

2015

Final Executive and Technical
Report for UR-Clark



Jodi Schilz

University of Wyoming

2/9/2015

Contents

Final Executive Report	4
Final Technical Report.....	17
Abstract.....	17
Introduction	18
Objectives	19
Specific Aim #1. Determine the toxicity of production bleed water before and after treatment with cupric oxide nanoparticles** for the removal of arsenic.	19
Specific Aim #2. Evaluate the ability of a cupric oxide nanoparticle removal system to decrease toxicity using a continuous flow method.	20
Specific Aim #3. Comparison of toxicity data compiled from Aims 1 and 2 with decontamination systems used in industry and analysis of the beneficial uses according to published water standards	21
Methods.....	21
Experimental design and brief experimental procedure for specific aim 1.....	21
Collection of production bleed water samples.....	21
Cupric Oxide nanoparticles**.....	22
Determination of production bleed water constituents.	22
Geochemical Modeling.....	22
Media.....	22
Cell Viability.....	23
Inhibitory Concentration 50 (IC50).	24
Flow Cytometry.....	24
Glutathione Oxidation.....	25
Oxidative Stress.....	26
TUNEL Assay.....	26
Data Analysis.....	27
Experimental design and brief experimental procedure for specific aim 2.....	28
Analysis of arsenic removal by cupric oxide nanoparticles using a continuous flow column.	28
Experimental design and brief experimental procedure for specific aim 3.....	29

Comparison of toxicity data.....	29
Experimental design and brief experimental procedure for bacterial analysis.....	29
Native PBW bacteria: Batch analysis of growth inhibition by CuO-NPs.	29
Flow-through analysis of growth inhibition by CuO-NPs of endogenous PBW bacteria.....	30
Metals.	31
Spiked E. coli growth inhibition: Flow-through analysis using CuO-NPs.	31
Timed Aliquots.	31
Copper and Metals.....	32
Identification of native bacteria in PBW.	32
Results.....	33
Technical Information.....	33
Collection and analysis of PBW.....	33
CuO-NP batch-treatment of PBW.....	37
Cytotoxicity evaluation of untreated and CuO-NP-treated PBW.....	46
Bactericidal Effects of CuO-NP.....	53
Batch assessment of native PBW bacteria.....	54
Identification of native PBW bacteria.....	55
Flow-through assessment of endogenous PWB bacteria.....	55
Flow-through assessment of spiked E. coli in buffered saline and PBW.....	58
Flow-through assessment of metal concentrations during bacterial studies.....	64
Flow-Through Metal Adsorption Data.....	68
Conclusions and recommendations.....	75
Summary of Findings.....	75
Appendix.....	82
Abstracts of resulting theses/dissertations.	82
Schilz, Jodi R., Investigating the ability of cupric oxide nanoparticles to adsorb metal contaminants from uranium in-situ recovery production bleed water and assessing the associated changes in cytotoxicity., PhD., Department of Biomedical Sciences/Reproductive Biology, May 2014.	82
Full citations for resulting publications and for manuscripts submitted for publication.	84
Schilz, J.R., Reddy, K.J., Nair, S., Johnsons, T.E., Tjakens, R.B., Krueger, K.P. and Clark S, Removal of Trace Elements by Cupric Oxide Nanoparticles from Uranium In Situ Recovery Bleed Water and Its Effects on Cell Viability. JoVE, In Press 2015.....	84
Scientific collaborators.....	84

Description of inventions..... 85

Other information that was specifically required by the terms of the award..... 85

