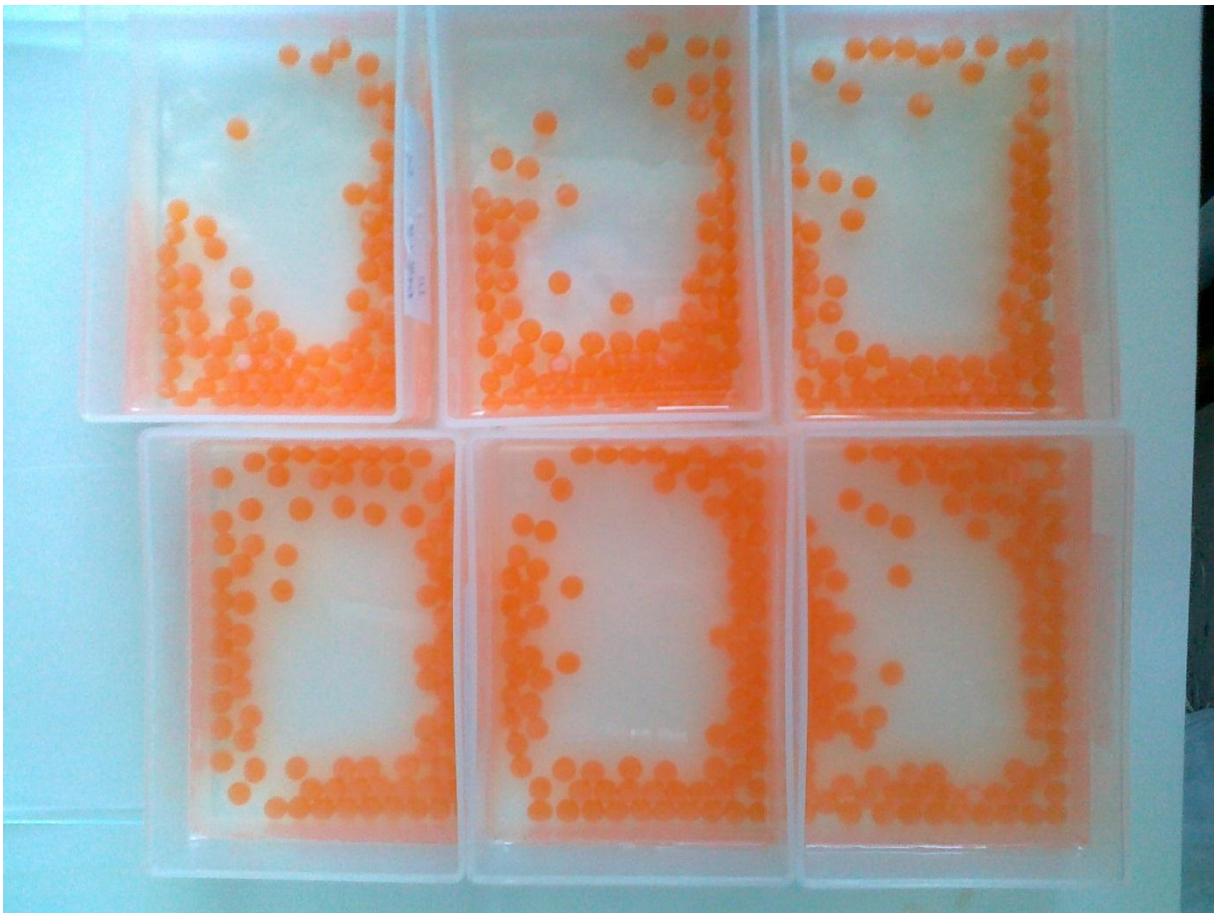

Figure 7: Exposure beakers after the addition of exposure solution and fertilized fish eggs.

2.2.3 Water characterization

Water samples from the egg exposure experiment were characterized to determine the concentration and the size of the particles, to which the fish eggs were exposed. Samples taken before and after the 24 hour exposure were fractionated according to size, using both filters and centrifugation. The first cut-off was 0.2 µm, which ensured that all particulate matter and aggregates were removed from the suspension (Table 4). This cut-off was acquired by filtering the suspension through a syringe membrane filter (25 mm syringe filter w/0.2 µm cellulose acetate membrane, VWR international). The filter was first conditioned by filtering 1 ml of solution which was discarded before the sampling. Non-dissolved matter was removed using two methods; simple high speed centrifugation (30 000 rpm; ~93 000 g) for 1 hour and membrane ultracentrifugation (3 kDa, Amicon ultra, ultracel®-3 kDa, regenerated cellulose, Millipore). The membrane centrifugation tubes were conditioned by using 1 ml of the sample solution and centrifuging (7500 rpm; ~5900 g) for 10 minutes. After the conditioning, the tubes were emptied and 4 ml of sample was added to each tube, before centrifuging at 7500 rpm for 40 minutes. Only total fractions were analyzed for depuration

water samples (96 and 192 hours). To prepare the water samples for the ICP-MS analysis, 0.5 ml of each sample were added 0.75 ml HNO_3 (69 % sub boiled ultrapure), 75 µl internal standard (IS) and diluted to 15 ml with Milli-Q water.

Table 4: Expected cut-off diameter of particles in suspension by different fractionation methods.

Cut-off	Method	Fraction
Total	No preparation	Particulate, colloidal, molecular, NP, (HMM and LMM)
<0.2 µm	Syringe membrane filtration	Some colloidal, NP, molecular (LMM, some HMM)
<~1-4 nm	Membrane ultracentrifugation or high speed centrifugation	Molecular (LMM)

Some water samples were also analyzed by TEM, using the same preparation procedure as described in section 2.1.2. These were UO_2, U_3O_8 and uranyl; total exposure solutions before and after exposure and <0.2 µm fraction before exposure. The size of the particles in the TEM images was measured manually.

2.2.4 Egg characterization

At each sampling point, three eggs from each replicate sample were taken out for total analysis. Three more eggs were sampled for dissection. The dissection of the eggs was performed using a syringe to extract the content inside the eggs (Figure 8). The exterior shell was discarded and the U content was analyzed. The eggs and the syringe extracts were weighed, resulting in the U quantification being per weight[2] unit, rather than per egg. After the eggs were weighed, 1 ml of HNO_3 (69 % sub boiled UltraPure) was added and the eggs were allowed to dissolve in this overnight. Before they were decomposed in the UltraClave (Appendix 1.1), 1 ml additional U.P. HNO_3, 1 ml Milli-Q and 100 µl IS was added. With each fortieth sample decomposed and analyzed, three blank samples containing the same matrix as the egg samples excluding the egg (2 ml U.P. HNO_3, 1 ml Milli-Q water and 100 µl IS), were also decomposed and analyzed. Three replicate samples (~0.2 g each) of certified reference material (CRM) were also decomposed and analyzed the same way as the eggs. This reference material refers to radionuclides in spinach (IAEA, 2009) containing a known amount of U activity per gram. The U concentrations in CRM analyzed were calculated from this (Appendix 2.3). The decomposed egg solutions were diluted to 20 ml before they were analyzed by the ICP-MS.

[2] Wet weight.

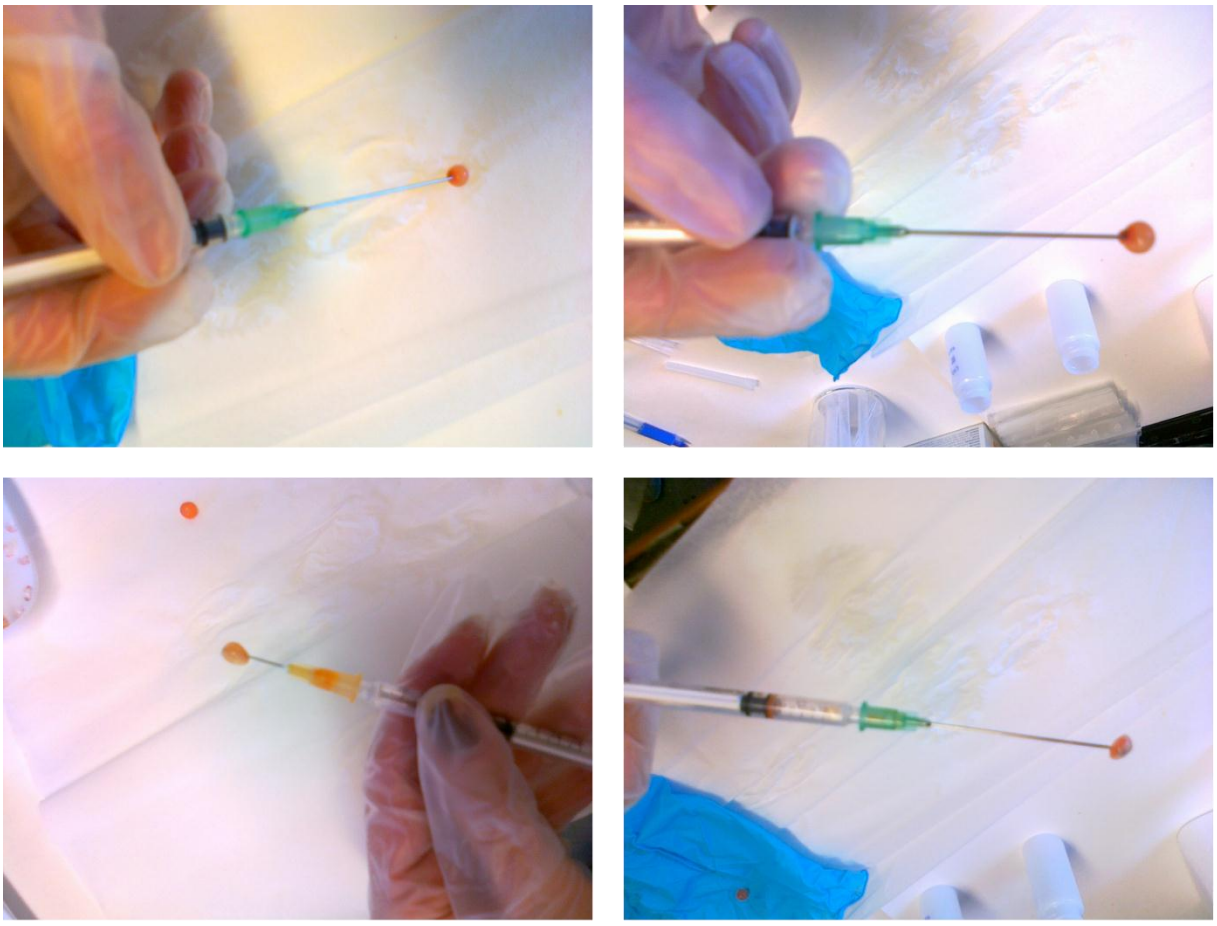

Figure 8: Dissection of fish eggs using a syringe.

2.2.5 ICP-MS

Standards containing the same concentration of IS and ultrapure HNO_3 as the samples, were made with 0, 20, 100 and 500 µg U/l. These were used to create a standard curve, for which the U concentrations of the sample solutions were measured against. The 20 µg/l standard was also measured between every tenth sample, to control for possible drift in the U measurements.

The ICP-MS used was a Perkin Elmer Sciex Elan 6000. After the instrument was started; the system was flushed with helium, to remove oxygen. A solution containing relatively high content of a variety of different elements (70-80 µg/l of Be, Mg, Co, Ni, In, Ce, Rh, Pb, Bi, U, Ba, Th) called "dual detector calibration", was used to calibrate the relation between the two detectors in the instrument to get a linear correlation between these. A "daily performance solution" containing the same elements, with a concentration of 10 µg/l, was used to calibrate the ionic lenses in the instrument before the samples were analyzed.

3 Results and discussion

3.1 Characterization of synthesized material

The first characterization of the synthesized material was carried out to get an overview of the particle size distribution, particle shape and concentration of the synthesized material. The results from this characterization were mainly used for selection and preparation of materials for further work. It was, however, evident from this characterization that the synthesis of two different kinds of U NPs was successful.

The U particles synthesized in the present work have not yet been characterized with respect to oxidation state and crystallographic structure. However, bulk samples of U particles produced according to Wang et al. (2008) in previous syntheses at UMB were determined by means of micro x-ray diffraction (µ-XRD) and quantitative micro x-ray absorption near edge spectroscopy (µ-XANES) to be UO_2 (Lind et al., 2010) and U_3O_8 (Lind O.C., pers. comm.), respectively. Based on these results the U NPs used in the present work are termed UO_2 and U_3O_8 pending further analysis.

3.1.1 TEM

Synthesized U particles, cleansed and fractionated coarsely by 30 minutes settling to remove the largest particles, were analyzed by TEM. This image show dialyzed and freeze-dried UO_2 and U_3O_8 NPs (Figure 9).

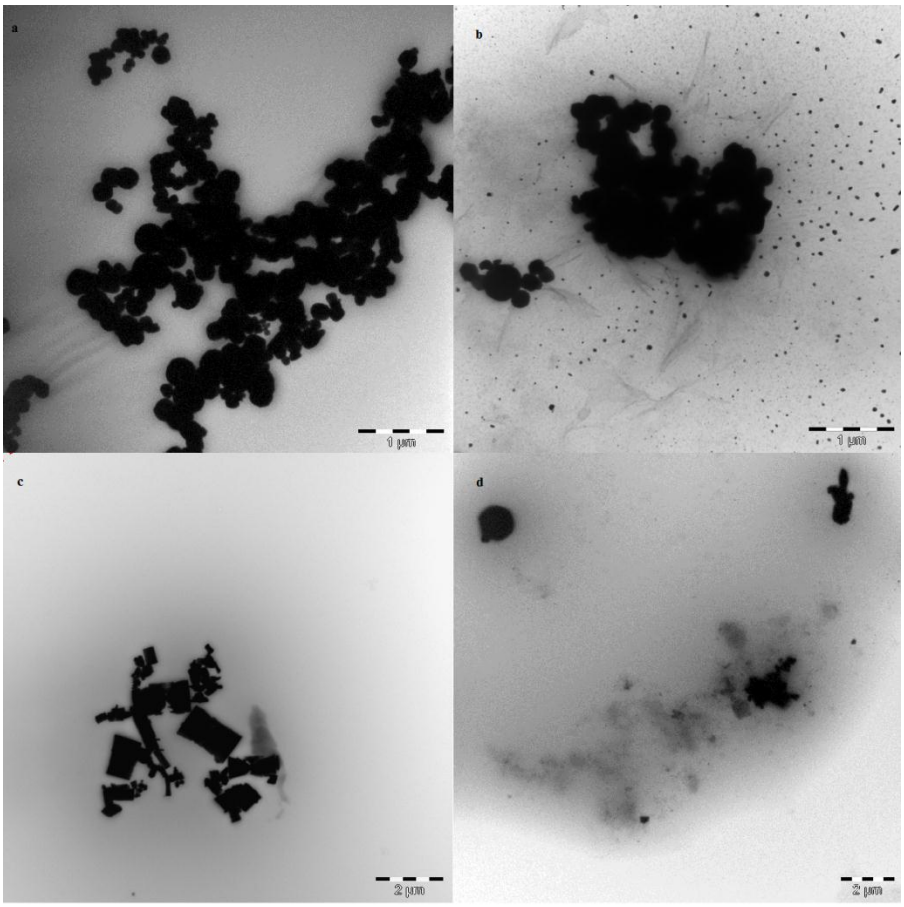

Figure 9: TEM images of the stock suspensions after NP synthesis and cleaning, all the suspensions in these images are supernatants of sedimented suspensions (30 minutes sedimentation) added Tween 20. a- Shows the UO_2 suspension cleaned by freeze-drying and b- shows the UO_2 suspension cleaned by dialysis. c- Shows the U_3O_8 suspension cleaned by freeze-drying and d- shows the U_3O_8 suspension that was subjected to dialysis. For a systematic description of pretreatment, see Figure 5.

