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Removal of Trace Elements by Cupric Oxide Nanoparticles from Uranium In situ Recovery Bleed Water and Its Effects on Cell Viability --Manuscript Draft--

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Corresponding Author:	Katta Jayaram Reddy University of Wyoming Laramie, WY UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	katta@uwyo.edu
Corresponding Author's Institution:	University of Wyoming
Corresponding Author's Secondary Institution:	
First Author:	Jodi Rene Schilz, Ph.D.
First Author Secondary Information:	
Other Authors:	Jodi Rene Schilz, Ph.D.
	Sreejayan Nair, Ph.D.
	Thomas E Johnson, Ph.D.
	Ronald B Tjalkens, Ph.D.
	Kem P Krueger, Ph.D.
	Suzanne Clark, Ph.D.
Order of Authors Secondary Information:	
Abstract:	In-situ recovery (ISR) is the predominant method of uranium extraction in the United States. During ISR, uranium is leached from an ore body and extracted through ion exchange. The resultant production bleed water (PBW) contains contaminants such as arsenic and other heavy metals. Samples of PBW from an active ISR uranium facility were treated with cupric oxide nanoparticles (CuO-NPs). CuO-NP treatment of PBW reduced priority contaminants, including arsenic, selenium, uranium, and vanadium. Untreated and CuO-NP treated PBW was used as the liquid component of the cell growth media and changes in viability were determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay in human embryonic kidney (HEK 293) and human hepatocellular carcinoma (Hep G2) cells. CuO-NP treatment was associated with improved HEK and HEP cell viability. Limitations of this method include dilution of the PBW by growth media components and during osmolality adjustment as well as necessary pH adjustment. This method is limited in its wider context due to dilution effects and changes in the pH of the PBW which is traditionally alkaline however, this method could have a broader use assessing CuO-NP treatment in more neutral waters.
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September 14th, 2014

Dear Editor
Journal of Visual Experiments

We were invited to submit our manuscript titled, "Removal of Trace Elements by Cupric Oxide Nanoparticles from Uranium *In situ* Recovery Bleed Water and Its Effects on Cell Viability" for possible publication. This manuscript is prepared by Jodi R. Schilz, K.J. Reddy, Sreejayan Nair, Thomas E. Johnson, Ronald B. Tjalkens, Kem P. Krueger, and Suzanne Clark.

Approximately 20% of the US electrical supply is provided by nuclear power and, based in part on national incentives to increase energy independence; nuclear capacity is expected to increase. In the US, uranium is predominately extracted through an in situ recovery (ISR) process. ISR technology produces significant volumes of production bleed water (PBW). The PBW consists of contaminants such as arsenic, selenium, vanadium and uranium, all of which are above regulatory levels and must be decontaminated before PBW can be discharged into the environment. Agencies including the ASTDR and NIEHS are placing more emphasis on studying the toxicity of environmentally relevant mixtures using *in vitro* testing to prioritize chemicals for further *in vivo* testing. The methods presented in our paper are a new and innovative way of treating complex environmental mixtures and incorporating the actual mixture before and after treatment into the cytotoxicity testing method. JoVE's unique multimedia format is ideal for our methods because it is a new innovative molecular biology/toxicology approach that would be better explained in a multimedia format.

Author Contributions:

Jodi Schilz obtained samples, conducted experiments, and collected and analyzed data. KJ Reddy, Suzanne Clark, Sreejayan Nair and Jodi Schilz wrote the grant that provided funding for this project. Thomas Johnson provided lab facilities during the first year of experiments. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Peer Reviewers (6 requested):

Dr. Saumitra Mukherjee, email: saumitramukherjee3@gmail.com

Dr. Neha Singh, email: hereisneha.singh@gmail.com

Dr. Morris Argyle, email: mdargyle@byu.edu

Carol Martinson, email: mysamuri@hotmail.com

Kyle McDonald, email: kyle.jeffrey.mcdonald@gmail.com

Dr. Pradip Bhattacharyya, email: no-reply@researchgate.net

Thank you for your consideration.

Sincerely

K.J. Reddy

Professor of Ecosystem Science and Management

University of Wyoming, WY

Visiting Research Scholar

Physics Department, Harvard University, MA

TITLE:

Removal of Trace Elements by Cupric Oxide Nanoparticles from Uranium *In Situ* Recovery Bleed Water and Its Effect on Cell Viability.

AUTHORS:

Jodi R. Schilz , K. J. Reddy, Sreejayan Nair, Thomas E. Johnson , Ronald B. Tjalkens, Kem P. Krueger , Suzanne Clark.

AUTHOR'S INSTITUTION(S)/AFFILIATION(S) FOR EACH AUTHOR:

Jodi R. Schilz
School of Pharmacy
University of Wyoming
Laramie, WY 82071 USA
jschilz@uwyo.edu

K.J. Reddy
Department of Ecosystem Science and Management
University of Wyoming
Laramie, WY 82071 USA
katta@uwyo.edu

Sreejayan Nair
School of Pharmacy
University of Wyoming
Laramie, WY 82071 USA
sreejay@uwyo.edu

Kem P. Krueger
School of Pharmacy
University of Wyoming
Laramie, WY 82071 USA
Kkruege1@uwyo.edu

Suzanne Clark
College of Pharmacy
California Northstate University
Elk Grove, California 95757
suzanne.clark.2014@gmail.com

Thomas E. Johnson
Department of Environmental and Radiological Health Sciences
Colorado State University
Fort Collins, CO 80523 USA
thomas.e.johnson@colostate.edu

Ronald B. Tjalkens

Center for Environmental Medicine
Colorado State University
Fort Collins, CO 80523 USA
tjalkens@rams.colostate.edu

CORRESPONDING AUTHORS:

K.J. Reddy
Department of Ecosystem Science and Management
University of Wyoming
Laramie, WY 82071 USA
katta@uwyo.edu

Suzanne Clark
College of Pharmacy
California Northstate University
Elk Grove, California 95757
suzanne.clark.2014@gmail.com

KEYWORDS:

Energy production; uranium *in situ* recovery; water decontamination; nanoparticles; toxicity; cytotoxicity; vitro cell culture

SHORT ABSTRACT:

Production bleed water (PBW) was treated with cupric oxide nanoparticles (CuO-NPs) and cellular toxicity was assessed in cultured human cells. The goal of this protocol was to integrate the native environmental sample into a cell culture format assessing the changes in toxicity due to CuO-NP treatment.