Final Budget **Error! Bookmark not defined.**

Bibliography 85

Final Executive Report

**Proprietary Information: Cupric oxide nanoparticles, K.J. Reddy. U.S. Patent # 7,235,179 & #7,897,052

In Wyoming, the uranium *in situ* leaching (ISL) process involved infusing native ground water with an oxidizing agent to produce a leaching solution known as the lixiviant. The lixiviant is then pumped through injection wells into the ore body to oxidize and mobilize the uranium. The uranium-rich lixiviant migrates through the pore spaces in the ore body, mobilizing uranium, as well as arsenic, selenium, vanadium, iron, manganese, radium, and other minerals. The lixiviant moves through the sandstone and is recovered by production wells. Production wells pump the uranium-rich lixiviant to the surface, where the uranium can be recovered by ion exchange, capturing uranium onto resin beads. (U.S. Nuclear Regulatory commission, 2009, sec. 2.4) After removal of uranium by ion exchange, about 1-3% of the lixiviant, now termed production bleed water (PBW), is removed from the circuit. PBW can, depending on location and regulations, be discharged to an evaporation pond or treated, usually by RO filtration, and the treatment waste (brine) is deep well injected. (U.S. Nuclear Regulatory commission, 2009, p. 2.4.3) In the U.S., uranium deposits exist in arid states, where water is of great value. Investigation of alternative treatments that would remove only toxic contaminants, allowing the re-use of PBW for agriculture or industry, while at the same time creating less waste for disposal, would benefit the community and industry.

Production bleed water is a complex mixture of heavy metals and salts that have been leached from the ore body. Mixtures pose their own challenges with respect to treatment, as well as assessment of their toxicity. One of our research objectives was to

use laboratory techniques to test effects of mixtures of environmentally important chemicals, such as arsenic, cadmium, chromium and mercury. Heavy metals often pose an issue for areas such as Wyoming, due to their naturally high levels in the soil and rock. One major challenge for water treatment, including mining waste water, is removal/reduction of toxic metals (U.S. Environmental Protection Agency, 2000, Chapter 3). Metal-contaminated waste water must be treated before discharge into the environment, due to the high solubility of metals in water, which allows them to diffuse into the environment, be absorbed by living organisms and plants, and enter the food chain (Bae, Gennings, Carter, Yang, & Campain, 2001; Buschmann et al., 2008; Cui et al., 2005).

Common techniques to treat metal-contaminated waste water are chemical precipitation, membrane filtration, ion-exchange, adsorption, coagulation-flocculation, flotation, electrochemical methods and reverse osmosis (Fu & Wang, 2011). Reverse osmosis (RO) is a continuous-flow water purification process that uses semipermeable membranes to separate dissolved solids from the solution, which, for ISR, is usually groundwater. RO uses hydrostatic pressure to overcome osmotic pressure, pushing water through a pore that is small enough to restrict the passage of larger molecules and ions. Due to the fine pore size, RO removes practically all constituents from the water, including minerals that would otherwise not be considered harmful (Kucera, 2010). Thus, the amount of solid waste from RO filtration is more substantial because it includes both toxic and non-toxic components. A removal method that prioritizes the toxic elements would reduce waste and increase the possible uses of this waste stream. This project applied nanoparticles to this waste water to investigate changes in the mixture toxicity after priority contaminants were removed.

Nanotechnology is revolutionizing many technologies and industry sectors, including information technology, energy, medicine, food safety, transportation, and environmental science (United States National Nanotechnology Initiative, 2014). Nanoparticles show unique size-dependent physical and chemical properties based on their large surface-area-to-size ratio. The reactive properties of nanoparticles are influenced by their chemical composition and shape.

There are organic nanoparticles (e.g. carbon nanotubes and polymeric micelles) and inorganic nanoparticles that are often made of metals (e.g. gold and copper) (Sanvicens & Marco, 2008). Nanoparticles are used as adsorbents, due to their high porosity and surface areas, which make them highly effective (Bhatnagar & Minocha, 2006). The function of these pores is to increase adsorption capacity (Shao, Lu, Zhang, & Pan, 2013). One of the greatest benefits of nanotechnology arises from the ability to incorporate nanoparticles into the structure of everyday materials, from eyeglasses to solar panels. These additions allow tailoring of the properties of materials, making them stronger, lighter, more durable and more efficient (United States National Nanotechnology Initiative, 2014). Nanotechnology is also being applied to the environmental remediation of drinking water in both large scale and portable applications. Cupric oxide nanoparticles researched and developed by K.J. Reddy, University of Wyoming, were used in this project due to their unique properties.

Cupric oxide nanoparticles (CuO-NPs) (Reddy, 2007, 2011) are new tools in the fight to treat contaminated groundwater. The ability of copper (II) oxide as an adsorbent for arsenic has been investigated (Goswami, Raul, & Purkait, 2011; Martinson & Reddy, 2009).