All the TEM images (Figure 9) were focused on aggregates of U particles, but even more important and interesting is the single small particles that could be seen in the two rightmost images (Figure 9) (dialysis cleaned suspension). These small particles suggest the presence of many NPs between 1-100 nm in diameter.

The TEM images of the U_3O_8 suspensions (Figure 9 c and d) show that U_3O_8 material tend to grow into rectangular rods, while the UO_2 particles (Figure 9 a) appears mostly as spherical particles. The images show that the suspensions cleaned with dialysis had many single small particles surrounding the aggregates and hence probably contained the particles with the

desired size distribution. These small single particles were found evenly distributed through the dialysis cleaned suspensions. The suspensions cleaned by freeze-drying had, on the other hand, considerably fewer of these single small NPs than the dialysis cleaned suspensions (Figure 9). The particles also tend to aggregate, however, in the suspensions where there were more single particles; these tend to be smaller than the average particles bound in aggregates (Figure 9 b).

A selection of the particle sizes was measured; and the average size and standard deviation of the particle suspensions was calculated (Table 5). Particles measured included both single particles in suspension and easily distinguishable particles that was part of an aggregate. Fewer particles from the freeze-dried suspensions were measured than from the dialysis treated suspension, this was because fewer single particles could be distinguished from the larger aggregates in these suspensions.

Table 5: Calculated average size and standard deviation of the synthesized U particles. The suspensions characterized for these calculations were dialysis or freeze-dried, added Tween 20 and sedimented for 30 minutes. The sizes were estimated from observations of particles in TEM images.

		Average Size (nm)	Standard deviation (nm)	n
Dialysis	**U_3O_8**	158	200	74
	UO_2	92	85	221
Freeze-dried	**U_3O_8**	530	260	17
	UO_2	220	138	24

Since n was not sufficiently high and the size distribution was large, the standard deviation was too great to conclude anything about the average size with any statistical significance. Nevertheless, there was still a good indication that there was a sufficient amount of NPs in the water and Tween 20 suspensions subjected to dialysis. This suspension also had considerable amounts of aggregates, but distinct particles of 15 to 300 nm were still observable in the aggregates and were included in the size distribution estimates. In addition to this, single NPs between 1-100 nm were evenly distributed through the suspension. The mean particle diameter was also smaller for the dialysis treated suspensions than for the freeze-dried particle (92 vs. 220 nm for UO_2 and 158 vs. 530 nm for U_3O_8), as should be expected. The dialysis cleaned particles were therefore used further in the egg exposure experiment, after the larger aggregates were subjected to ultrasonic agitation for dispersion purposes.

The reason why the dialysis cleaned suspension contained smaller particles than the freeze-

dried suspension might be because these particles were held in suspension through the whole experiment. The freeze-drying forced the particles to lump tightly together, which should induce aggregation. The chemical and physical environment surrounding the particles was also changed considerably during this process (e.g. temperature and humidity). Some of these changes might have induced the particles to form bonds and bridges between each other, which might not break when resuspended. Even though the bulk aggregates were on the micrometer scale, they might still have NP behavior, as long as the entities in the aggregate/agglomerate were on the nanometer scale (Handy et al., 2008). This means that experiments carried out with the dialysis treated suspension were still classified as NP studies, because most of the primary particles in the aggregate/agglomerate were small enough to be termed NPs.

3.1.2 ICP-OES

The ICP-OES (Perkin-Elmer, Optima 5300 DV) analysis performed before the exposure experiment was carried out to get an indication of the U concentration in the supernatant following 30 minutes sedimentation and centrifugation, individually. This was also carried out to be able to produce exposure solutions with desired concentration. Due to the fact that all exposure solutions were thoroughly characterized before exposure, the ICP-OES analysis of the stock solution was only carried out to give a rough estimate of the concentration, and with no replicate samples (Table 6.)

Table 6: Results from analysis of stock suspensions containing synthesized U particles. The analysis was performed using ICP-OES.

	Sedimented for 30 minutes		Centrifuged 2000 rpm for 70 min	
	UO_2 suspension (mg U/L)	**U_3O_8 suspension (mg U/L)**	**UO_2 suspension (mg U/L)**	**U_3O_8 suspension (mg U/L)**
Average	531	546	23	17
STD	1.8	0.7	1.4	1.0
LOQ	0.20	0.20	0.20	0.20
LOD	0.060	0.060	0.060	0.060

Because the average U measured were well above the limit of detection (LOD) and the limit of quantification (LOQ) for U, the U in the suspensions could be determined with reasonable degree of confidence (Table 6). Because the U concentration in the supernatant from the centrifugation was only 3-5 % of the U concentration in the supernatant from the sedimentation, it was decided to use the sedimented supernatant for the exposure experiment (Table 6). This was probably a result of aggregation induced by centrifugation (Hassellöv et al., 2008).

3.2 Statistics

Minitab and excel was used to perform the statistical calculations and analysis on the data in this experiment. First a normality test was conducted on the results acquired from the ICP-MS analysis, to find out if the data had a normal distribution. It was found that almost all replicates from each variable had a normal distribution. The exception was the U concentrations in the control eggs not containing U and the size distribution of the synthesized U particles. The reason for this can be that the measured U concentrations in these solutions were low and small variations in measured concentration could make the statistical distribution fluctuate from normal distribution. Because n in this experiment is small (3 for water samples and 9 for egg samples), small variations in measured concentration could have great influence on both the normal distribution and the standard deviation.

Because most of the data matched a normal distribution, an ANOVA one-way variance analysis was conducted, the results from this is presented in graphs with 95 % confidence intervals (CI). The 95 % CI means that there is a 95 % chance that the real concentration or size is in the CI. This also means that there is a 5 % chance it is not, and hence a 5 % chance that the conclusion based on such an analysis is wrong. This analysis also forms the basis for discerning whether or not there is significant difference in U concentration of the different groups.

3.3 Characterization of exposure water

The results from the water samples analyzed were also processed statistically with Minitab. The average of the different measurements used to make the graphs and statistical analysis in this section can be seen in Appendix 2.1. The synthetic Maridalsvann used in the experiment was adjusted to pH ~7 before adding eggs and U.

3.3.1 ICP-MS analysis of total uranium concentrations in exposure water

A graph showing mean total U concentration in exposure water with a 95 % CI was created (Figure 10). Two additional samples were taken after the exposure, these represent NP control samples. These control samples were U NP suspensions[3] placed in the same beakers as the exposure water, but not added fish eggs. These were included to find out how stable the

[3] The same suspensions used for exposure of fish eggs.

suspensions were, and to which degree the eggs changed the speciation of the NP suspensions.

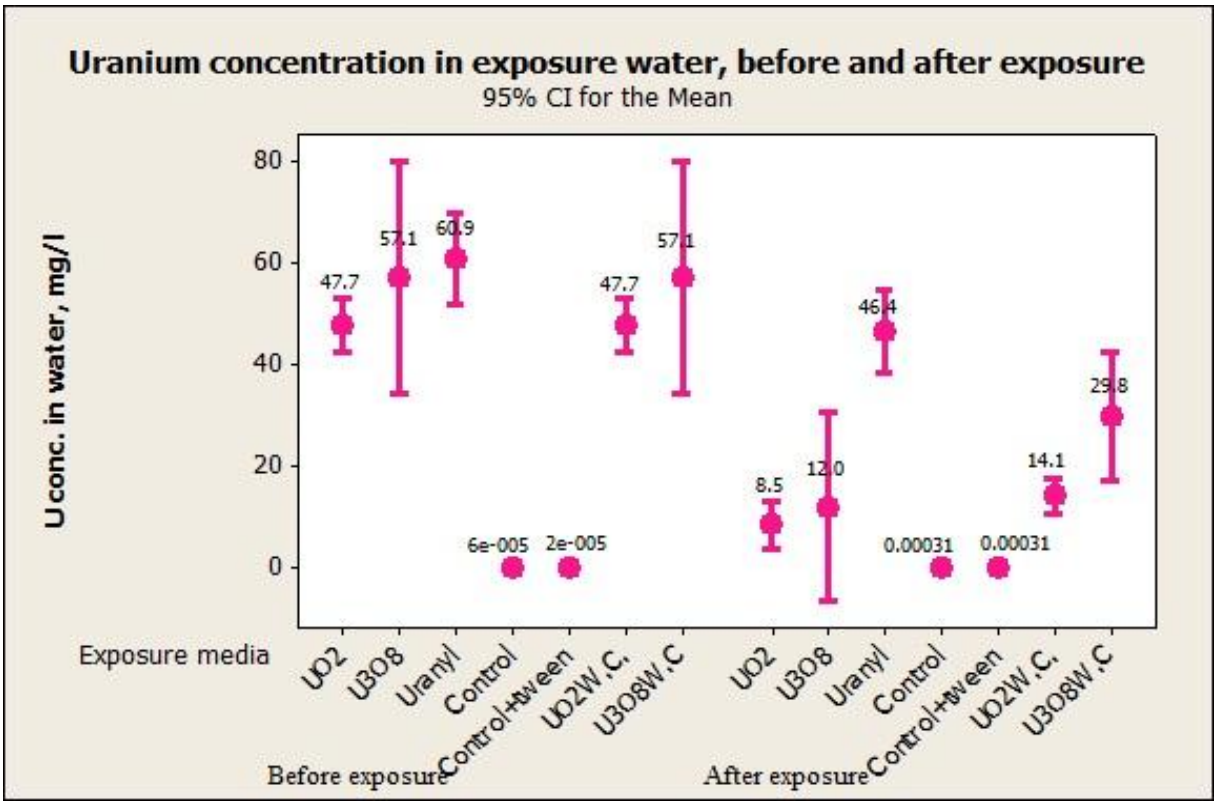

Figure 10: Results of measured U concentration in total water samples before and after exposure, the results are presented as mean values with a 95 % CI (n=3). UO2W.C. and U3O8W.C. refers to the NP control samples. These are the exposure suspensions of UO2 and U3O8

Measured U concentrations in U_3O_8 suspensions varied widely (Figure 10) reflecting uneven distribution of U and a small number of replicates.

Before exposure, the suspensions showed no significant difference in the U concentrations between UO_2, U_3O_8 and uranyl, at 48±2.1, 57±9.2 and 61±3.6 mg U/l, respectively. This was expected since similar amounts of U were added to each exposure suspension. There was a slightly higher mean value of U in the uranyl solution (Appendix 2.), but this was not statistically significant (i.e., there is overlap of the 95 % CI). All the U concentrations were significantly different from levels in control water, as well as the control plus Tween 20 water. The U concentration in the uranyl water before and after exposure could not be distinguished with any statistical significance (61±3.6 vs. 46±3.3 mg/l), but the tendency was a slight decrease in U concentration after exposure. The wide 95 % CI for U_3O_8 (57±10 mg/l) made it difficult to show statistically significant differences in the three different exposure solutions: before, after and without eggs. But the tendency was that the total concentrations drop after exposure (~80 % decrease), and more so with eggs than without. The concentration of U in

the UO_2 suspension after exposure was considerably lower (~80 %) than the concentrations measured before exposure. The UO_2 suspension did not, however, seem to be as affected by the eggs as the U_3O_8 suspension. The UO_2 water not exposed to eggs had a slightly higher U concentration than the exposure water (9±1.9 vs. 14±1.4 mg U/l, the latter without eggs), but the difference was smaller than what was the case for the U_3O_8 suspension (12±7.5 vs. 30±5.1 mg U/l, the latter without eggs). Sorption of U and deposition of U NPs to the container walls and deposition of U NPs on the eggs surface shells was observed and can explain some of the U decrease measured after exposure. Some of this decrease can also be accounted for by the uneven distribution and precipitation/sedimentation of heavy particles in suspension. Uneven distribution of U particles was also observed before exposure, but this effect was reduced by shaking the suspension prior to sampling. This could, however, not be carried out prior to sampling after exposure because the eggs were present and the containers were open (Figure 7). A minor fraction of the decreased U concentrations in the water could also to some extent be accounted for by the uptake of U in and on the eggs (Table 7).

The approximate total uptake of all the eggs in each beaker was calculated to see if this could contribute to a decrease in U concentration in exposure solutions (Table 7). The calculations were performed by first finding the total amount of U taken up by the eggs in each beaker; this was found by multiplying the mean µg U/(g fish egg(w.w.)) after 24 hours with the mean weight of the fish eggs and the amount of fish eggs in each beaker. The approximate decrease of U in each exposure suspension was then found by dividing this number by the water volume in each beaker.

Table 7: The approximate sum of U uptake in each beaker after 24 hours and the resulting approximate decrease in U concentration of the water in each beaker as a result of this.