LONG ABSTRACT:

In-situ recovery (ISR) is the predominant method of uranium extraction in the United States. During ISR, uranium is leached from an ore body and extracted through ion exchange. The resultant production bleed water (PBW) contains contaminants such as arsenic and other heavy metals. Samples of PBW from an active ISR uranium facility were treated with cupric oxide nanoparticles (CuO-NPs). CuO-NP treatment of PBW reduced priority contaminants, including arsenic, selenium, uranium, and vanadium. Untreated and CuO-NP treated PBW was used as the liquid component of the cell growth media and changes in viability were determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay in human embryonic kidney (HEK 293) and human hepatocellular carcinoma (Hep G2) cells. CuO-NP treatment was associated with improved HEK and HEP cell viability. Limitations of this method include dilution of the PBW by growth media components and during osmolality adjustment as well as necessary pH adjustment. This method is limited in its wider context due to dilution effects and changes in the pH of the PBW which is traditionally alkaline however, this method could have a broader use assessing CuO-NP treatment in more neutral waters.

INTRODUCTION:

Approximately 20% of the US electrical supply is provided by nuclear energy and, based in part on national incentives to increase energy independence, US nuclear capacity is expected to increase ¹. Worldwide growth of nuclear energy also is expected to continue, with much of the growth occurring outside the US ². As of 2013, 83% of US uranium was imported, but 952,544 metric tons of reserves exist in the US ^{3,4}. In 2013 there were 7 new facility applications and 14 restart/expansion applications between Wyoming, New Mexico, and Nebraska ⁵. In the US, uranium is predominately extracted through *in situ* recovery (ISR) processes ⁶. ISR causes less land disruption and avoids creating tailing piles that can release environmental contaminants ⁷. ISR uses water-based oxidizing solutions to leach uranium from the underground ore body, after which the uranium is extracted from the leachate through an ion exchange process ⁸. To maintain a negative water balance in the ore body, a portion of the leachate, called production bleed water (PBW), is bled off. A portion of the PBW is decontaminated using reverse osmosis (RO) and re-introduced into the mining process, but PBW also could have beneficial industrial or agricultural uses, if toxic contaminants can be reduced to acceptable levels determined by state regulatory agencies for surface and groundwater ⁹. Currently, most ISR uranium facilities use RO to remove contaminants from PBW. However, RO processing is energy intensive and produces toxic waste brine, which requires regulated disposal.

Many water decontamination methods exist, including adsorbents, membranes, and ion exchange. Of these, adsorption is the most commonly used, and recent development of improved nanoparticle synthesis has enhanced the capabilities of adsorbent-based water decontamination processes ¹⁰. Cupric oxide nanoparticles (CuO-NPs) previously had not been extensively studied on uranium ISR PBW, but in recent studies of contaminant removal from groundwater, CuO-NPs were found to have unique properties, including not requiring pre- or post-water treatment steps (e.g., adjusting pH or redox potential) and performing well in different water compositions (e.g., in different pHs, salt concentrations, or competing ions) ¹¹. In addition, CuO-NPs are easily regenerated by leaching with sodium hydroxide (NaOH), after which the regenerated CuO-NPs can be reused. Details of CuO-NP trace metal filtering capabilities from natural waters have been previously published ¹¹⁻¹⁴.

Although useful for water treatment, metal oxide nanoparticles can be toxic to living organisms, but the extent of the toxicity depends, in part, on nanoparticle characteristics and constituents ^{10,15,16}. Therefore, it is important to study simultaneous contaminant removal and nanoparticles toxicities before field applications. The current study determined the capability of CuO-NPs to remove PBW priority contaminants (including arsenic, selenium, vanadium and uranium), and assessed the effect of CuO-NP treatment on PBW cytotoxicity.

PBW was collected from an active ISR uranium facility and utilized to determine the efficacy of CuO-NP treatment in priority contaminant removal. PBW cytotoxicity before and after CuO-NP treatment also was assessed. PBW is a complex geological (industrial/environmental) mixture and both the National Institute of Environmental Health and Science (NIEHS) and the Agency for Toxic Substances & Disease Registry (ASTDR) are placing emphasis on studying the toxicity of environmentally relevant mixtures, including mixtures as they exist in nature or industrial settings, as well as promoting *in vitro* testing to prioritize chemicals for further *in vivo* testing ¹⁷⁻¹⁹. Studies of chronic, low-dose mixture exposures are challenging because chronic

exposure to a low dose mixture not produce obvious effects, at least not in the short time frame of most laboratory studies. Similarly, most *in vitro* studies of chemical mixtures expose cells to a defined lab-made mixture of 2 or more metals ^{20,21}. These studies provide baseline information, but simplified mixtures do not replicate the complex antagonistic and synergistic interactions that may occur in a native, environmental sample, where the full range of mixture components is present.