CuO-NPs may prove to be more versatile than conventional adsorbents such as oxides of aluminum, iron and manganese. CuO-NPs do not require pH adjustment or oxidation of water for arsenic removal, and CuO-NPs remove both arsenite and arsenate in the presence of the competing anions phosphate, silicate and sulfate (Reddy, McDonald, & King, 2013). Also, CuO-NPs can be regenerated and re-used, reducing reagent costs and the amount of spent media in need of disposal (Reddy et al., 2013).

Metals are the most abundant elements in the environment. Some metals are essential to biological systems (copper, manganese, iron), while other metals (arsenic, cadmium, vanadium) have been linked to cellular damage, inflammation and cancer (Flora, 2011; Leonard, Harris, & Shi, 2004). In general, the principles of metal toxicity are thought to be due to the simultaneous insults of increased reactive species formation and the depletion of antioxidant protection (Franco, Sánchez-Olea, Reyes-Reyes, & Panayiotidis, 2009).

There were two interconnected goals to this project. The first was to test the efficacy of CuO-NPs to remove contaminants from ISL PBW. We knew that CuO-NPs could remove arsenic and selenium from groundwater (Reddy & Roth, 2012). However, ISR PBW is more complex than most groundwater and is often called “enriched” groundwater. The basic components of groundwater are present in PBW (i.e., metals and salts), but at higher concentrations and at a lower pH. The decreased pH is one of the challenges associated with the decontamination of PBW. The second goal was to demonstrate changes in the cytotoxicity of PBW, before and after CuO-NP treatment, through *in vitro* cell culture methods. A third goal was added to the project to investigate the possible bacteriostatic

properties of CuO-NPs and their ability to remove metal contaminants and influence bacterial growth - both during the same treatment step.

Results
PBW Elements

Results showed that PBW contained 30-plus components that are leached from the ore body during uranium extraction and that then remain in the PBW after the uranium has been removed by ion exchange. Table 1 shows PBW component concentrations (Schilz et al., 2015).

Elements	Concentration (mg/L)	Elements	Concentration (mg/L)	Anions	Concentration (mg/L)
Aluminum	<0.001	Mercury	0.00033	Alkalinity-Bicarbonate	1960
Antimony	<0.001	Molybdenum	0.004	Sulfate	610
Arsenic	0.020	Nickel	0.011	Chloride	32
Barium	0.110	Platinum	<0.001	Ammonia-N	<0.1
Beryllium	<0.001	Potassium	9.9	Nitrate-N	<0.05
Boron	0.075	Selenium	1.3	Nitrite-N	<0.05
Cadmium	<0.001	Silicon	12	Fluoride	<0.1
Calcium	190	Silver	<0.001	Phosphate	0.3
Chromium	<0.001	Sodium	690	TDS	2610
Cobalt	0.003	Strontium	3.4	Conductivity (µmhos/cm)	3560
Copper	0.020	Thallium	<0.001	pH (units)	6.9
Iron	<0.020	Thorium	<0.001		
Lead	<0.001	Uranium	0.820		
Lithium	0.096	Vanadium	0.910		
Magnesium	36	Zinc	0.006		
Manganese	0.280				

Table 1 – Concentrations of elements in PBW.

PBW Elements Concentrations after Treatment

Treatment of PBW with CuO-NP removed priority contaminants, including arsenic, selenium, uranium and vanadium (Table 2). The average arsenic concentration was reduced by 87% [(from 0.017 to 0.002 mg/L (two-tailed paired t-test, $p < 0.0001$)]. CuO-NP treatment also significantly reduced selenium (30%), uranium (78%), and vanadium (92%), and phosphate (85%) ($p < 0.05$). CuO-NP treatment also reduced the phosphate concentration. Copper concentration increased from 0.0015 to 0.926 mg/L after CuO-NP treatment.