Beaker		U taken up by total eggs in beakers (µg U)	Decrease of U in water because of uptake in eggs (mg U/l)
UO2	I	1146.35	7.64
	II	568.80	3.79
	III	1422.00	9.48
U3O8	I	1072.91	7.15
	II	745.27	4.97
	III	761.82	5.08
Uranyl	I	1172.60	7.82
	II	1235.32	8.24
	III	1050.09	7.00

The estimations of total U uptake in all fish eggs (Table 7) showed that some of the U decrease in the water samples taken after exposure could be attributed to U uptake into and on the eggs. The calculations showed that uptake into eggs could be responsible for up to a ~9.5 mg/l decrease in U concentration in the UO_2 suspension (this was ~24 % of the total U decrease, Figure 10).

3.3.2 Relative distribution of U in water fractions, measured by ICP-MS

The measured U concentrations in the different samples fractionated before and after exposure, were used to obtain a relative (%) distribution of U in the water (Figure 11 and Figure 12).

Before exposure

The distribution of U in different size fractions of water sampled before exposure was compared to the U concentration in the total water (see Figure 10), resulting in a relative (%) distribution of U in the different fractions (Figure 11).

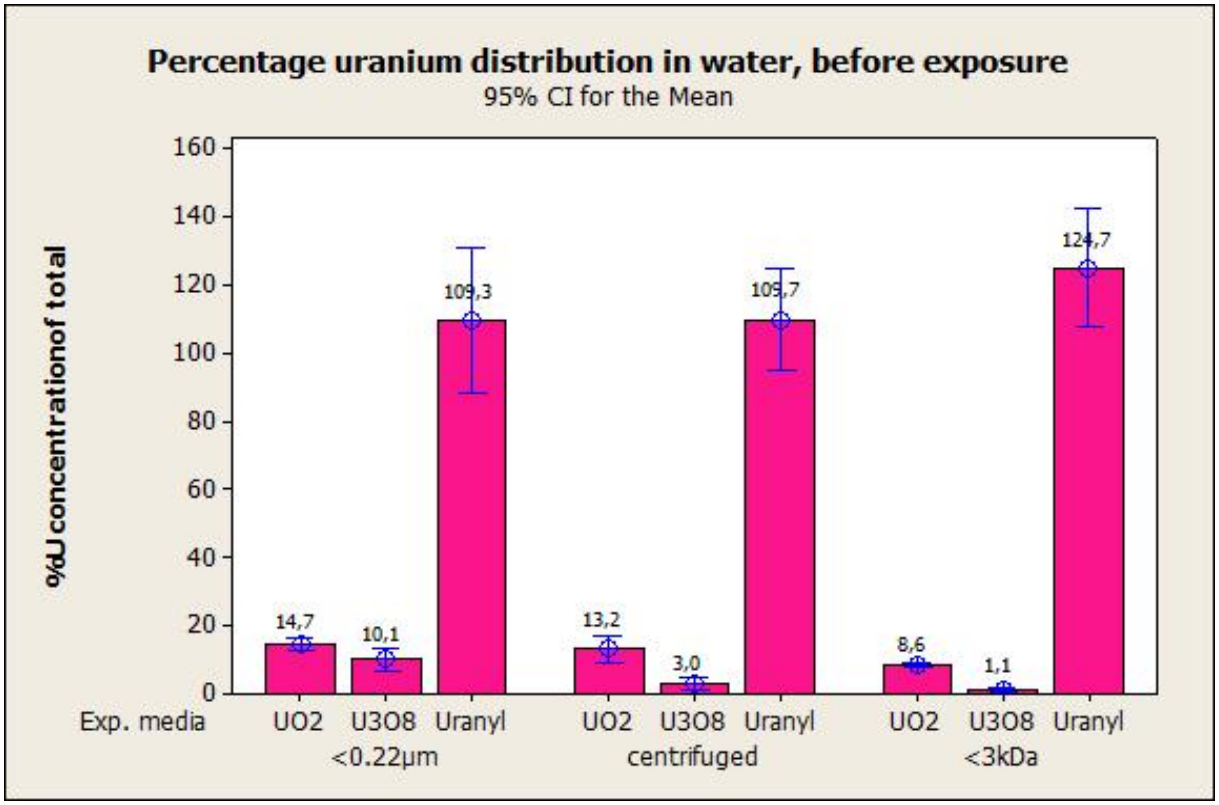

Figure 11: Percentage distribution of U before exposure in different fractions compared to the U concentration measured in total water samples. The percentage was calculated with the mean values and is presented with a 95 % CI. Where 3 kDa=~1-3 nm.

It was evident that for uranyl, 100 % of the U was present in the <3 kDa size fraction (~1-3 nm, Figure 3), hence probably as dissolved ionic species (Figure 11). On the other hand, the relative fraction of U of U NP in the <0.2 µm fraction and <3 kDa fractions, as well as the supernatant after centrifugation, were all under 20 % (Figure 11).

The U concentration was somewhat smaller in the <3 kDa (~<1-3 nm) fraction than in the <0.2 µm fraction for the UO_2 exposure solution (9 vs. 15 %, respectively). The concentration of U in the fraction between these two size cut offs, represents U particles in NP size region (1-100 nm). In the U_3O_8 solution, the U concentration in the fraction <0.2 µm was somewhat less than that for UO_2 (10 vs. 15 %, respectively). There was also little U_3O_8 <3 kDa (1 %). The centrifuged fractions for the two NP suspensions also varied a great deal (13 % for UO_2 vs. 3 % for U_3O_8). This could either be attributed to different size distributions for the U particles in the two solutions, or to different shapes of the particles. Because UO_2 particles have a round shape and U_3O_8 particles are rod shaped (Figure 9), they can behave differently when centrifuged, and move into different size categories. More rod shaped particles might also be filtered than round particles, when using filter fractionation methods. This is because the rod shaped particles can have one dimension that is greater than the filter cut-off even though another dimension has a diameter under 100 nm.

Since the fraction of U in the NP suspensions <0.2 µm and >3 kDa was only between 6 and 10 %, it is unclear whether the fish eggs were actually exposed to a significant amount of NPs at all. But these distributions could also be a result of clogging, aggregation and/or agglomeration of the particles during fractionation. Filtration is known to cause clogging, and centrifugation often causes particles to aggregate and agglomerate. These factors might have caused many of the initial NPs in the exposure solution to end up in larger size fractions, and hence not be measured as NPs. But as a worst case scenario, one might assume that at least 6 % of the total exposures could be classified as NPs.

After exposure

The relative (%) distribution of U in different fractions was also calculated after exposure, and a bar chart was created (Figure 12).

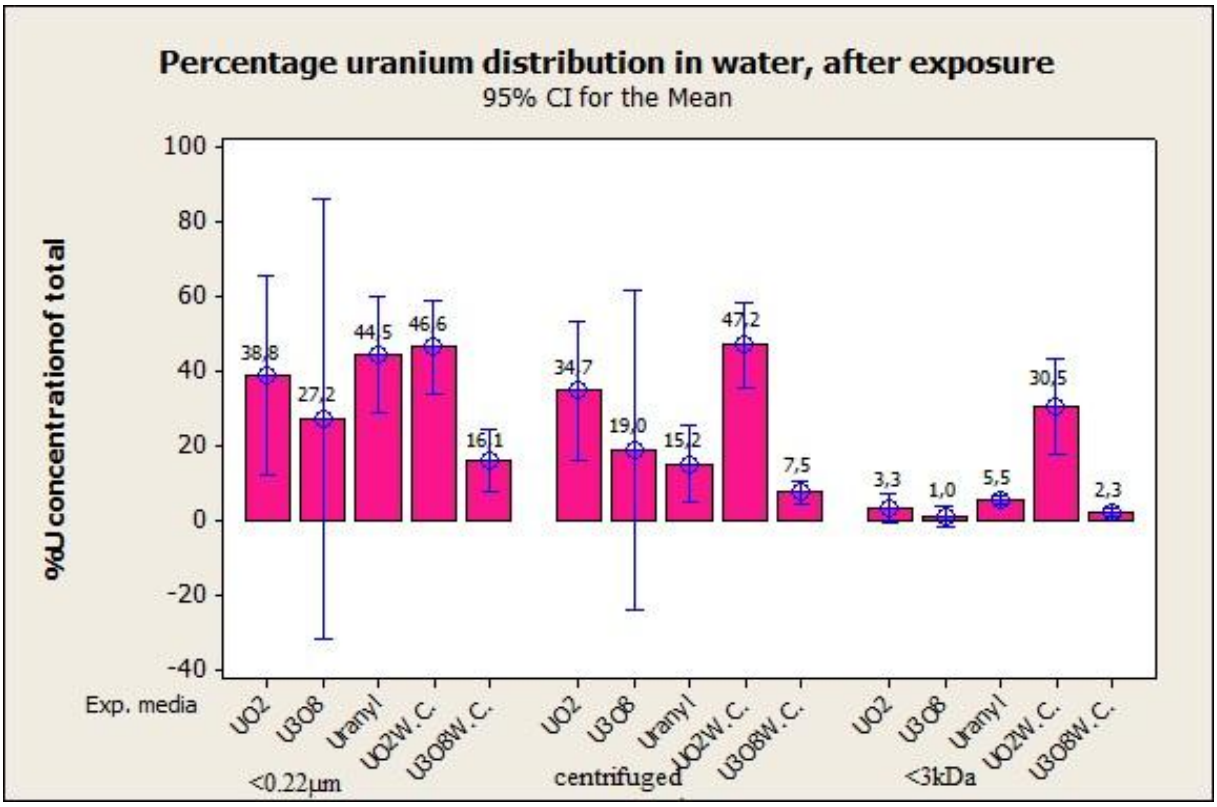

Figure 12: Relative (%) distribution of U after exposure in different fractions compared to the U concentration measured in total water samples. The percentage was calculated with the mean values and is presented with a 95 % CI (n=3). Where 3 kDa=~1-3 nm.

The relative (%) fraction of U in the <0.2 µm and the centrifuged fraction was difficult to determine due to large CI intervals (Figure 12), the trend was however an increase of U material in these fractions compared to measurements before exposure. This increase was slightly higher in the UO_2 suspension (39 vs. 15 %) than in the U_3O_8 suspension (27 vs. 10 %) in the <0.2 µm fraction. The increase in the centrifuged fraction was also slightly higher for the UO_2 suspension (35 vs. 13 %) than for the U_3O_8 (19 vs. 3 %). This relative increase of U in the <0.2 µm and the centrifuged fractions, can be explained by precipitation, sorption of larger particles and aggregates to the container walls and egg surfaces, aggregation and sedimentation. However, these fractions also grew in the UO_2 suspensions i.e., (46 vs. 14 % for <0.2 µm fraction and 47 vs. 13 % for the centrifuged fraction) and the U_3O_8 (16 vs. 10 % for the <0.2 µm fraction and 8 vs. 3 % in the centrifuged fraction) NP control groups, which were not exposed to fish eggs. This shows that the variations in speciation before and after

exposure can be both a function of time as well as the presence of fish eggs. The U concentration in all the uranyl fractions decreased during the exposure (from 100 % to 45 %, 15 % and 6 % in <0.2 µm, centrifuged and <3 kDa fractions respectively). This indicates particle formation, sorption or interaction with organic matter during the exposure period in the uranyl solutions.

These results can be seen in comparison to a U fish exposure experiment conducted by Song et al. (2012) at UMB. In this experiment juvenile Atlantic salmon were exposed to uranyl added to water from Maridalsvannet. The chemical analysis showed that 90 % of U in suspension was found in the <0.45 µm fraction and this fraction was relatively stable throughout the experiment of 48 hours (Song et al. 2012). In the present experiment, 100 % of the U was in the dissolved fraction before exposure (<3 kDa), while after exposure, only 6 % of U was present in this fraction. The fraction of U in <0.2 µm also decreased from 100 % to 45 % after exposure. This can indicate a difference in U behavior in water from Maridalsvannet and the synthesized analogue, or fish eggs and spermatozoa can change the speciation of U more readily than live fish.

3.3.3 Size distribution of uranium particles in exposure water determined by TEM

UO_2, U_3O_8 and uranyl suspensions both before and after exposure, as well as the <0.2 µm fraction before exposure, were analyzed by TEM (Figure 13 - Figure 21). There are TEM images with different magnification, and these were placed in descending order (e.g. Figure 13 A has greater magnification than Figure 13 B), the images are not necessarily from the same part of the TEM grid.

Total fraction before exposure

TEM images of the UO_2, U_3O_8 and uranyl exposure solution before exposure were taken using different magnifications (largest magnification: x 180k) (Figure 13-Figure 15).

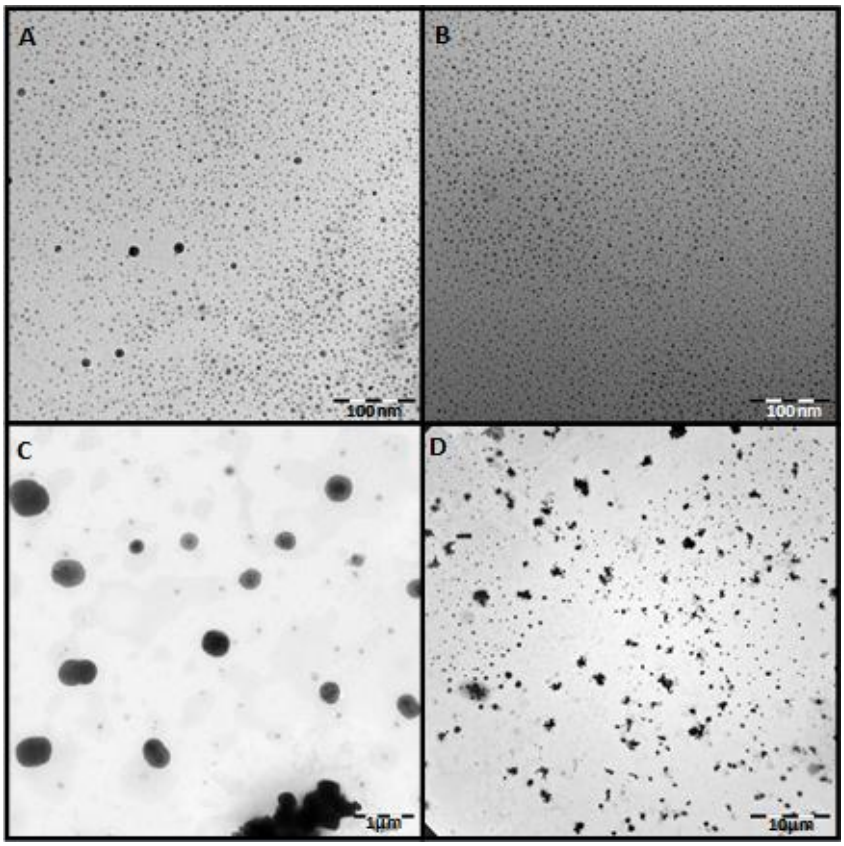

Figure 13: TEM images of UO_2 exposure suspension, samples taken before exposure with different magnifications (bars from 100 nm-10 µm). Uranium concentration in suspension is ~47 mg/l.