The goals of this study were to examine alternate contaminant removal processes for PBW and to evaluate the effect of (CuO-NP) treatment on PBW cytotoxicity using cultured human cells. The results could benefit the uranium industry through the development of more efficient or environmentally friendly methods for contaminant removal. This study provides the first evidence that reduction of priority contaminants in PBW by CuO-NPs reduces cytotoxicity in mammalian cells ²².

PROTOCOL TEXT:

All samples were collected at the uranium liquid processing building of a uranium ISR facility in Wyoming.

1. Production Bleed Water (PBW)

1.1) Collect two types of water samples from an ISR uranium facility: PBW and reverse osmosis (RO) water. Collect PBW from a monitoring tap after the ion exchange process but before reverse osmosis decontamination. Collect RO samples after the PBW is decontaminated by reverse osmosis treatment.

NOTE: Lixivant is transported in pipelines from multiple well fields to the uranium liquid processing building, where it is collected in a column and prepared for ion exchange. Approximately 1-3% of the lixiviant after ion exchange is removed from the circuit and termed production bleed water (PBW). PBW is re-used in the mining processes or decontaminated/demineralized with RO filtration.

1.2) Collect water samples in high density polyethylene (HDPE) bottles with zero head space according to standard operating procedures for sample collection and analysis of the Wyoming Department of Environmental Quality (WYDEQ) ²³.

1.3) Measure temperature and pH on-site and transport samples on ice to keep them cool. Store all samples at 4°C.

1.4) Store PBW at 4°C. Keep the PBW solution cool until after the concentrated Eagle's minimum essential media (EMEM-10X) is added during media preparation as instructed in the following protocol.

NOTE: PBW is an oxidized solution that will precipitate if allowed to freeze or warmed to room temperature. After dilution the PBW solution is sufficiently dilute that it will not precipitate when heated to 37°C before application to cells and during incubation.

2. Preparation of CuO Nanoparticles (CuO-NPs)

2.1) Combine a pure ethanolic solution containing 250 mL of 0.2 M $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 250 mL of 0.4 M sodium hydroxide (NaOH), and 5 g polyethylene glycol (PEG) in a round-bottom flask with six mm borosilicate glass balls.

2.2) Place the solution in a modified microwave oven and allow it to react under reflux at ambient air pressure for 10 min at 20% power (intervals of 6 s on, 24 s off).

2.3) Cool the solution to room temperature (20°C), then decanted into 50 mL conical tubes, leaving the glass balls.

2.4) Centrifuge the solution in the 50 mL conical tubes at 1000 x g for 30 min, decanted, and then wash the CuO-NPs with a sequence of 300 mL hot water (60-65°C), 100 mL ethanol, and 100 mL acetone.

2.5) Dry the CuO-NPs to room temperature (20°C) in the 50 ml conical tubes.

2.6) Scrape the CuO-NPs out of their tubes into a mortar. Cover the CuO-NPs with tin foil and heat the CuO-NPs to 110°C in an oven to remove the remaining liquid. Combine CuO-NPs into one batch and weigh the CuO-NPs.

NOTE: The preparation of CuO-NPs and CuO-NP treatment of PBW were conducted in Water Quality Laboratory of Ecosystem Science and Management, University of Wyoming. CuO-NP synthesis followed the procedure of Martinson and Reddy (2009)¹¹.

3. Treatment of PBW with CuO-NPs

3.1) Add 50 mg (1 mg/ml) of CuO-NP to a 50 mL conical tube followed by 50 mL of PBW. Seal the tube and reacted for 30 minutes on a bench top orbital shaker at 250 rpm.

3.2) Centrifuge sample tubes at 250 x g for 30 min and then filter the supernatant using a 0.45 μm syringe filter. Alter the centrifuge speed and time can depending on the nanoparticle to ensure the CuO-NPs become compact in the centrifuge tube.

4. Elemental Analysis

4.1) Prepare Untreated (control) and CuO-NP-treated PBW samples for elemental analysis as follows.

4.2) Acidify aliquots (40 mLs) of CuO-NP-treated and untreated PBW with trace metal grade nitric acid to a pH of 2.0. Analyze acidified PBW aliquots for cations by inductively coupled plasma-mass spectroscopy (ICP-MS) as described in¹³.

4.3) Prepare unacidified aliquots (20 mLs) of CuO-NP-treated and untreated PBW and analyze the unacidified aliquots for anions by ion chromatography (IC) as described in¹³.

NOTE: Aliquots were analyzed by the Wyoming Department of Agriculture Analytical Services, Laramie WY 82070. A description of the IC and ICPMS procedure can be found in Reddy and Roth, 2009¹³.

5. Preparation of cell culture media using PBW

5.1) Use two control (EMEM-1X and RO+media) and eight PBW test media solutions (four concentrations each of untreated PBW and CuO-NP-treated media) in the viability studies. Overviews of the solutions are as follows:

5.1.1) For EMEM-1X control, purchase Eagle's minimum essential media (EMEM-1X) with L-glutamine and sodium bicarbonate already added. Add fetal bovine serum (FBS) and antibiotics per manufacturer's instructions.

NOTE: EMEM-1X is purchased diluted to the proper concentration for cell growth and containing L-glutamine and sodium bicarbonate. EMEM-1X requires the addition of fetal bovine serum (FBS) and an antibiotic mix of penicillin and streptomycin (50 I.U./ml penicillin and 50 µg/ml streptomycin). EMEM-1X is used as a control media because it is the manufacturer's recommended growth media for both cell types used in this study. Concentrated EMEM-10X is diluted with RO or PBW to produce the test solutions. Concentrated EMEM-10X when purchased does not contain L-glutamine or sodium bicarbonate so these are added in addition to the fetal bovine serum (FBS) and an antibiotic mix of penicillin and streptomycin.