Element (mg/L)	Average, SD. & Significance		
	Before Treatment	After Treatment	
Arsenic	0.0175 ± 0.001	0.002 ± 0.0005	*
Selenium	1.75 ± 0.071	1.25 ± 0.058	*
Copper	0.0015 ± 0.001	0.926 ± 0.438	*
Calcium	101.5 ± 82.731	105.75 ± 15.882	
Strontium	3.3 ± 1.131	1.5215 ± 0.455	*
Magnesium	44.5 ± 2.121	47.25 ± 1.708	
Sodium	610 ± 0.000	627.5 ± 27.538	
Uranium	0.9755 ± 0.035	0.208 ± 0.037	*
<i>Manganese</i>	<i>0.00475 0.006</i>	<i>0.331 ± 0.032</i>	*
Barium	0.037 ± 0.025	0.019 ± 0.010	
Potassium	12 ± 0.000	12 ± 0.816	
Silicon	11.5 ± 0.707	11.5 ± 0.577	
Vanadium	1.25 ± 0.071	0.1025 ± 0.021	*
Aluminum	0.037 ± 0.010	0.011 ± 0.008	*
Boron	0.085 ± 0.003	0.0885 ± 0.003	
Molybdenum	0.004 ± 0.000	0.00375 ± 0.001	
Phosphate	0.35 ± 0.071	0.05 ± 0.00	*
Sulfate	805 ± 21.213	807.5 ± 15.000	
Chloride	46.5 ± 0.707	55.25 ± 8.180	
Conductivity	3125 ± 143.350	3190 ± 62.282	
pH	7.315 ± 0.092	7.36 ± 0.051	

Table 2 – Changes in element concentrations after treatment of PBW with CuO-NPs

Flow-through Experiments

Our studies demonstrated that CuO-NPs remove priority contaminants from ISR uranium PBW using a batch treatment method (Schilz, 2013). Further studies show that CuO-NPs are also effective in a flow-through reactor column method. Although pH did affect the release of copper, CuO-NPs were effective at two different pHs encountered in the industrial samples, showing consistent removal of arsenic, selenium and vanadium, despite the solution pH.

Bacterial Experiments

The research described here investigates the ability of CuO-NPs to reduce metal contaminants from a complex waste water solution, while decreasing the ability of the bacteria to grow and replicate after treatment. Two types of bacteria are investigated: bacteria native to the PBW (present from the ISR process) and *E. coli* spiked into phosphate buffered saline (PBS), or into PBW, as an indicator of the efficacy of CuO-NP water treatment to decrease viability of coliform bacteria. Experiments were conducted using batch (endogenous) and flow-through (endogenous and exogenous bacterial addition) methods. The results presented here are preliminary and require confirmation, but they showed a reduction in both endogenous bacterial number and reduction in *E. coli* spiked into PBW. This suggests that a one-step process may be possible with CuO-NPs. This may also be true, regardless of the underlying mechanism by which the bacteria are removed (e.g., via direct bacteria-CuO-NP interactions or through the effect of increased (?released?) copper

on bacteria survival). A limiting factor may be the high level of copper that is released in some situations, for levels can surpass EPA-allowed levels for drinking water.

***In Vitro* Testing**

Several *in vitro* cytotoxicology methods were identified in the literature as being potentially useful. The first step toward understanding how an agent will react in the human body often involves cell culture studies. Cell culture studies can be quick, easy to reproduce and inexpensive, especially when compared to *in vivo* animal studies (Lewinski, Colvin, & Drezek, 2008). Cytotoxicity can be assessed using assays of viability, reactive oxygen species, DNA damage and eventual cell death. Reactive oxygen species disrupt cellular processes, leading to cellular stress and dysfunction. ROS is assessed either directly by measuring the amount of ROS present in a cell or indirectly by measuring the secondary effects of oxidative stress. A common result of cellular stress and toxicity is DNA damage. DNA damage and cellular stress lead to programmed cell death or suicide. Viability is an indicator of overall cell health, including metabolic health.

Cell viability measured with the MTT assay showed decreased viability of both cell types when grown in untreated PBW. Treatment of the PBW with CuO-NP and subsequent removal of the elements listed above improved cell viability in a concentration-dependent manner. A picture of healthy cells grown in normal media is shown in Figure 1 (A), unhealthy cells grown in untreated PBW (B) and cells grown in CuO-NP treated PBW (C). Cells grown in PBW treated with CuO-NPs have healthier morphology, better attachment and growth compared to cells grown in untreated PBW.