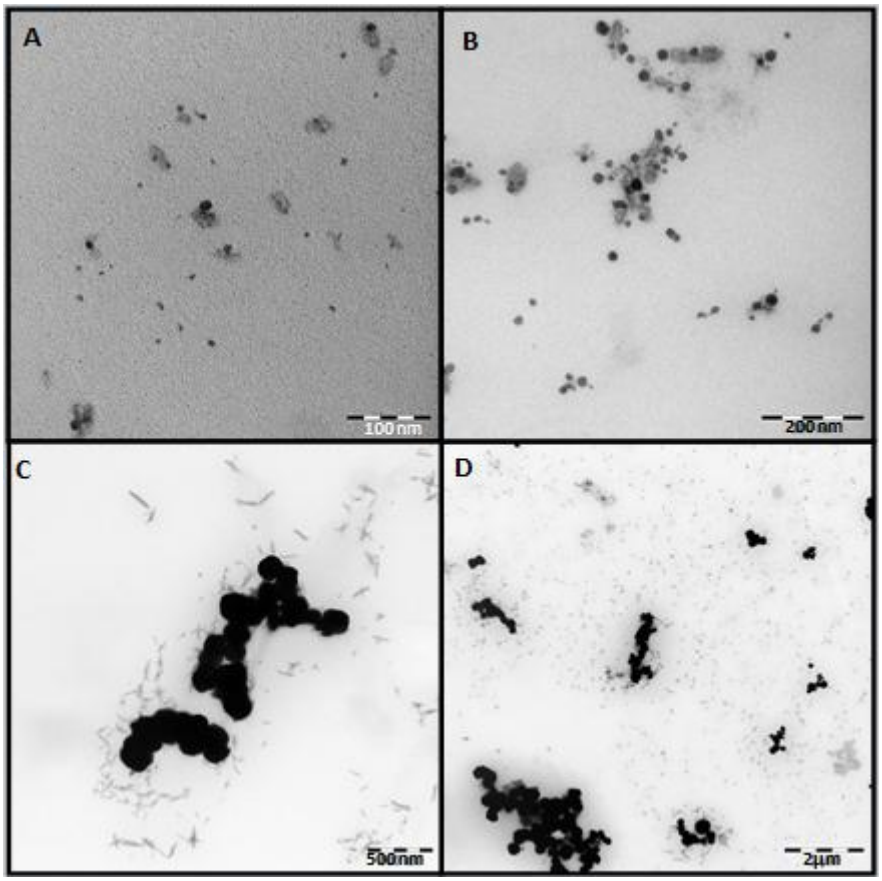

Figure 14: TEM images of U_3O_8 exposure suspension, samples taken before exposure with different magnifications (Bars from 100 nm–2 µm). Uranium concentration in suspension is ~57 mg/l.

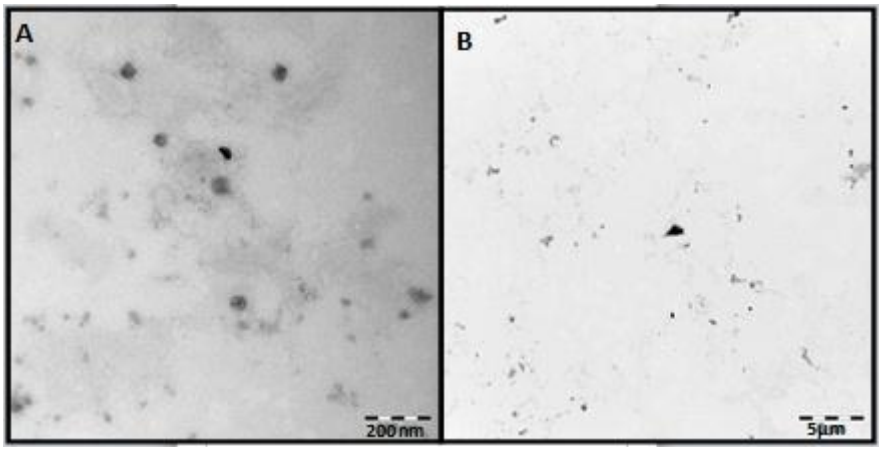

Figure 15: TEM images of uranyl exposure solution, samples taken before exposure with different magnifications (Bars from 200 nm–5 µm). Uranium concentration in suspension is ~61 mg/l.

Image A and B in (Figure 13) shows UO_2 NPs with diameters from about 1 to 20 nm. Image C and D (Figure 13), which both have lower magnification than images A and B, shows that the size distribution of particles in this suspension ranged from ~1 nm to several micrometers. Some of the larger aggregates and agglomerates (Figure 9) seem to have dissolved and numerous small particles (~1-20 nm) were observed in the suspension (Figure 13). This can be a result of the ultrasonic agitation and the dilution in synthetic Maridalsvann. The low salinity in the water might also have contributed to stabilization of the particles.

The U_3O_8 suspension in the TEM image (Figure 14) has many small, rod shaped particles. This confirms the formation of U_3O_8, as these NPs are rod shaped, as opposed to UO_2 NPs which are round. The largest aggregates/agglomerate also seems to have dissolved some in this suspension.

Image A of the uranyl suspension (Figure 15) shows some small particles between ~10-50 nm in diameter, while the particles in image B have diameters of ~100-1000 nm. Even though no particles should be present in the uranyl solution (only readily soluble uranium salt has been added), there are still some particles with various sizes in the TEM images (Figure 15). This can be either unsolved U salt, salt clusters from the water, contaminants or a combination of these. Because no X-ray microanalysis has been performed on the suspensions, there is no way to know the composites of the observed particles.

<0.2 fraction before exposure

TEM images of <0.2 µm fraction of NP suspensions and uranyl solution before exposure were also taken (Figure 16 - Figure 18). These images show particles with the same shapes as images of suspensions before filtration (Figure 13 - Figure 15); the alteration is that the larger aggregates and particles were filtered out.

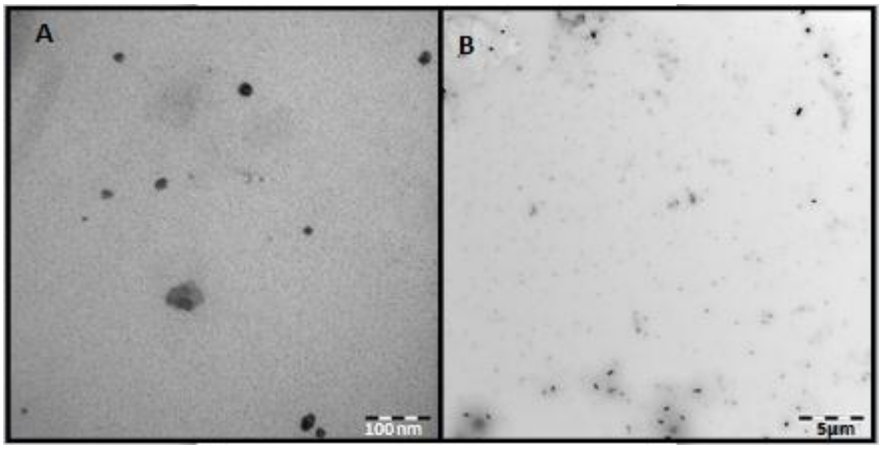

Figure 16: TEM images of UO_2 exposure suspension, filtered through a 0.2 µm filter, samples taken before exposure with different magnifications (Bars from 100 nm–5 µm). Uranium concentration in suspension is ~7 mg/l.

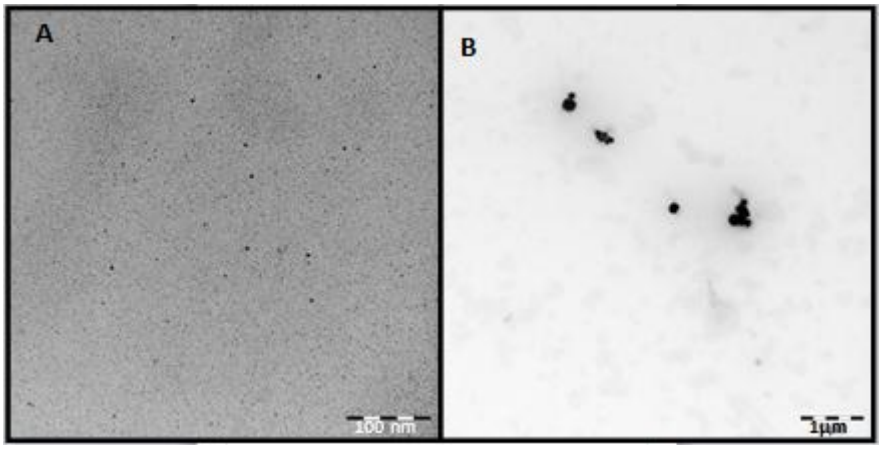

Figure 17: TEM images of U_3O_8 exposure suspension, filtered through a 0.2 µm filter, samples taken before exposure with different magnifications. B- shows aggregates present in the bulk solution; A- shows the smaller NP's distributed throughout the suspension (bars from 100 nm –1 µm). Uranium concentration in suspension is ~6 mg/l.

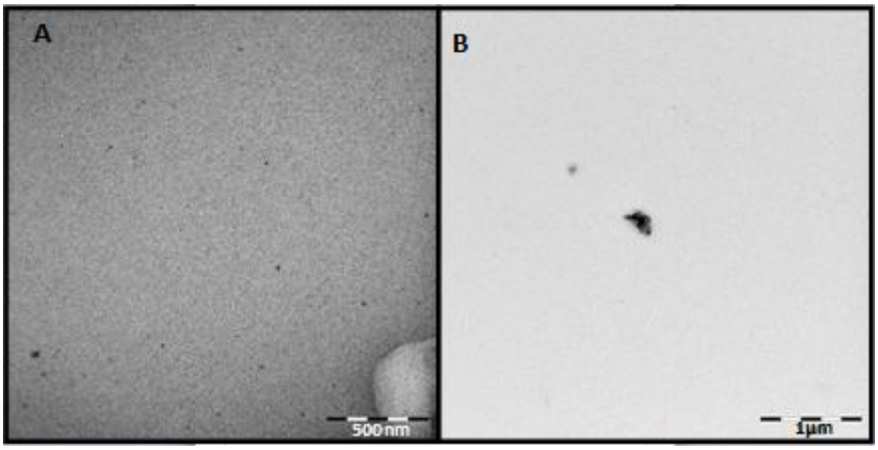

Figure 18: TEM images of uranyl exposure solution, filtered through a 0.2 µm filter, samples taken before exposure with different magnifications (bars from 500 nm-1 µm). Uranium concentration in suspension is ~66 mg/.

Two images of UO_2 (Figure 16), U_3O_8 (Figure 17) and uranyl (Figure 18) suspensions are shown with two magnifications, with the larger magnification to the right. The TEM images of the U NP fractions <0.2 µm (Figure 16 and Figure 17) shows that there were fewer small particles (~1-20 nm) evenly distributed through the suspension in this fraction than in the total fractions (Figure 13 and Figure 14). This can be because the 0.2 µm filters can clog causing smaller particles to be retained. These results can also suggest that the measurements of U content in each fraction of the water (Figure 11 - Figure 12), might not present correct U distribution. The U in the size range <0.2 µm might therefore be larger than what was found using filter fractionation and ICP-MS.

Total fraction after exposure

After exposure, suspensions of both NPs and uranyl were analyzed by TEM, two images with different magnifications are displayed for each suspension, the rightmost images with largest magnification (Figure 19-Figure 21).

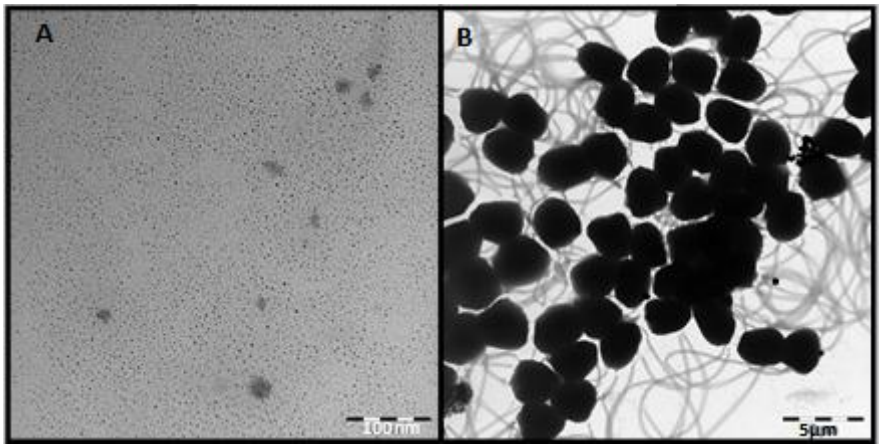

Figure 19: TEM images of UO_2 exposure suspension, water sampled after exposure with different magnifications (bars from 100 nm-5 µm). Uranium concentration in suspension is ~8.5 mg/l.