5.1.2) For RO control use the ISR facility RO water to prepare the PBW solutions using the same protocol as the PBW test media only substitute RO for PBW.

5.1.3) Dilute untreated PBW into four test concentrations before mixing with the cell culture media components. Prepare the four different concentrations of untreated PBW solutions by mixing untreated PBW with RO in the following combinations: 100% (pure PBW + no RO water), 75% (375 mL of PBW + 125 mL RO water), 50% (250 mL of PBW + 250 mL of RO water) or 25% (125 mL of PBW + 375 mL of RO water).

5.1.4) Dilute CuO-NP-treated PBW into four test concentrations before mixing with the cell culture media components. Prepare the four different concentrations of CuO-NP-treated PBW solutions by mixing PBW (pre-treated with 1 mg/ml CuO-NP for 30 min) with RO in the following combinations: 100% (pure PBW + no RO water), 75% (375 mL of PBW + 125 mL RO water), 50% (250 mL of PBW + 250 mL of RO water) or 25% (125 mL of PBW + 375 mL of RO water).

5.2) Prepare 250 mL of RO+media, untreated PBW+media and CuO-NP-treated PBW+media concentration by adding 25 ml of concentrated EMEM-10X to 190 ml of the 100% RO and the 100%, 75%, 50% or 25% of the premade untreated or CuO-NP-treated PBW concentrations created in step 6.1.3 and 6.1.4.

5.3) Adjust the pH of each solution to 7.4 with NaOH or HCL.

5.4) Supplement each concentration of untreated and CuO-NP-treated PBW as well as RO+media with the following standard components: 25ml (10%) fetal bovine serum (FBS), 2.5 ml L-glutamine, 0.55 g NaHCO₃ and 1.25 ml Pen/Strep (50 I.U./ml penicillin and 50 µg/ml streptomycin).

5.5) Adjust the osmolality of each concentration of untreated PBW+media, CuO-NP-treated PBW+media and RO+media to 290-310 mOSM/kg by adding RO water and measure using an osmometer.

5.6) Filter each solution using a 0.22 µm vacuum filter unit, and store at 4°C.

NOTE: Due to slight variations in the amount of RO water used to adjust osmolality, vary final media concentrations within a 5% range, with untreated PBW+media concentrations at 56%, 44%, 29% and 16.5% and CuO-NP-treated PBW+media concentrations at 53%, 45%, 30% & 17%.

6. Cell Viability

NOTE: Given that kidney and liver are target organs of heavy metal toxicity or cytotoxicity, employ cultured human embryonic kidney (HEK293) cells (HEK) and human hepatocellular carcinoma (HepG2) cells (HEP) testing methods²⁴⁻²⁶.

6.1) Prepare a culture of HEK and HEP cells 2-3 days before plating the 96-well plates used in the experiment per manufacturer's instructions.

6.2) Measure cell viability using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay.

NOTE: The MTT assay protocol was modified from Meerloo et al (2011)²⁷.

6.2.2) Obtain MTT in powder form. Add phosphate buffered saline (PBS) to make up a stock concentration of 50mg/mL. Agitate the solution for 2 hours and then filter with a 0.45 µm syringe filter and aliquot into 1.5ml freezer safe tubes. Protect tubes from light and stored at -20°C.

6.3) Remove HEK and HEP cells from their culture dishes using trypsin, centrifuge at 1000 x g for 5 minutes and decant the trypsin. Add 5ml of PBS and mix cells to obtain a single cell solution. Then, apply 20 µl of the single cell solution to a hemocytometer to obtain a cell count per milliliter of solution. Centrifuge the cells again at 1000 x g for 5 minutes and decant the PBS used to rinse the cells. Add the appropriate amount of EMEM-1X to adjust the concentration of cells to 500 cell/100 µl (100 µl/well).

6.4) Fill the perimeter wells of the plate with 200 µl PBS to control for evaporation.

6.5) Seed cells at a density of 500 cells/well adding 100 µl to each well, except for the perimeter wells (which are not plated with cells).

NOTE: Seeding density for HEK and HEP cells is based on experimental growth curves that allow the peak of growth to occur around days 4-5. Prepare growth curves for all cell lines to estimate seeding density.

6.6) Incubate cells for 24-hours at 37°C allowing them to recover (form tight adhesions to the plate) before performing baseline MTT readings of cell density.

6.7) Perform baseline MTT readings of cell density by removing the seeding media from the first column (not including the perimeter) and adding 100 µL of MTT (5 mg/ml in media) to the wells for one hour.

6.8) After one hour, remove the MTT and add 100 µL of dimethyl sulfoxide (DMSO) to dissolve the MTT-formazan produced by viable cells (20 min).

6.9) Read the optical density (OD) of the first column at an absorption wavelength of 570 nm to obtain a baseline reading.

6.9.1) Use baseline readings to ensure all plates were seeded correctly and that cells are growing consistently between plates. Remove the DMSO from the column being tested before incubating for the next 24h.

NOTE: If DMSO is left in the plate overnight it pulls moisture from the adjacent column, causing a reduction in the media volume.

6.10) Warm the test solutions (i.e., the EMEM-1X, RO, untreated PBW and CuO-NP-treated PBW media solutions) to 37°C in a water bath.

6.11) Remove the seeding media from the rest of the plate (not including the perimeter or the first column which was used for the baseline reading) and replaced with 100 µL of EMEM-1X, RO+media, untreated PBW+media concentrations or CuO-NP-treated PBW+media concentrations (one solution per plate). Incubate cells in their test concentrations or control solutions for a total of seven days (Days 2-8).