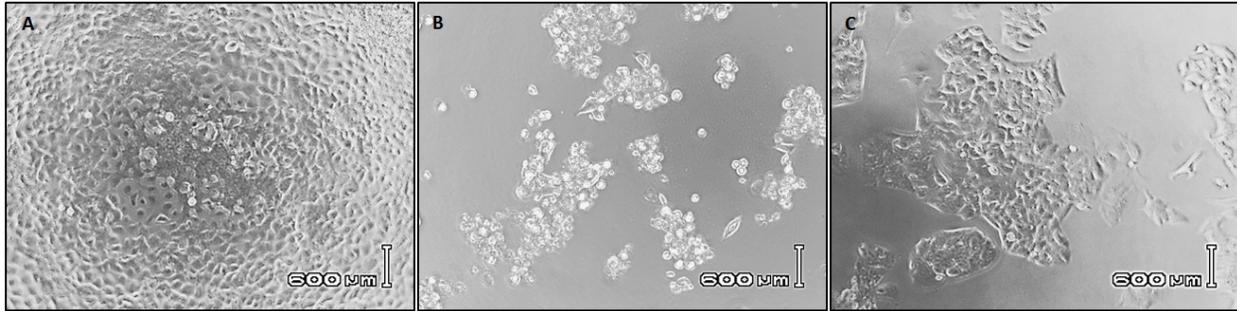


Figure 1 – Cellular growth of human kidney cells in normal growth medium (A), in PBW (B), and in PBW after treatment with CuO-NPs (C). Imaged with bright field microscopy (20X).

Measurements of reactive oxygen species (ROS) showed no significant difference in the amount of ROS produced between untreated or CuO-NP-treated PBW. Measurements of antioxidant consumption also showed no significant difference between untreated PBW+media and CuO-NP-treated PBW: Neither the DCFDA or GSH/GSSG ratio assays showed a significant difference between treated and untreated PBW, however CuO-NP-treatment did not increase the amounts of oxidative stress or GSH oxidation. The removal of contaminants such as uranium, selenium and vanadium may allow interactions of the remaining metals, inducing similar amounts of oxidative stress and antioxidant depletion as untreated PBW. Another explanation is that induction of ROS production and GSH oxidation may have occurred at an earlier time point. Metals can produce both apoptosis (programmed cell death) and necrosis (destructive cell death) (Sabolić, 2006). Flow cytometry analysis assessing apoptosis and necrosis revealed that a large number of cells are rendered necrotic by either the treatment or the extraction methods used to harvest the cells for analysis. However, for apoptosis, the percentage of cells undergoing apoptosis (Annexin V+) increased significantly with time in both the HEK and HEP cells exposed to

untreated PBW, whereas CuO-NP treatment reduced the number of cells undergoing apoptosis. DNA damage assay showed an increase in DNA fragmentation in HEK and HEP cells exposed to untreated PBW+media compared to CuO-NP treated PBW+media. However the levels of DNA damage were very low in both test media. Morphology results previously reported (Schilz, 2013) showed detachment of apoptotic bodies from the culture surface in HEK cells. Therefore, TUNEL methods may not accurately reflect DNA damage, due to removal of the fragmenting DNA during the staining procedure.

pH

The pH of the PBW was the greatest issue faced during experimentation. pH affected the amount of copper released and the amount of uranium removed. There also is a considerable difference in the amount of copper released from the CuO-NP at the pH of 6.67, compared to the higher pH 7.36. As was seen in batch method, at the lower pH, significantly higher amounts of copper are released from the CuO-NPs (5570 µg/L), well over the EPA maximum contamination limit (MCL) for copper (1300 µg/L) (United States Environmental Protection Agency, 2011). Also, the longer the flow-through process continued, the more copper was released from the CuO-NPs. Also, uranium removal was increased at a higher pH. However, removal of the other priority contaminants was not significantly affected by the pH.

Conclusions

Our study is the first to test the efficacy of CuONPs in the removal of priority contaminants from PBW and its effect on cultured human cells grown in the actual

environmental sample: a complex mixture of metals produced during uranium ISR. Treatment of uranium ISR PBW with CuO-NPs effectively removed priority contaminants, including arsenic, uranium, selenium, and vanadium. CuO-NP treatment altered the cytotoxicity of PBW, assessed by human cell culture methods.