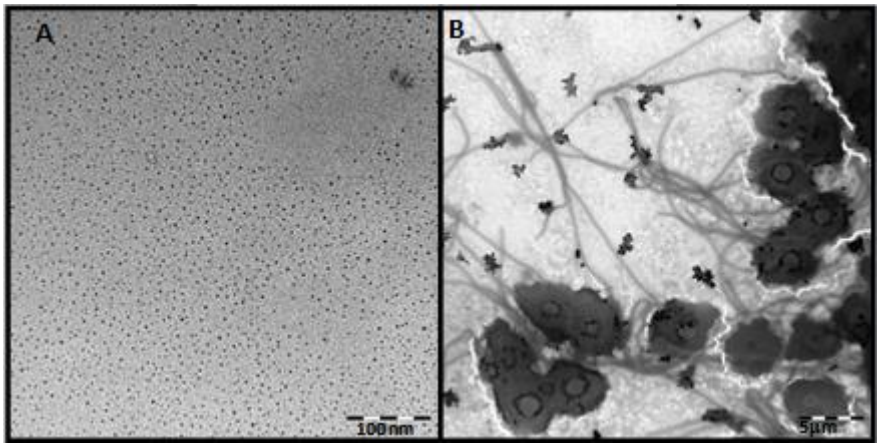

Figure 20: TEM images of U_3O_8 exposure suspension, water samples after exposure with different magnifications (bars from 100 nm-5 µm). Uranium concentration in suspension is ~12 mg/l.

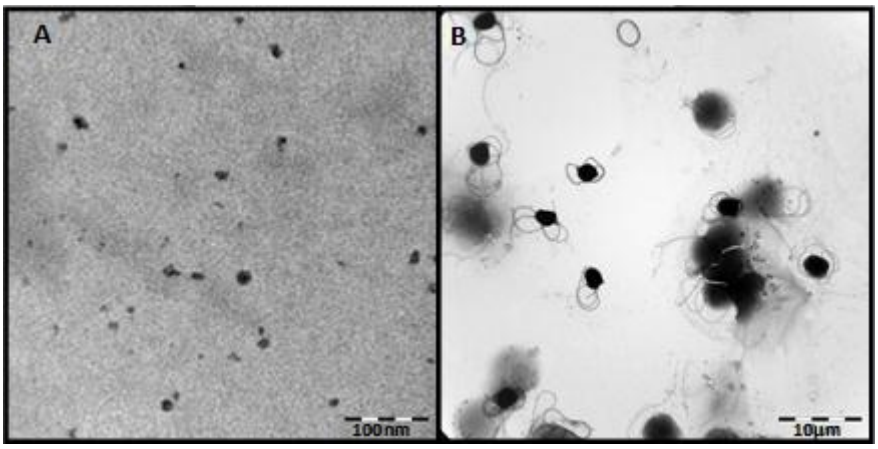

Figure 21: TEM images of uranyl exposure solution, water sampled after exposure with different magnifications (bars from 100 nm-10 µm). Uranium concentration in suspension is ~46 mg/l.

All the TEM images of suspensions sampled after exposure show both clusters of organic material, as well as threads of these (Figure 19 - Figure 21). Especially in the UO_2 and U_3O_8 suspensions after exposure, TEM images show a tendency of U particles clustering around the organic material (Figure 19 and Figure 20). The aggregation of UO_2 particles around organic polymers were also observed in the biogenic formation experiment with *Shewanella putrefaciens* CN32 executed by Lee et al. in 2010. These images also indicate that the speciation of the particles change as a function of time and because of the organic addition to the solution. The smaller particles (~1-20 nm) are less affected by the organic addition (Figure 19 A and Figure 20 A). It can however be difficult to know what is actually a U particle and what is organic matter in the TEM images of the U NPs after exposure (Figure 19 B and Figure 20 B). Some of the particles in U_3O_8 suspension (Figure 20) were diffuse, indicating lighter particles than the high contrast particles (black appearance) shown in the TEM images of suspension sampled before exposure (Figure 14). These more diffuse particles might therefore be organic; the same particles are, however, not seen in the other suspensions (Figure 19 and Figure 21). The clusters seen around the organic threads in the UO_2 suspensions (Figure 19) have a much more distinct black color than the clusters seen in the U_3O_8 suspension (Figure 20), these also have a regular round shape and look like the U particles seen in the suspension before exposure (Figure 13). These particles are therefore most likely U particles. Some similar round black particles can also be seen in the TEM image of the uranyl solution after exposure (Figure 21 B). This solution was only added U ions, and should not contain U particles. There were, however, fewer of these particles in the uranyl solution than in the UO_2 suspension. The addition of organic matter to the uranyl solution

might have induced the formation of some U particles (UO_2) from U ions, indicating that organic matter can act as a template for particle growth.

Particle size measurements and calculations

TEM images were used to measure the size of the particles in the different suspensions, mean particle diameter and standard deviation was calculated (Appendix 3) and histograms of the logarithmic values of the measured particle diameters was created to visualize the particle size distribution (Figure 22).

The mean size of the UO_2 NPs synthesized was 198±345 nm. Wang et al. (2008) achieved a mean diameter of 100 nm when using the same synthesizing method as used in this experiment, but these particles had a relatively uniform size distribution. This result differs from the result obtained during the present synthesis, where the mean diameter size was somewhat higher than the what Wang et al. obtained, but the resulting distribution of particles were not uniform, and had a large standard deviation (345 nm). The larger mean diameter was mainly due to some larger particles increasing the mean diameter size. It therefore seems plausible that material synthesized in this experiment contained smaller UO_2 particles than those synthesized by Wang et al. (2008). The mean size of synthesized U_3O_8 particles in this experiment was 85±169 nm, in comparison to particle diameters ranging from 80-100 nm synthesized by Wang et al. (2008). The mean diameter of the synthesized particles in these two experiments is thus similar. The TEM images of the synthesized U_3O_8 particles (Figure 14) in the present experiment, however, reveal numerous particles with diameters smaller than 80 nm. These results indicate that cleaning the synthesized particles by dialysis created a product containing smaller particles, but also a wider size distribution in the synthesized particles, than particles treated with by freeze-drying (Wang et al. 2008).

The TEM images are not random, how many particles are in the images and in which order of magnitude the images are, is subjective. The mean value and also the relative size distribution of the particles are therefore subject to large uncertainties. These numbers should therefore only be an indication of the particle sizes and the particle size distribution.

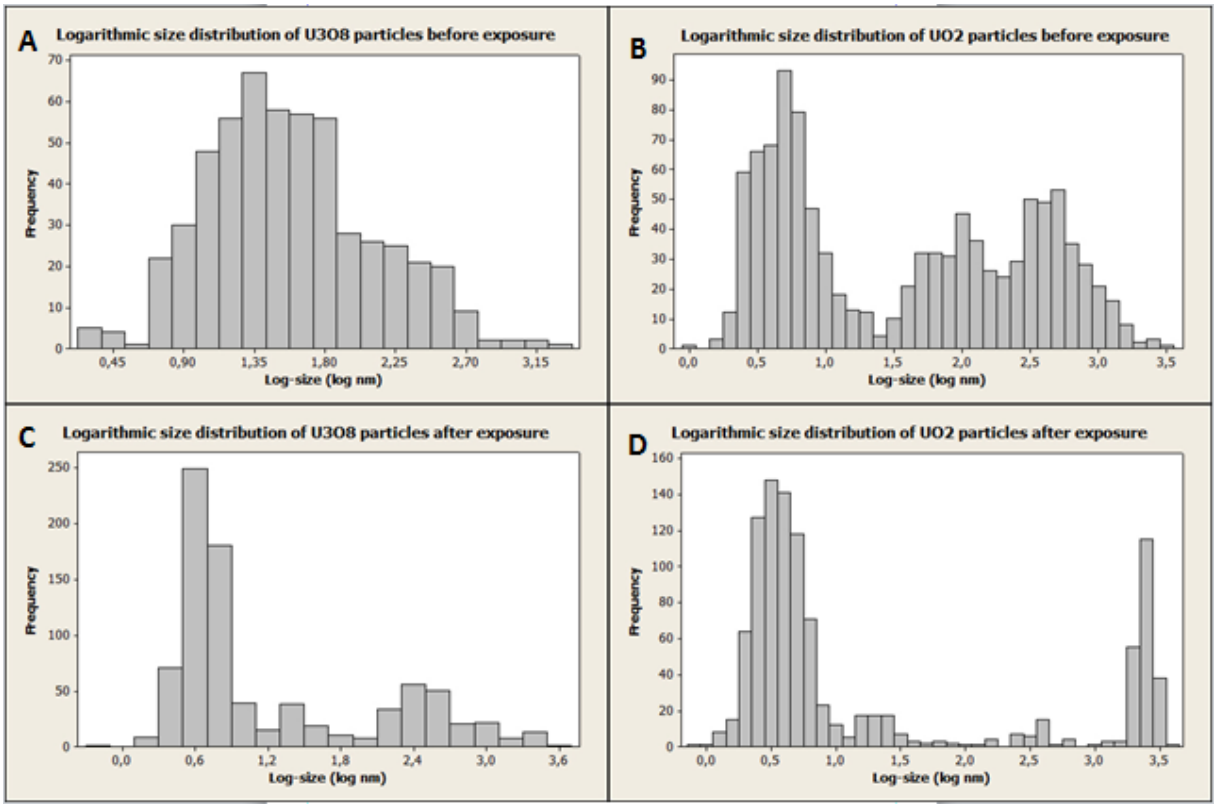

Figure 22: Logarithmic size distribution of U particles as observed in TEM. A-U_3O_8 particles before exposure, mean=85 nm and n=540. B- UO_2 particles before exposure, mean=198 nm and n=1060. C-U_3O_8 after exposure, mean=152 nm and n=838. D- UO_2 particles after exposure, mean=519 nm and n=1057

The sizes of the U3O8 particle rods were measured over the shortest part of the particles. The U3O8 particles measured before exposure had a log-normal size distribution (Figure 22 A). On the other hand, the logarithmic size distribution shows that the UO2 particles measured both before and after, as well as the U3O8 particles measured after exposure, had two particle size peaks (Figure 22). In all the graphs, these peaks occur <100 nm and >250 nm. This trend could also be seen by comparing the percentage of particles between 1-100nm (Figure 23), and the mean diameter size (Appendix 3). The solutions analyzed before exposure shows that the U3O8 particles both had a smaller mean diameter size and a larger percentage of the particles between 1-100 nm than is the case for the UO2 solution (85 nm and 80 % vs. 198 nm and 61 %, for U3O8 and UO2 respectively). The relative amount of particles with diameter size between 1-100 nm was more equal in the two NP suspensions after exposure (75 % for U3O8 vs. 76 % for UO2). For the UO2 suspension, the relative (%) amount of particles between 1-100 nm was larger after the exposure than before (76 vs. 61 %), this was also the case for the mean particle diameter (519 vs. 198 nm). This can indicate that the particles above 100 nm in diameter more readily clusters as a response to the addition of organic matter and/or as a function of time. This caused the particles above 100 nm to be fewer but larger, leading to a larger percentage of the particles being below 100 nm and the mean diameter to

increase. The percentage of particles between 1-100 nm in the U3O8 solution was on the other hand smaller after the exposure than before (75 vs. 80 %). The particles in the U3O8 suspension also had an increase of the mean diameter after exposure (152 vs. 85 nm). This can indicate that the clustering and growth of the particles after the addition of the organic matter was more uniform according to particle size in the U3O8 solution than in the UO2 solution.

According to the European Commisions definition of nanomaterials, 50 % of the particles should be in the size range 1-100 nm. The relative (%) amount of particles between 1-100 nm has therefore been calculated (Appendix 3) and presented in a bar chart (Figure 23).

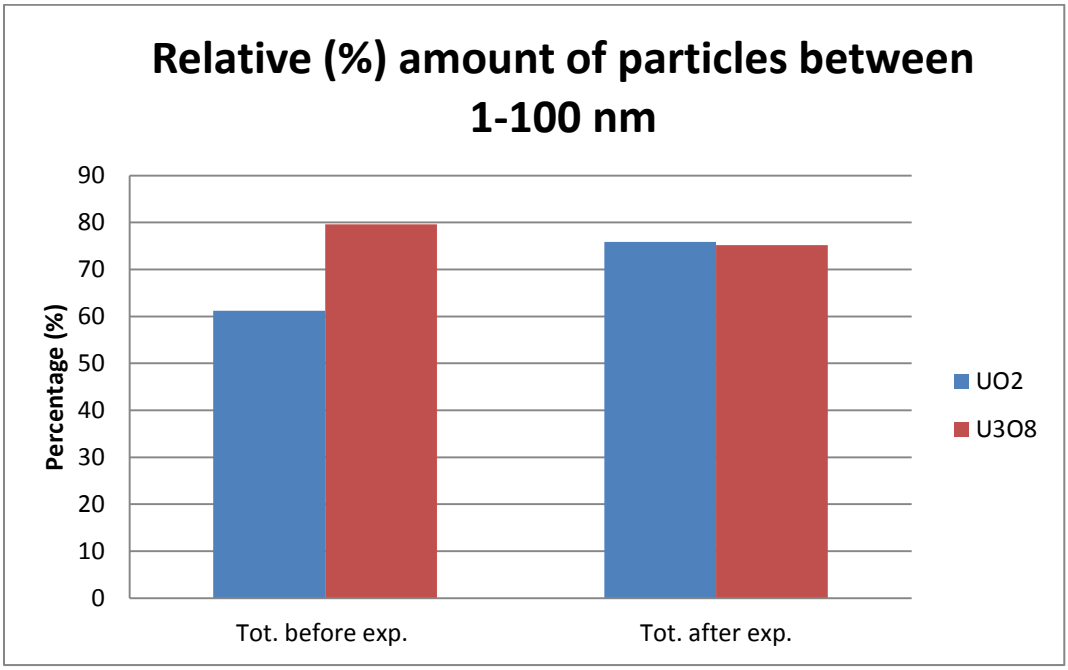

Figure 23: Percentage of measured particles within size range 1-100 nm in U NP exposure suspensions.

At first sight it can look as though there are more large particles over 100 nm than smaller NPs in the exposure suspensions (Figure 13 - Figure 21). The 50 % limit in the European commission definition, calculates the particle distribution according to number of particles, and not according to mass. Even though a lot of the space and the mass were taken up by larger particles (>100 nm), the smaller particles (<100 nm) were more numerous (Appendix 3). The relative (%) amount of particles between 1-100 nm in the NP suspensions calculated (Figure 23), were above 50 %. This means that the synthesized material could be defined as nanomaterial.