NOTE: There 10 plates total: 1 EMEM-1X, 1 RO+media, 1 of each untreated PBW+media concentration (56%, 44%, 29% and 16.5%) and one plate of each CuO-NP-treated PBW+media concentration (53%, 45%, 30% & 17%) per experiment per cell line.

6.12) Each day following baseline MTT reading, remove the control and test solutions (listed in the note under 6.11) from the next column of their respective plate (e.g. Day 2 test and control media are removed from row 3, wells B-G; Day 3: row 4, wells B-G etc.) and repeat the MTT protocol as described in steps 6.7-6.9 above.

6.13) Repeat the protocol every day for seven days. Average the OD results for each row (6 wells) and reported against time to generate a seven-day growth curve.

6.14) To assess the effect of copper chelation on cell viability in CuO-NP-treated PBW+media follow the same procedure as above, except add 100 μ M of D-penicillamine to control and test solutions before adding the solutions to their respective plates. Perform data analysis using a scientific graphing software.

7. Geochemical Modeling

7.1) Download Visual MINTEQ version 3.0/3.1 a freeware from the following website <http://www2.lwr.kth.se/English/Oursoftware/vminteq/>.

NOTE: Visual MINTEQ is a freeware chemical equilibrium model for the calculation of metal speciation, solubility equilibria, sorption etc. for natural waters. In addition it is used to predict ion speciation, ion activities, ion complexes and saturation indices which is compared to the concentration of elements before and after treatment (mass spectroscopy results) to examine possible mechanisms of element removal²⁸.

7.2) Open the program and input the mass spectroscopy data from step 4, including pH, alkalinity and the concentrations of different elements, into the program.

NOTE: Given that groundwater is oxidized during *in-situ* uranium extraction process, use oxidized species of arsenic, vanadium, and uranium for input.

8. Inhibitory Concentration 50 (IC₅₀)

8.1) Calculate the IC₅₀ for the untreated and CuO-NP-treated PBW+media concentrations by first averaging the viability (OD averages) on day 5 of three separate runs.

8.2) Subtract day five viability averages of the untreated and CuO-NP-treated PBW+media concentrations from day five viability averages of EMEM-1X to calculate viability differences. Then divide the viability differences by the average viability on Day 5 in EMEM, and multiply by 100 to get percent inhibition.

8.3) Subtract the percent inhibition from 100 (EMEM-1X viability) to get the percent viability for each untreated and CuO-NP-treated PBW+media concentration.

8.4) Input into scientific graphing software by setting EMEM-1X at a concentration of one and a percent viability of 100; transform all concentrations into log scale ($X = \text{Log}(X)$) and perform nonlinear regression with least square fit analysis.

9. Data Analysis

9.1) Compare concentrations of elements in untreated and CuO-NP-treated PBW with a two-tailed, paired, Student T-test.

9.2) Calculate the areas under the curve (AUC) by using the growth curve data collected over seven days and analyze the variance with repeated measures analysis of variance (ANOVA), followed by Tukey's post hoc comparison between all groups (n=3).

9.3) Compute the IC_{50} by using data from day five of the growth curve for both untreated and CuO-NP-treated PBW+media solutions (described above). P values of <0.05 are considered significant.

NOTE: For the purpose of statistical analysis, mass spectroscopy values of half the detection limit was assigned to ions concentrations levels below that limit²⁹.

REPRESENTATIVE RESULTS:

PBW component concentrations and pH in untreated and CuO-NP-treated PBW are reported in Table 1. Martinson and Reddy (2009), reported that the point of zero charge of the CuO-NP is estimated at 9.4 ± 0.4 . Given that the pH of PBW was 7.2-7.4, in these conditions, water donates protons to CuO-NPs, causing the nanoparticle surface to be positively charged allowing for the adsorption of negatively charged species. CuO-NP treatment removed priority contaminants from PBW, including arsenic, selenium, uranium and vanadium. The average arsenic concentration was reduced by 87% [from 0.0175 to 0.002 mg/L (two-tailed paired t-test, $p < 0.0001$)]. CuO-NP treatment also significantly reduced selenium (30%), uranium (78%), vanadium (92%), and phosphate (85%) ($p < 0.05$).

Speciation modeling results, reported in Table 2, support the analytical results: 99% of total dissolved arsenic in PBW is present as $HAsO_4^{2-}$ and $H_2AsO_4^-$ and 94% of total dissolved selenium in PBW is present as SeO_4^{2-} . These species are negatively charged, hence capable of adsorbing to CuO-NPs. Speciation modeling predicted that 99% of vanadium species in PBW are negatively charged, also promoting adsorption to CuO-NPs. However, speciation modeling predicted only 35.5% of the uranium species are negatively charged, which would limit adsorption to CuO-NPs. Analysis of saturation indices predicted that no species of arsenic-, selenium-, uranium- or vanadium-containing minerals were near saturation (i.e., mineral precipitation) levels, supporting adsorption to CuO-NPs, versus precipitation.

To assess if expected concentrations of priority contaminants are in the media made from untreated and CuO-NP-treated PBW, samples of undiluted control media (EMEM-1X), 56% untreated PBW+media and the 53% CuO-NP-treated PBW+media were analyzed by ICP-MS. To make the PBW+media solutions, the original PBW was diluted by the addition of media growth components and RO water for osmolality adjustment, as described above. Undiluted control media (EMEM-1X) is a commercial product supplied with L-glutamine and sodium bicarbonate (pre-added). Copper and selenium concentrations in control EMEM-1X were slightly elevated as expected because they are essential for cell growth, but arsenic, uranium and vanadium were negligible, reported in Table 3. Preliminary studies showed that, arsenic, selenium and vanadium concentrations were reduced by CuO-NP treatment and that the decrease was represented in the concentrations in the CuO-NP-treated PBW+media. The measured concentration of uranium in the CuO-NP-treated PBW+media was decreased compared to untreated PBW, and this decrease was more pronounced than predicted by Visual MINTEC v.3 modeling. Copper levels rose in CuO-NP-treated media as expected.