Studies of chronic, low-dose mixture exposures are challenging. Although short-term, high level exposures to chemicals produce well-recognized acute syndromes, long-term, low-dose exposure may not produce obvious clinical effects, at least not initially, until an “excess” is accumulated and normal function is lost (Goyer, 2004). Also, most *in vitro* studies of chemical mixtures expose cells or tissues to a lab-made mixture of 2 or more metals, and often use concentrations that are above typical environmental levels (Bae et al., 2001; Wang & Fowler, 2008). However, these simplified mixtures do not duplicate possible antagonistic and synergistic interactions that may occur in a native, environmental sample, where the full range of mixture components is present.

In vitro testing of heavy metals has shown that metals can induce apoptosis and necrosis, produce oxidative stress, and decrease antioxidant levels (Flora, 2011; Valko et al., 2007; Valko, Morris, & Cronin, 2005a). Studies have attempted to quantify the toxicity of a complex waste mixture at environmentally relevant concentrations using whole organisms, such as fish, plants, rats and *Daphnia magna* (Antunes, Pereira, & Gonçalves, 2007; Farombi, Akintunde, Nzute, Adedara, & Arojojoye, 2011; Klauck, Rodrigues, & Basso da Silva, 2013). Also, several labs have investigated the ecotoxicological impact of water contaminated from abandoned uranium mines (Antunes, de Figueiredo, et al., 2007; Antunes, Pereira, et al., 2007; Marques, Gonçalves, & Pereira, 2008). However, to our knowledge, there are no

reports of comparisons of the cytotoxicity of uranium waste water before and after treatment with CuO-NPs on cultured cells.

Similarly, most studies of nanoparticles have focused on their inherent toxicity by applying the nanoparticles, themselves, directly to organisms or cells. For example, several studies report that nanoparticles, when directly applied, can be toxic to bacteria, cultured cells, and animals, and also may have unexpected effects in the environment (Baek & An, 2011; Chen et al., 2006; Hoskins, Cuschieri, & Wang, 2012; Lowry et al., 2010). Although these direct-applications studies are important, most are not focused on testing the end product of nanoparticle treatment, such as water treatment. For example, as discussed above, nanoparticles can decontaminate waste water, due to their increased surface area and high activity levels (Hua et al., 2012). Ideally, water decontamination processes using nanoparticles will develop methods to minimize the escape of nanoparticles (or breakdown NP components) into the final product, in this case the treated water. However, complete NP confinement is difficult, plus metal oxide nanoparticles such as CuO-NPs are subject to pH-dependent leaching of ions.

Water quality can be impacted by both biological and chemical contamination, including bacteria and heavy metals. Bacterial contamination can cause a wide range of waterborne diseases, and metals can produce acute and chronic toxicities. However, certain metals can have bactericidal or bacteriostatic properties, such that they can kill or slow the growth of pathogenic bacteria at metal concentrations that are safe for humans and animals. Furthermore, for certain bacterial-contaminated waters, metal-based treatment

process may provide an advantage, through the action of the very metals used in treatment. Thus, water contaminated with both bacteria and heavy metals may benefit from metal-based nanoparticle treatment methods, for the nanoparticles may adsorb metal contaminants and also affect the ability of the bacteria to grow and reproduce.

CuO-NPs seem to be excellent adsorbents for arsenic and vanadium, regardless of pH. Vanadium is a contaminant in yellow cake in Wyoming and attempts to remove the vanadium during the ISR process have not been successful. CuO-NPs could be useful in ISR vanadium removal, but the pH of the ISR water is normally ~6.9, which causes high concentrations of copper to be pulled from CuO-NPs. Altering the pH of the ISR water would affect the ability of the ion exchange resin to absorb uranium, and therefore more research is needed to assess if CuO-NPs are a viable vanadium removal method in ISR leachate.

Although the pH of ISR water may limit the use of CuO-NPs as a vanadium removal system, this problem would not be encountered in groundwater contaminated with metals. Groundwater pH is usually close to neutral and therefore would not cause release of high concentrations of copper from CuO-NPs during treatment. CuO-NPs have been shown to remove arsenic from groundwater (Reddy & Roth, 2012). Further research will confirm the ability of CuO-NPs to remove vanadium, selenium and arsenic from groundwater, making CuO-NPs an innovative tool for spot-treatment of contaminated groundwater.