The relative (%) distribution of U in the water (Figure 11 - Figure 12) showed that less than 20 % of the U in the UO_2 and U_3O_8 suspensions had a diameter <0.2 µm. This was however a mass distribution and the TEM images showed another story. In these images it was evident that there were numerous particles <0.2 µm present in the suspensions and more than 60 % of the particles measured had a diameter under 100 nm both before and after exposure. These particles were thus so small that they contributed much less to the measured concentration of U in the solution than the larger particles. The percentage distribution of U according to mass and according to number of particles, can therefore be quite different. But as discussed earlier, much more than the particles >0.2 µm in size might have been retained during the filtration. These results also demonstrate the importance of complementary techniques.

3.4 Egg exposure experiment

3.4.1 Microscope

Images of eggs exposed to different media were taken by an ordinary light microscope (Figure 24).

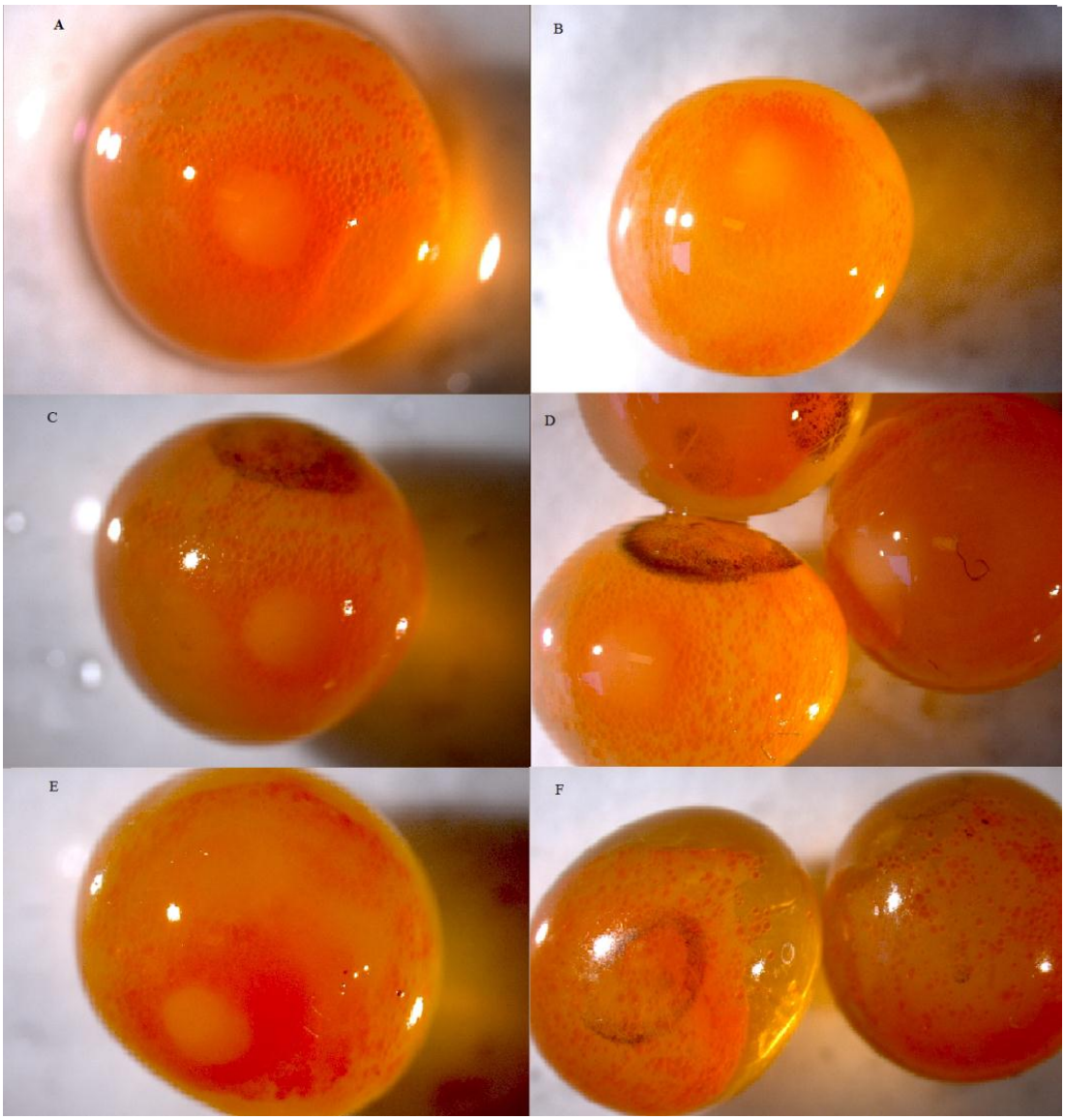

Figure 24: Images taken with a light microscope of eggs exposed for 24 hours. A - Control eggs, exposed to Maridalsvann. B - Control eggs exposed to Maridalsvann and Tween. C - Egg exposed to U_3O_8 exposure solution. D - Eggs exposed to UO_2 exposure solution. E - Eggs exposed to uranyl solution. F - Unfertilized eggs. (Images taken by Kleiven, M.)

Eggs viewed with light microscope all had a round area slightly lighter than the eggs, except the eggs which were unfertilized (Figure 24 F, Table 8). This light area is the cell lump, which will eventually develop into a fetus. Fertilizing degree of sampled eggs was found by counting the unfertilized eggs (Table 8).

Eggs exposed to U NPs (Figure 24 C and D) had black circles on the surface; this indicates adsorption of U particles on the egg shell. All the eggs exposed to U (Figure 24 C, D and E) had a slightly irregular shape, compared to the more round shape of the eggs only exposed to water (Figure 24 A) and water plus Tween 20 (Figure 24 B). This could suggest that the development of the egg shells could have been affected by U concentration in the water, regardless of the U speciation.

Table 8: Amount of eggs in each beaker, amount of fertilized eggs out of three samples from each beakers and the mean swelling value of three sample eggs from each beaker is displayed in the table. The swelling degree where measured by weighing the eggs after 24 hours.

Exposure media	Replicate no.	Total amount of eggs in exposure beakers	Fertilized Yes (Y)/ No (N) (n=3)	Swelling (n=3) (g)	
				Mean	Std.
Control	I	65	3xY	0,142	± 0,0047
	II	60	3xY	0,144	± 0,0065
	III	72	3*Y	0,151	± 0,0060
Control + Tween 20	I	85	3*Y	0,147	± 0,0056
	II	63	3*Y	0,146	± 0,0064
	III	81	2*Y & 1*N	0,145	± 0,0095
Uranyl	I	67	3*Y	0,144	±0,0077
	II	77	3*Y	0,145	± 0,0026
	III	72	3*Y	0,146	± 0,0032
UO2	I	131	1*Y, 1*N & 1*?	0,143	± 0,0044
	II	65	3*Y	0,149	± 0,0042
	III	75	3*Y	0,138	± 0,0035
U3O8	I	140	2*Y & 1*N	0,141	± 0,0051
	II	73	1*Y & 2*N	0,145	±0,0021
	III	80	3*Y	0,140	±0,0029

Some more of the eggs sampled from the groups exposed to U_3O_8 were unfertilized compared to the eggs sampled from the two control groups (3 vs. 0 and 1). This was probably random, and not because of the U addition in the water. This can be said because the eggs were fertilized before they were added to the exposure solutions. There seem to be no difference in swelling in the eggs exposed to the different exposure solutions (Table 8) as was the case with *C. Carpio* eggs exposed copper in Jezierska's et al. (2008) experiment.

3.4.2 Uranium concentration in fish eggs, measured by ICP-MS

The U concentration in the decomposed egg solutions together with the weight of each egg, were used to calculate the U content per gram egg (Appendix 2.2). The average measurement of the blank samples was used to calculated limit of detection (LOD) and limit of quantification (LOQ) (see Appendix 2.4). Some of the U concentrations measured in the

dissected eggs exposed to U_3O_8 for 3 hours were below LOQ, so even though U was detected in the samples, it is too little to give a quantitative measurement (the measured values were still used in the graphs). The measured values of U in the CRM samples correspond well with the known added amount in the samples (Appendix 2.3). This indicates that the method and the sample preparation lead to accurate measurements of U.

The data of the total U content in fish eggs has been grouped into different exposure media and sampling times to allow for comparison of U uptake in eggs as a function of time, or exposure media and was represented in a graph (Figure 25).

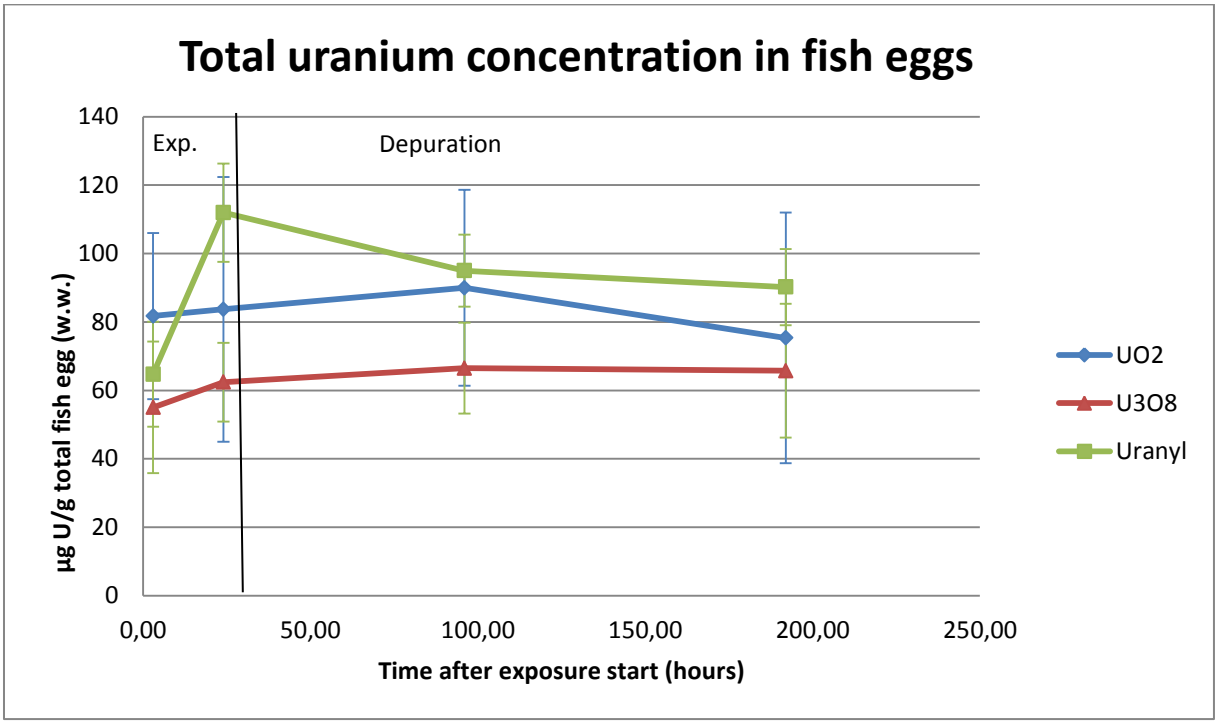

Figure 25: Concentration of U in total egg samples exposed to different exposure media after 3, 24, 96 and 192 hours (n=9). The content is shown as mean U µg/g egg (wet weight), measured with ICP-MS, with standard deviation error bars.

There was no significant difference in the total U concentration in the fish eggs of the three U exposure groups after three hours (82, 55 and 65 µg U/(g egg(w.w)) for UO_2, U_3O_8 and uranyl respectively). After 24 hours a difference was however observed, the levels in the uranyl group (112 µg U/g egg(w.w)) was slightly higher than in the UO_2 group (84 µg U/g egg(w.w)) and significantly higher than the U_3O_8 group (62 µg U/g egg(w.w)) (Figure 25). All measured U concentrations after exposure were also significantly different from the controls. The U contents of the eggs seemed to be quite stable and unchangable in the eggs exposed to U NPs

(Figure 25). The U NPs were disposed on the exterior shell of the eggs (Figure 24), and without any natural water flow in the exposure beakers, the U concentration of the eggs did not decrease. This also suggests that the U NP uptake on or into the eggs occured during the first 3 hours. The U ions, however, have another pattern of uptake. The U contents of the eggs exposed to uranyl, seemed to increase steadily for the first 24 hours (65 after 3 hours and 112 µg U/g egg(w.w) after 24 hours) and a slight decrease of U was seen after 192 hours. The U concentration in the eggs exposed to U_3O_8 was lower than the the U concentration in the uranyl exposed eggs after 24 and 96 hours.This suggests that the U ions were taken up by the eggs continusly with steady supply of U ions in the water. Because of the solubility of the ions however, depuration might be more efficient with U ions than with U NPs.

These results does not take into account whether the U penetrated the eggs, or whether it was only disposed on the surface of the egg shell. This was tested by measuring the U contents of the eggs interior (Figure 26). The U concentration in the dissected eggs exposed to U NPs (UO_2 and U_3O_8) could not be distinguished from the U concentration of the control eggs (Figure 26). This was because the U concentration in the eggs exposed to uranyl was so large, compared to the contents in the eggs exposed to U NPs. A separate graph was therefore made excluding the eggs exposed to uranyl (Figure 27).