To determine the ability of CuO-NP treatment to ameliorate cytotoxicity of PBW on mammalian cells, viability was assessed in cells exposed to solutions of PBW+media before and after CuO-NP treatment. Both HEK (**Figure 1A**) and HEP (**Figure 1B**) cells were exposed to different

concentrations of untreated or treated PBW+media for up to seven days. In cells grown in untreated PBW+media, viability was impaired in a concentration-dependent manner, whereas CuO-NP treatment improved cellular viability in both cell lines. The integrated AUC in **Figure 1C** shows that HEK cells grown in CuO-NP-treated PBW+media were more viable compared to untreated PBW+media at the three highest concentrations (29%, 44% and 56%). HEP cells showed slightly different viability: only the two highest concentrations of untreated PBW+media (44% and 56%) showed impaired viability compared to CuO-NP-treated PBW+media (**Figure 1D**). The more dilute concentrations of PBW were less toxic to HEP cells, and cell viability less affected by treatment. The viability of both HEK and HEP cells grown in 16.5% untreated PBW+media was not significantly different from cells grown in 53% CuO-NP-treated PBW+media ($p < 0.05$). Thus, CuO-NP treatment appeared to ameliorate the cytotoxicity of PBW, with viability near control levels. As discussed above, CuO-NP treatment of PBW is associated with an increase in copper concentrations. The increase was expected, based on earlier results by Reddy and Roth (2012), in which they used CuO-NPs to remove arsenic from groundwater. The increase in copper is dependent on the specific water chemistry of the PBW, but remained below EPA MCL of 1.3 mg/L. However, it was important to rule out that the increase in copper concentrations contributed to improved viability (i.e., in addition to, or instead of, the decrease in priority contaminants). Accordingly, the copper chelator D-penicillamine was added to EMEM-1X control, RO+media control, untreated and CuO-NP-treated PBW+media solutions, and then MTT viability growth curve were generated, as described above. Copper chelation did not significant affect viability of either HEK or HEP cells incubated in RO+media control, untreated and CuO-NP-treated PBW+media (results not shown).

The half maximal inhibitory concentration (IC_{50}) was calculated from day five growth of HEK and HEP cells grown in untreated PBW+media (**Table 4A**) and CuO-NP-treated PBW+media (**Table 4B**). For HEK cells grown in untreated PBW+media, the IC_{50} value was 1.264 (log % PBW). Thus, the untreated PBW+media would have to be diluted to 18.38% to get to a 50% decrease in viability. For HEK cells grown in CuO-NP-treated PBW+media, the IC_{50} value was 2.744 (log % PBW). This result suggests that theoretically the cytotoxicity of the solution was reduced to the extent that treated PBW+media would need to be concentrated by 500% (log % PBW = 2.744) to produce a similar 50% decrease in viability. For HEP cells grown in untreated PBW+media, the IC_{50} was 1.243 (log % PBW). This would require a dilution of the PBW+media to 17.5% to produce a 50% decrease in viability. In contrast, for HEP cells grown in CuO-NP-treated PBW+media, the IC_{50} was 5.327 (log % PBW). This value likely was so large, because the viability of the cells in CuO-NP-treated PBW+media was not significantly different from cells grown in EMEM-1X (control). Bright field imaging, illustrated in Figure 2, of both HEK and HEP cellular growth on day five. Cell number and attachment in the CuO-NP-treated PBW+media (**Figure 2E, F**) were improved compared to untreated PBW+media (**Figure 2C, D**).

TABLES AND FIGURES:

Figure 1: Growth Curves. Growth curves were used to assess the viability and growth of the cultures during treatment. Growth curves for HEK (A) and HEP (B) cells grown in four dilutions of PBW+media compared to 53% CuO-NP-treated PBW+media (upper panels). EMEM-1X control (EMEM) ▼, RO ◆, 53% CuO-NP-treated ★, 16.5% untreated PBW ●, 29% untreated PBW ▲, 44% untreated PBW ■, 56% untreated PBW ●. Area under the curve

(AUC) analysis of HEK (C) and HEP (D) 7 day growth curve data (lower panels). * $p < 0.05$ compared to EMEM control, # $p < 0.05$ compared to RO control, § $p < 0.05$ compared to 53% CuO NP-treated PBW-media. (Compared using a two-tailed ANOVA with Tukey's post hoc analysis, $n = 3$).

Figure 2: Cell morphology before and after CuO-NP treatment. Bright field microscopy (20X) of HEK (left column) and HEP (right column) cells at day 5, grown in: EMEM-1X control (EMEM) (A, B), 56% untreated PBW+media (C, D) and 53% CuO-NP-treated PBW+media (E, F) was used to examine cell morphology. HEK and HEP cells grown in EMEM-1X control (EMEM) (A, B) show healthy, near-confluent growth. HEK and HEP cells grown in untreated PBW+media have reduced numbers and appear detached (C, D). HEK and HEP cells grown in CuO-NP-treated PBW+media show better attachment and healthy, more confluent cells (E, F).