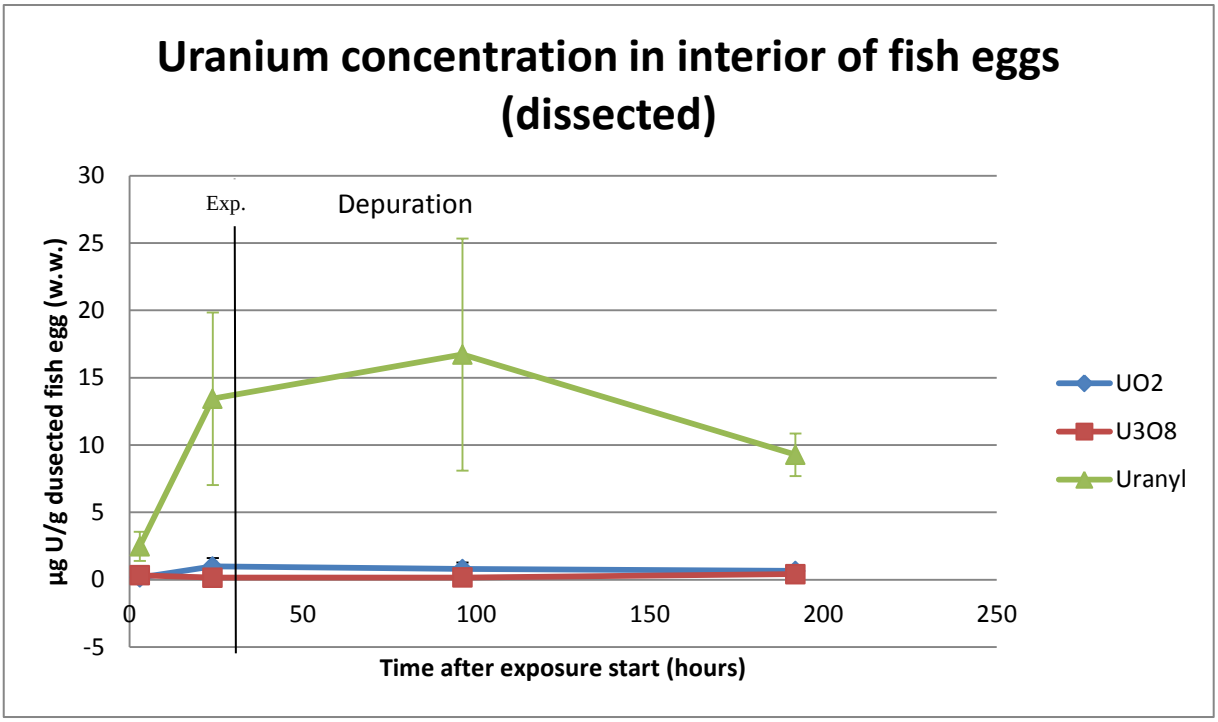

Figure 26: Concentration of U in dissected egg samples exposed to different exposure media after 3, 24, 96 and 192 hours (n=9). The content is shown as mean µg/g egg (wet weight), measured with ICP-MS, with standard deviation error bars.

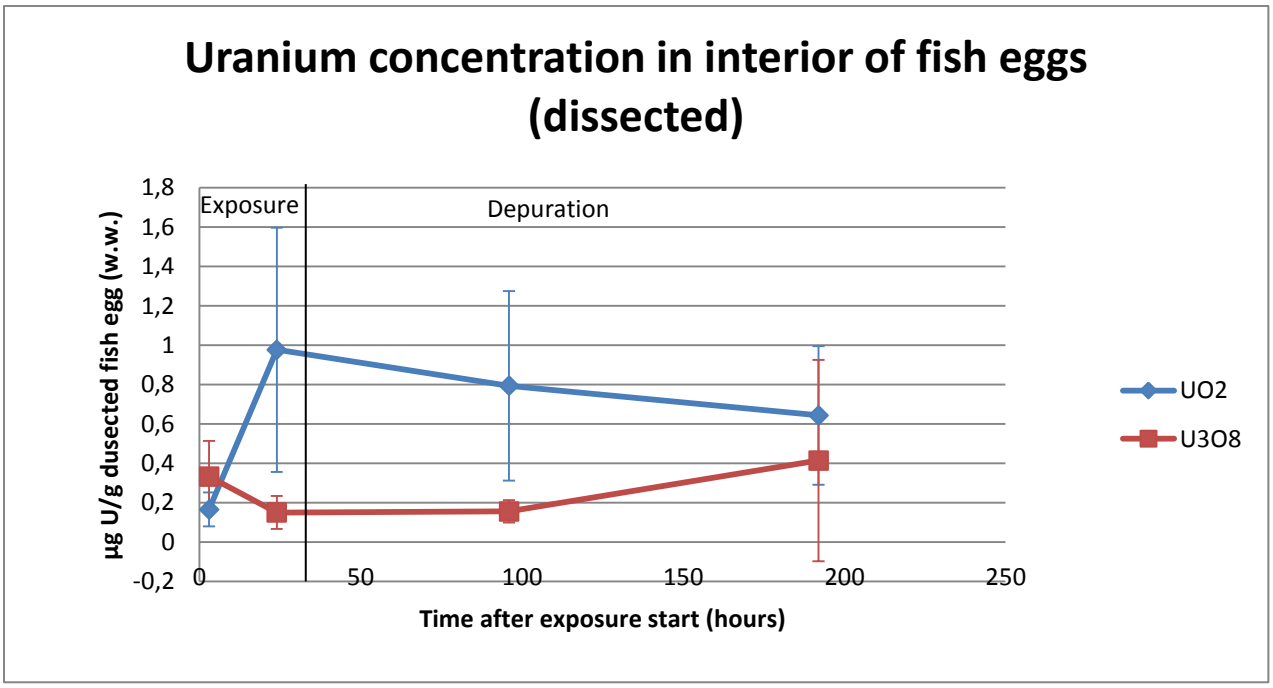

Figure 27: Concentration of U in dissected egg samples exposed to UO_2 and U_3O_8 exposure media after 6 hours, 24 hours, 3 days and 7 days (n=9). The content is shown as mean µg/g egg (wet weight), measured with ICP-MS, with standard deviation error bars.

The uptake of U into the eggs interior during the first 3 hours could almost not be distinguished from the control eggs for the NP exposed groups, but the eggs in the uranyl group showed a slight U uptake (Figure 26). The concentration of U in the dissected eggs, exposed to uranyl, increased to 13 µg U/(g egg(w.w.)) during the first 24 hours. The U contents from the measurements after 24, 96 and 192 hours could not be distinguished from each other with any statistical significance for any of the groups (Figure 26). The trend for the U concentration in the eggs exposed to uranyl was, however, a ~20 % increase from 24 to 96 hours and a decrease of ~40 % from 96 to 192 hours. This suggests that uranyl was first deposited on the egg shell, and entered the egg even after the eggs had been removed to clean water. With time, some of the U leached out from the eggs again, causing the U concentration to fall slightly (although changes were not significantly different).

After 3 hours, little or no U was taken up in either the eggs exposed to UO_2 or U_3O_8 (0.16 and 0.2 µg U/(g egg(w.w.)), respectively) (Figure 27). The U concentration in the eggs exposed to UO_2 was, however, statistically higher than the control eggs after 24, 96 and 192 hours, although they were not different from each other. It can therefore seem as though the eggs did not take up U as NPs during the first three hours after fertilization, but the U NPs was merely adsorbed on the eggs exterior shell during this period (Figure 24). Some U is taken up as a function of time by the eggs exposed to both UO_2 and U_3O_8, as was also the case for the eggs exposed to U ions. The same trends are seen in the eggs exposed to U ions and UO_2 NPs, but ~93 % more U was taken up in the eggs exposed to uranyl versus the eggs exposed to UO_2. The eggs exposed to U_3O_8 did however take up little or no U with the highest mean value at 192 hours (0.41 µg U/g egg(w.w)). This is consistent with the results from Michabata's (1981) and Beattie and Pascoe's (1978) exposure experiment, where the majority of the metal taken up by the eggs, were found in the chorion and egg membrane. This is also expected due to the "water-hardening" of the eggs when exposed to freshwater (Potts & Rudy JR., 1968).

The relative (%) concentration of U inside each egg, compared to the total U concentration in the egg, was calculated (see Appendix 2.2). The relative concentration of U inside the NP exposed eggs, were all under ~1 %. The percentages seem somewhat higher in the uranyl exposed eggs, but even these did not exceed 20 % U in the interior of the eggs. The relative U concentration in the interior of the eggs exposed to UO_2 seemed to increase some during the first 24 hours (from ~0.1 % after 3 hours to ~0.5 % after 24 hours), but it is difficult to say if

this changed during the depuration period due to large deviations. In the eggs exposed to U_3O_8 it was hard to distinguish the relative (%) amount of U inside the eggs at the different sampling times from each other. This can be because the measured U values in the dissected eggs were low (from <loq to 0.6 µg U/g egg(w.w)) and hence small variations could cause large uncertainties. The relative amount of U in the interior of the eggs exposed to uranyl increased during the first 24 hours (from ~2 % at 3 hours to ~8 % at 24 hours), further, a slight increase was seen after 92 hours (~11 %). The percentage had however decreased on the seventh day of depuration (~5 %).

Overall the results show that the eggs exposed to U NPs took up less U into the interior of the eggs than eggs exposed to U ions. This was expected, as the ions are LMM, and considered to be much more bioavailable than HMM which are thought to be virtually inert in biological uptake (Salbu, 2000). The HMM used in this experiment was, however, NPs who are considered to have different properties than other HMM particles. Even though the uptake of U in eggs was higher for eggs exposed to U ions than U NPs, the NPs were not biologically inert, and uptake of these in eggs was observed. The uptake study of silver NPs performed by Lee et al. (2007), showed both active and passive transport of NPs into *Danio rerio* eggs. This suggests either that silver NPs behave differently in biological uptake than U NPs, or the size of the particles in the U suspensions were larger than the silver particles used by Lee et al. (2007). This might do the penetration through the egg shell more difficult and the NP uptake smaller.

3.5 Uranium depuration in clean water

Uranium concentration was also measured in water used for depuration (Table 9) (Appendix 2.1).

Table 9: Shows the U concentration in depuration water after 3 and 7 days (96 and 192 hours after exposure start) of depuration, this table is extracted from Appendix 2.1.

Average U concentration in depuration water				
Exposure media	**After 3 days depuration**		**After 7 days depuration**	
	Average U concentration, mg/l	**Standard deviation**	**Average U concentration, mg/l**	**Standard deviation**
UO2	0.08	0.093	0.07	0.014
U3O8	0.017	0.005	0.03	0.012
uranyl	0.31	0.078	0.7	0.26
control	ND*	-	-0.00001	0.000035

**ND- No detection*

The U concentrations in the depuration water (Table 9), originates from the leaching of U from the eggs previously exposed to U. The U concentration in the depuration water for egg exposed to U was all higher than the U measured in the control water after both 3 and 7 days. The U concentration in the water added eggs exposed to uranyl was considerably higher than the other two water samples exposed to U, this concentration also increased from 3 to 7 days. This strengthens the belief that the depuration of U from eggs exposed to uranyl was more rapid than for the eggs exposed to U NPs.

3.6 Bioconcentration factor (BCF)

Bioconcentration factor (BCF) was calculated for the U concentration of the water analyzed before exposure, versus the U concentration of the eggs sampled after 3 and 24 hours (Table 10). The calculations were performed with Equation 2.

Table 10: Calculated bioconcentration factors of the U concentration in the eggs, versus the U concentration in the surrounding water for both dissected and total eggs, before and after exposure (total water fraction). The BCF before were calculated with the water analyzed before exposure versus the egg samples taken after 3 and 24 hours.

	Average bioaccumulation factors			
	Total, 3 hours (l/g)	Dissected, 3 hours (l/g)	Total, 24 hours (l/g)	Dissected, 24 hours (l/g)
UO2	1.72	0.0035	1.78	0.11
U3O8	1.00	0.0035	1.12	0.017
Uranyl	1.07	0.041	1.85	0.26

The calculated BCF shows that the uptake of U by eggs increases over time (Table 10). It also shows that the BCF calculated for the whole eggs was ~40 % higher for the eggs exposed to UO_2 than for the other eggs after 3 hours. It does seem as though there was not a considerable difference in bioaconcentration factor for the whole eggs after 24 hours. For the dissected eggs, on the other hand, the BCF was larger for the eggs exposed to uranyl than for the eggs exposed to U NP by ~90 % after 3 hours and by ~57 % (UO_2) and ~93 % (U_3O_8) after 24 hours. Apparently, more UO_2 was present on the eggs surface after 3 hours than U_3O_8 or uranyl. It was also evident that the eggs took up more U into the interior when exposed to uranyl, than to U NP. The BCF, in dissected eggs, was a factor of ten higher for the eggs exposed to UO_2 than U_3O_8, after 24 hours; this suggests that UO_2 was more bioavailable to the eggs than U_3O_8. This was different to what was seen in a similar study on availability of U NP to earthworms (Basnet, 2011), possibly due to a greater fraction being present as ionic or suspended NP fraction.

4 Conclusion

The particles synthesized were found to be in the nanomaterial definition according to the European Commission's guide line. Hence it can be concluded that two types of U NPs were successfully synthesized. The TEM images show that most of the UO_2 were present as circular particles, while the U_3O_8 formed rectangular rods. It was also found that the preferable preparation method for the synthesized particles was dialysis, as opposed to freeze-drying. This treatment resulted in a suspension containing more particles in the NP fraction, than the suspension treated with freeze-drying. It was also found that Tween 20 reduced aggregation of the particles, and that ultrasonic agitation successfully dissolved aggregates and agglomerates. The TEM images of the U NPs showed the tendency for larger particles to form aggregates and agglomerates more readily than smaller particles. Based on TEM observations of suspensions after exposure, it appears that organic matter induced particle growth and aggregation, both in the NP suspensions, and in the uranyl solution. TEM micrographs of 0.2 µm filtrated suspensions showed that many of the smaller particles (<100 nm) also were retained in the filter, probably because of clogging.

Overall the measurement of U showed large variation, especially in the NP suspensions. This made it difficult to say anything certain about the U concentration in the water. All of the U in the solution containing uranyl, was in a LMM form before exposure, but after exposure, more was as the HMM form. This indicates that uranyl was unstable in the exposure solution and supports the hypothesis that the organic matter induces particle formation. In the NP suspension, a much greater fraction of U was in a HMM form. A large part of the U in the NP suspensions (85-90 %) was >0.2 µm in size before exposure, although the exact percentage is not known due to possible filter clogging. Similar results were obtained by centrifugation, although co-settling of small particles with larger aggregates is of concern. The centrifuged (supernatant) fraction remained relatively stable during exposure.

Both of the synthesized materials had large numbers of small particles (1-100 nm) in suspension. Even though the concentration of particles in the >0.2 µm fraction was measured to be high (~85-90 % before exp. and ~60-80 % after exp.), the number of particles between 1-100 nm was above 60 % for both suspensions both before and after exposure. According to the European Commission's definition, the synthesized material is therefore nanomaterial.

Uranium in the water does not seem to have affected the swelling or fertilization of the eggs. It does however appear as though it altered the round regular shape of the eggs, but whether this is of concern for the development of the eggs cannot be said on the basis of the present findings. Measurement of U in fish eggs showed good precision and comparison with CRM showed good accuracy. The NP exposed whole eggs took up most of the U during the first 3 hours, and most of this U was deposited on the outer shell of the eggs. Most of the U that was taken up into the interior of the eggs exposed to U NP, was taken up between the 3 and 24 hour sampling. The BCF of dissected eggs exposed to UO_2 after 24 hours was about ten times greater than the same BCF for U_3O_8; this suggests higher bioavailability for UO_2 towards fish eggs than U_3O_8. The eggs exposed to uranyl, on the other hand, took up U during the whole exposure of 24 hours, uranyl exposed eggs also took up a higher relative amount into the interior of the eggs. The BCF was however largest for the UO_2 exposed eggs, but this was only the case for the whole eggs. For the dissected eggs, the BCF was largest for the uranyl exposed eggs.

The conclusion is hence that U measured in eggs exposed to U NPs, is found mostly on the exterior of the eggs, as deposited particles on the eggs surface, rather than being taken up by the eggs. Eggs exposed to uranyl take up more U into the eggs than the eggs exposed to U NPs. This supports the hypothesis that uranyl is much more bioavailable than U NPs. The eggs exposed to uranyl, seemed to release U more rapidly during depuration than the eggs exposed to U NPs. This can be different in nature, as the NPs deposited on the eggs exterior might decrease as a response to natural water flow.

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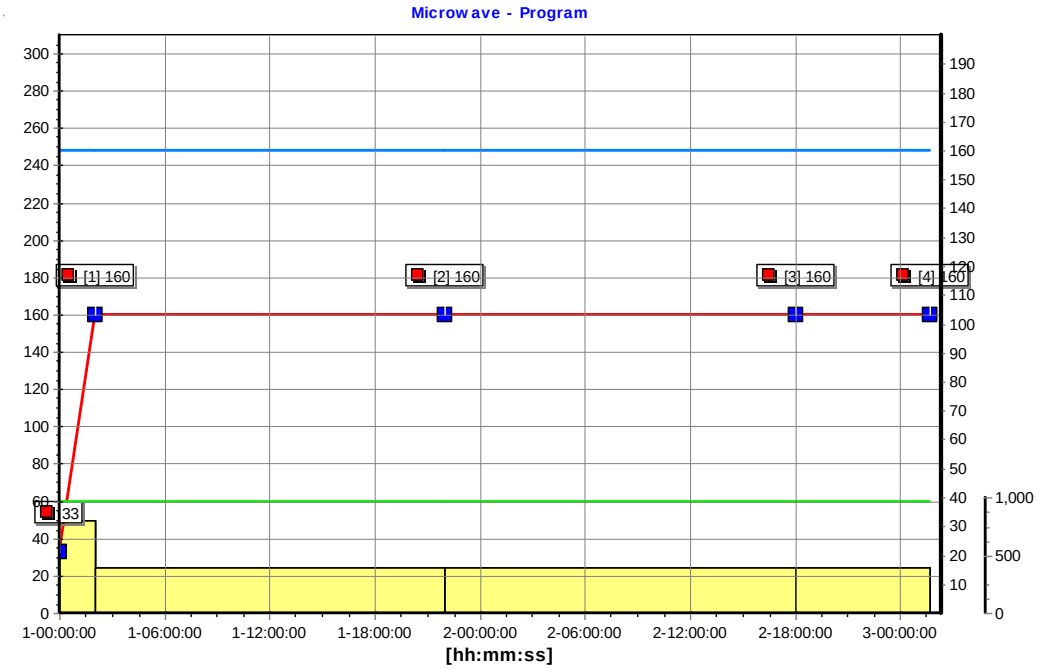
Appendix

Appendix 1

UltraClave program

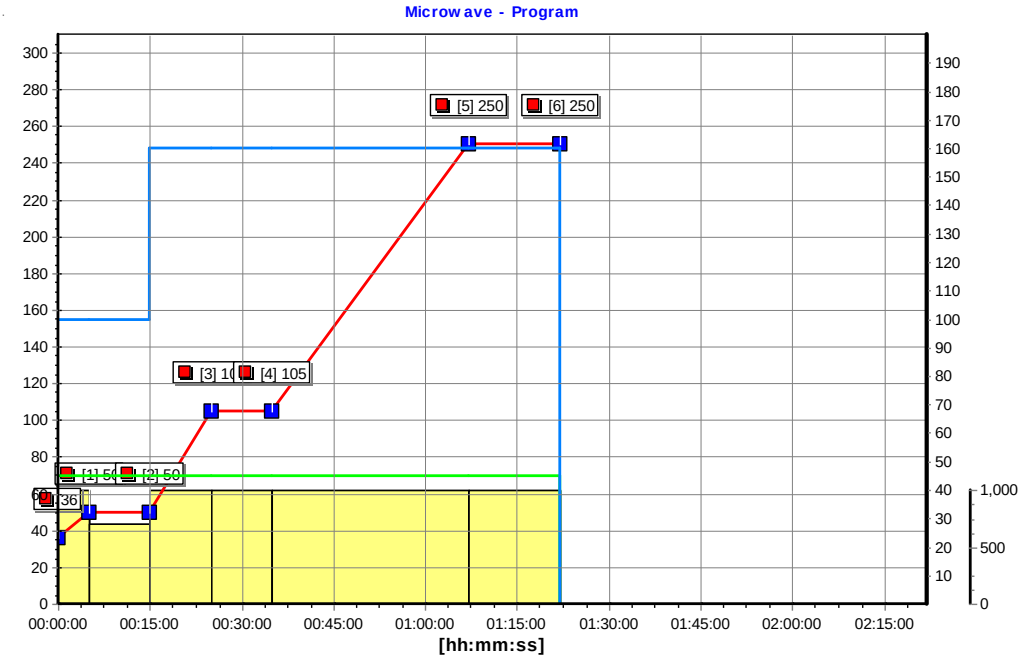
Appendix 1.1

The figure shows the microwave program used to synthesize U NPs. The diagram shows the temperature, power, time and pressure during synthesis



Appendix 1.2

The figure shows the temperature, graph, time and power in the microwave program used to decompose egg and water samples.



Appendix 2
ICP-MS measurements

Appendix 2.1
Mean measured U concentrations in water samples, resulting from ICP-MS analysis.

Fraction	Exposure media	Before exposure		After exposure		After 3 days depuration		After 7 days depuration	
		Average U concentration, mg/l	Standard deviation	Average U concentration, mg/l	Standard deviation	Average U concentration, mg/l	Standard deviation	Average U concentration, mg/l	Standard deviation
Tot	UO2	48	2.1	9	1.9	0.08	0.093	0.07	0.014
Tot	U3O8	57	9.2	12	7.5	0.017	0.0050	0.03	0.012
Tot	uranyl	61	3.6	46	3.3	0.31	0.078	0.7	0.26
Tot	control	0.00006	0.0	0.0003	0.00041	-0.00267	0.0	-0.00001	0.000035
Tot	control + tween	0.000020	0.000017	0.0003	0.00020	-	-	-	-
Tot	UO2 Water contr.	-	-	14	1.4	-	-	-	-
Tot	U3O8 water contr.	-	-	30	5.1	-	-	-	-
<0.2 µm	UO2	7.00	0.059	3.16	0.082	-	-	-	-
<0.2 µm	U3O8	5.7	0.27	2.1	0.40	-	-	-	-
<0.2 µm	uranyl	66	3.2	21	1.5	-	-	-	-
<0.2 µm	control	0.0005	0.00023	0.00016	0.000017	-	-	-	-
<0.2 µm	UO2 Water contr.	-	-	6.52	0.099	-	-	-	-
<0.2 µm	U3O8 water contr.	-	-	4.7	0.32	-	-	-	-
Centr.	UO2	6.3	0.50	2,86	0,095	-	-	-	-
Centr.	U3O8	1.7	0.11	1.4	0.46	-	-	-	-
Centr.	uranyl	67	5.1	7	1.6	-	-	-	-
Centr.	control	0.00011	0.000062	0.0	0.00047	-	-	-	-
Centr.	UO2 Water contr.	-	-	6.6	0.76	-	-	-	-
Centr.	U3O8 water contr.	-	-	2.2	0.17	-	-	-	-
< 3 kDa	UO2	4.1	0.15	0.26	0.070	-	-	-	-
< 3 kDa	U3O8	0.61	0.049	0.07	0.018	-	-	-	-
< 3 kDa	uranyl	75.8	0.25	2.6	0.20	-	-	-	-
< 3 kDa	control	0.00009	0.0	0.00003	0.000030	-	-	-	-
< 3 kDa	UO2 Water contr.	-	-	4.2	0.39	-	-	-	-
< 3 kDa	U3O8 water contr.	-	-	0.67	0.022	-	-	-	-

Appendix 2.2

Mean measured U content in total and dissected eggs, from ICP-MS analysis. The values were obtained by dividing the U content measured by the ICP-MS was divided by the weight of the eggs.

Specie	Replicate	Sampling time	Mean µg U/g tot. egg	std.	Mean µg U/g diss. Egg	std.	Av. % U in eggs interior (Udiss/Utot%)
UO2	I	3 h	66	9.3	0.21	0.096	0.2
UO2	II	3 h	80	31	0.13	0.068	0.1
UO2	III	3 h	100	19	0.2	0.10	0.1
U3O8	I	3 h	31	5.3	0.4	0.20	0.6
U3O8	II	3 h	68	4.2	<Loq	0.067	-
U3O8	III	3 h	70	13	<Loq	0.24	-
Uranyl	I	3 h	70	20	3.5	0.50	3.1
Uranyl	II	3 h	70	14	2	1.1	1.5
Uranyl	III	3 h	53	6.6	2.0	0.93	1.8
0	I	3 h	0.01	0.011	0.0032	0.00095	-
0	I	3 h	0.005	0.0014	0.005	0.0040	-
0	III	3 h	0.003	0.0019	0.006	0.0034	-
UO2	I	24 h	60	26	1	1.1	0.4
UO2	II	24 h	60	20	0.6	0.17	0.6
UO2	III	24 h	130	27	1.3	0.2	0.6
U3O8	I	24 h	53	3.3	0.14	0.075	0.1
U3O8	II	24 h	70	14	0.2	0.13	0.1
U3O8	III	24 h	65	2.5	0.12	0.025	0.1
Uranyl	I	24 h	120	13	18	6.7	10.1
Uranyl	II	24 h	110	13	11	1.7	6.0
Uranyl	III	24 h	100	8.8	11	8.3	7.1
0	I	24 h	0.0040	0.00078	0.006	0.0013	-
0	II	24 h	0.005	0.0037	0.011	0.0082	-
0	III	24 h	0.0032	0.00087	0.02	0.019	-
0+Tween	I	24 h	0.005	0.0019	0.01	0.010	-
0+Tween	II	24 h	0.002	0.0013	0.004	0.0012	-
0+Tween	III	24 h	0.0020	0.00049	0.004	0.0012	-
UO2	I	3 d	60	20	0.47	0.090	0.6
UO2	II	3 d	110	15	1.1	0.47	0.7
UO2	III	3 d	100	34	0.8	0.60	0.4
U3O8	I	3 d	70	24	0.12	0.032	0.1
U3O8	II	3 d	62	7.1	0.21	0.017	0.2
U3O8	III	3 d	68	4.8	0.13	0.075	0.1
Uranyl	I	3 d	100	11	25	9.7	16.8
Uranyl	II	3 d	96	8.4	14	4.8	8.9
Uranyl	III	3 d	90	11	11	1.2	8.1
0	I	3 d	0.005	0.0041	0.008	0.0029	-
0	II	3 d	0.003	0.0013	0.0035	0.00080	-
0	III	3 d	0.0021	0.00059	0.0032	0.00062	-
UO2	I	7 d	47	8.6	0.2	0.077	0.3
UO2	II	7 d	70	31	0.7	0.13	0.9
UO2	III	7 d	110	26	1.0	0.17	0.5
U3O8	I	7 d	60	12	0.11	0.026	0.1
U3O8	II	7 d	60	30	0.5	0.63	0.7
U3O8	III	7 d	80	15	0.6	0.66	0.6
Uranyl	I	7 d	83	5.8	9	2.2	4.7
Uranyl	II	7 d	84.8	0.97	9	2.3	5.5
Uranyl	III	7 d	103	9.1	9.3	0.19	4.9
0	I	7 d	0.0040	0.00058	0.005	0.0013	-
0	II	7 d	0.0028	0.00012	0.0029	0.00033	-
0	III	7 d	0.0026	0.00031	0.0033	0.00052	-

**The control samples are really below LOD, but since the concentrations were used in the statistical analysis, the numbers are displayed in the table.*

Appendix 2.3

Values for ^{238}U in certificate for the CRM (IAEA, 2009), specific activity for ^{238}U, amount of CRM added to the three samples, calculated ^{238}U in these samples, using the values for ^{238}U in certificate for the CRM and the specific activity for ^{238}U, and the mean measured ^{238}U in the samples with ICP-MS.

	CRM U238
Bq/g in CRM	0.00095
Bq/g in U	12455
Mean added CRM, g	0.2009330
Mean U in CRM samples, µg	0.01533
Mean measured U	0.015 ± 0.0011

Appendix 2.4

Mean U concentration in blank samples measured with ICP-MS, calculated std, Loq (10xstd) and Lod (3xstd).

	U concentration mg/l
Mean U in blanks	0.0181
Std	0.020813
Loq	0.208134
Lod	0.06244

Appendix 3

Particle diameter sizes of; UO_2, U_3O_8 and uranyl, measured from TEM images. Presented as mean, minimum, maximum and std. The number percentage of particles between 1-100 nm is also calculated and shown in the table. (The particles observed in the uranyl suspensions might, however, not consist of U. This could be determined by x-ray microanalysis.)

Statistical Function	**Total samples before exp.**			**<0.2 samples before exp.**			**Total samples after exp.**		
	UO2	**U3O8**	**Uranyl**	**UO2**	**U3O8**	**Uranyl**	**UO2**	**U3O8**	**Uranyl**
Count, (number of particles)	1060	540	109	356	689	12	1057	838	277
Mean, (nm)	198	85	164	77	46	71	519	152	249
Minimum (nm)	0.9	1.8	17.2	5.4	1.5	9.8	0.8	0.7	4.7
Maximum (nm)	3535.8	2270.1	2145.1	1011.0	2682.8	286.0	3713.4	3402.6	3691.6
Standard Deviation (nm)	345	169	251	103	132	77	1001	419	668
Percentage of particles between 1-100 nm (%)	61	80	61	78	85	83	76	75	85