Table 1: Analysis of cations and anions before and after CuO-NP treatment. Average element concentrations before and after treatment with CuO-NP. Significance between the concentration of CuO-NP-treated and untreated PBW are designated as * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$. A blank cell indicates no significant difference. Chloride concentrations ranged between 46.5 ± 0.707 and 55.25 ± 8.180 . Aluminum, boron, and molybdenum concentrations were low and showed no significant change due to CuO-NP treatment. Manganese concentrations were not consistent.

Table 2: Species modeling using Visual MINTEQ ver. 3.0 software. Visual MINTEQ ver. 3.0 software (KTH Royal Institute of Technology, Valhallavägen, Sweden) was used to calculate metal speciation of the PBW components listed in Table 1. (aq) = aqueous as opposed to the solid form of that species.

Table 3: Concentrations of contaminants in media. Concentrations of priority contaminants (mg/L) in EMEM-1X control (EMEM), untreated PBW, CuO-NP-treated PBW, untreated PBW+media and CuO-NP-treated PBW+media after adding media components ($n = 3$) were assessed to ensure changes in contaminant concentration due to treatment were represented in untreated PBW+media and CuO-NP-treated PBW+media applied to cells.

Table 4: Calculation of IC_{50} . The IC_{50} represents the concentration of untreated PBW+media or CuO-NP-treated PBW+media that is required for a 50% inhibition of viability. The percent viability on day 5 for HEK and HEP cells exposed to dilutions of untreated PBW+Media (A) or CuO-NP-treated PBW+Media (B) were used to calculate the half maximal inhibitory concentration (IC_{50}).

DISCUSSION:

Previous studies reported that CuO-NPs removed arsenic from groundwater^{11,13,30,31}. This study supports these previous findings and also reports that CuO-NPs remove additional contaminants from PBW. This study also confirms previous reports that CuO-NPs are effective at arsenic removal, despite the presence of other contaminants and potential competing ions¹¹. Speciation modeling predicted that 97% of vanadium species in PBW are negatively charged, allowing for adsorption to CuO-NPs, and batch treatment removed 92% of vanadium.

This is the first study to investigate the effects of removing specific contaminants from PBW using CuO-NP, and then assess the changes in cytotoxicity associated with the removal. The results demonstrate that investigating the changes in cytotoxicity of complex mixtures using an *in vitro* approach may be possible, but these methods are not without limits. PBW could not be used full strength on the cells, because to survive, cultured cells require a defined growth media and specific osmolality. PBW+media could also not be used on the cells without pH adjustment. The pH of the PBW was 7.31 before and 7.36 after treatment however; the addition of growth media components reduced the pH to approximately 6.8, depending on the dilution. Ph adjustment is a normal step in the preparation of cell culture media however; adjusting the pH of the PBW+media may have altered the molecular interactions of the element species with media components. Untreated and CuO-NP-treated PBW were combined with concentrated EMEM-10X growth media in various proportions to obtain the test solutions (PBW+media). ICP-MS analysis was performed on test media to verify that the concentrations of metals significantly affected by CuO-NP-treatment (arsenic, copper, selenium, uranium, vanadium) were at expected concentrations after dilution by media components and osmolality adjustment. The decrease in arsenic, selenium, and vanadium after CuO-NP-treatment is reflected in the concentration differences between untreated PBW+media and the CuO-NP-treated PBW+media. Uranium concentrations are higher in the CuO-NP-treated PBW+media than predicted. ICP-MS data (**Table 1**) suggests that more uranium was removed from PBW during CuO-NP treatment than predicted by modeling. Speciation modeling (**Table 2**) predicted that at pH 7.3, only 35.5% of the uranium species are negatively charged. The model predicts that the major uranium species, calcium uranyl carbonate ($\text{Ca}_2\text{UO}_2(\text{CO}_3)_3$), is neutral.

The observed 78% removal of uranium was likely due to a combination of uranium adsorption and precipitation (as a calcium uranyl carbonate mineral). Based on the geochemical modeling, the percentage of uranium removed by adsorption is less than calculated allowing for a higher concentration in the CuO-NP-treated PBW+media. The mechanism of uranium removal by CuO-NP-treatment is unclear and requires further investigation. An increase in the concentration of calcium, potassium and magnesium was expected when PBW was added to EMEM-10X however; CuO-NP-treatment did not produce a significant change in these elements so no difference was seen in untreated vs. CuO-NP-treated PBW+media. The technique of combining the actual environmental with media components was successful in representing the changes seen in element concentrations due to treatment; however the oxidized nature of the PBW limited how the PBW+media could be made. In an attempt to increase the maximum concentration of the elements in test media, powdered cell culture media was originally mixed with untreated and CuO-NP-treated PBW to make PBW+media. The powdered media often resulted in precipitation of calcium salts and it increased the osmolality of the PBW+media which required a greater dilution with RO water, producing concentrations close to those obtained with liquid 10X media. These issues are most likely PBW-specific due to its oxidative state and may not be an issue with other less sensitive mixtures.

The MTT assay was chosen to assess cytotoxicity because it is considered a recognized standard high-throughput assay that evaluates the overall health of cells by measuring mitochondrial activity. This method has advantages and disadvantages. The 96-well format is useful for obtaining multiple data points however; the majority of the cells at day 5 were unhealthy looking, rounded and no longer attached to the plate. The photos in **Figure 2** were taken before

the media was removed using suctioning; suctioning off the media, and then adding the MTT solution may have removed unattached cells or detached poorly adherent cells, contributing to the overall plateau of the MTT signal after Day Two seen with untreated PBW. The assumption is that floating cells are dead or dying and only the attached cells are assessed using this method. It is also important to consider the limitations of the MTT assay with respect to studies using nanoparticles.

Previous studies have reported that, when directly applied to cultured cells, nanoparticles may have inherent toxicity, beyond their base chemical properties, depending on their unique physical characteristics such as size and shape^{32,33}. In this current study, we did not apply the CuO-NPs directly onto the cells. Instead, cells were exposed to PBW that had been previously treated with CuO-NPs, centrifuged to remove the majority of the CuO-NPs and then filtered twice to remove more CuO-NPs before the PBW was used to prepare PBW+media. The MS results showed an increase in copper after treatment. This could be copper ions that were dissolved from the nanoparticles during treatment or CuO-NPs that may have passed through the centrifugation/filtration steps to remain in the treated PBW used to make the PBW+media. CuO-NPs range in size from 12-18 nm with a BET measured surface area of $85 \pm 1 \text{ m}^2/\text{g}$ ¹¹ but are known to aggregate and based on the minimal increase in copper concentrations in the treated PBW, most of the copper regardless of the source is removed after centrifugation and filtration. Visual confirmation of improved cell health and confluence support the MTT assay results of improved viability due to CuO-NP treatment of the PBW (Figure 2). Future studies using other methods can evaluate (or characterize) similar confounding effects caused by CuO-NPs.

Human Embryonic Kidney (HEK 293) and Human Hepatocellular Carcinoma (HEP G2) cells were chosen for toxicity testing. These are standard cell lines that are clinically relevant to heavy metal organ toxicity^{24,25,34-40}. A low seeding density was used for the MTT assays. Cells were seeded at 500 cells/well, allowed to recover for 24h, and then exposed to the test media. The low seeding density was necessary to achieve a growth curve with a log phase around day 5, before becoming over-confluent and stationary on day 6 or 7. Chakraborty et al., (2010) reported that in a study of cadmium toxicity on cultured kidney proximal tubule cells (PTC), confluency and proliferation status (proliferating vs. quiescent) affected the response to cadmium exposure: sub-confluent proliferating cells showed more cytotoxicity than confluent (quiescent) cells. HEP and HEK cells exposed to PBW at higher concentrations (greater confluency) similar to those used for other assay (results not shown) did not show the robust changes in the morphology seen with the MTT assay. Further investigation into the changes in cytotoxicity using non-adherent cell lines or protocols that harvest and collect all cells (e.g. flow cytometry) is needed.

Another limitation of the MTT method in studies using nanoparticles is that some types of nanoparticles may interfere with cellular nutrition. Cell culture media typically contain added protein sources, such as fetal bovine serum (FBS), to supplement cell growth. Studies have shown that metal oxide nanoparticles can deplete important growth components in FBS, due to the increased absorption capacity of nanoparticles. Metal oxide nanoparticles have been shown to link to FBS through an interaction with calcium⁴¹. Depending on the pH of the solution, metal nanoparticles can carry a positive or negative charge. Cytotoxicity studies have shown that metal nanoparticles added to cell culture media adsorb cations, including Ca^{2+} , and then remove FBS/serum albumin through binding of the NP- Ca^{2+} complex to the calcium binding

sites on proteins in the FBS. This decreases the concentration of Ca^{2+} and FBS from the media, essentially starving the cells and increasing the cytotoxicity attributed to the nanoparticles⁴¹. Furthermore, pre-exposure of nanoparticles to FBS/ Ca^{2+} coated the nanoparticles, decreasing their cytotoxic effect. However, we did not directly expose the media to CuO-NPs. Also, no significant decrease in Ca^{2+} concentrations were seen in PBW after treatment with CuO-NPs, indicating no significant absorption of Ca^{2+} onto the CuO-NPs priming them to bind with the FBS. However, the concentration of calcium in the PBW is high enough that a nanoparticle-induced decrease may not have been apparent. It is still unlikely that the CuO-NPs used in this study are absorbing large amounts of calcium during processing, because there was no decrease in arsenic absorption capabilities of the CuO-NPs in PBW, which contains high levels of calcium compared to earlier studies with groundwater with a lower calcium concentrations¹³.

The data demonstrate that CuO-NPs remove arsenic, selenium vanadium and uranium, and this is associated with improved HEK and HEP cell viability in the MTT assay. The mechanism(s) by which viability is improved has yet to be determined, but could be due to removal of priority contaminants by CuO-NP, among other mechanisms. The current study also demonstrates that standard cell culture methods can be used to assess the efficacy of a nanoparticle ISR water treatment method, potentially allowing a range of mechanistic studies to be completed, before moving into the more costly and time-consuming *in vivo* animal studies. In addition, CuO-NPs may prove to be more versatile for mining processes and for the treatment of metal mixtures than conventional adsorbents like oxides of aluminum, iron, titanium, and manganese, since 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. Also, CuO-NPs can be regenerated and re-used, reducing reagent costs and the amount of spent treatment waste byproducts in need of disposal¹².

Potential limitations of the MTT protocol include the low cell density at time of exposure, detachment of cells and loss of signal, cell starvation and possible direct exposure of the cells to CuO-NP altering MTT reactivity. Cell density and detachment issues could be addressed by using an alternative test such as flow cytometry, which allows for higher seeding densities as well as the collection of all cells (i.e., both floating and attached). Cell starvation questions could be assessed by measuring growth factor concentrations in the media periodically during treatment. Future work will focus on applying the current protocol to different cytotoxicity assays which will address if possible CuO-NP exposure altered assay activity, measurements of cell starvation during treatment and also testing the ability of CuO-NPs to remove contaminants and affect the cytotoxicity of other types of complex mixtures, such as waste from superfund sites and waste disposal ponds. Such studies would also address whether the methods were robust in various settings.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure
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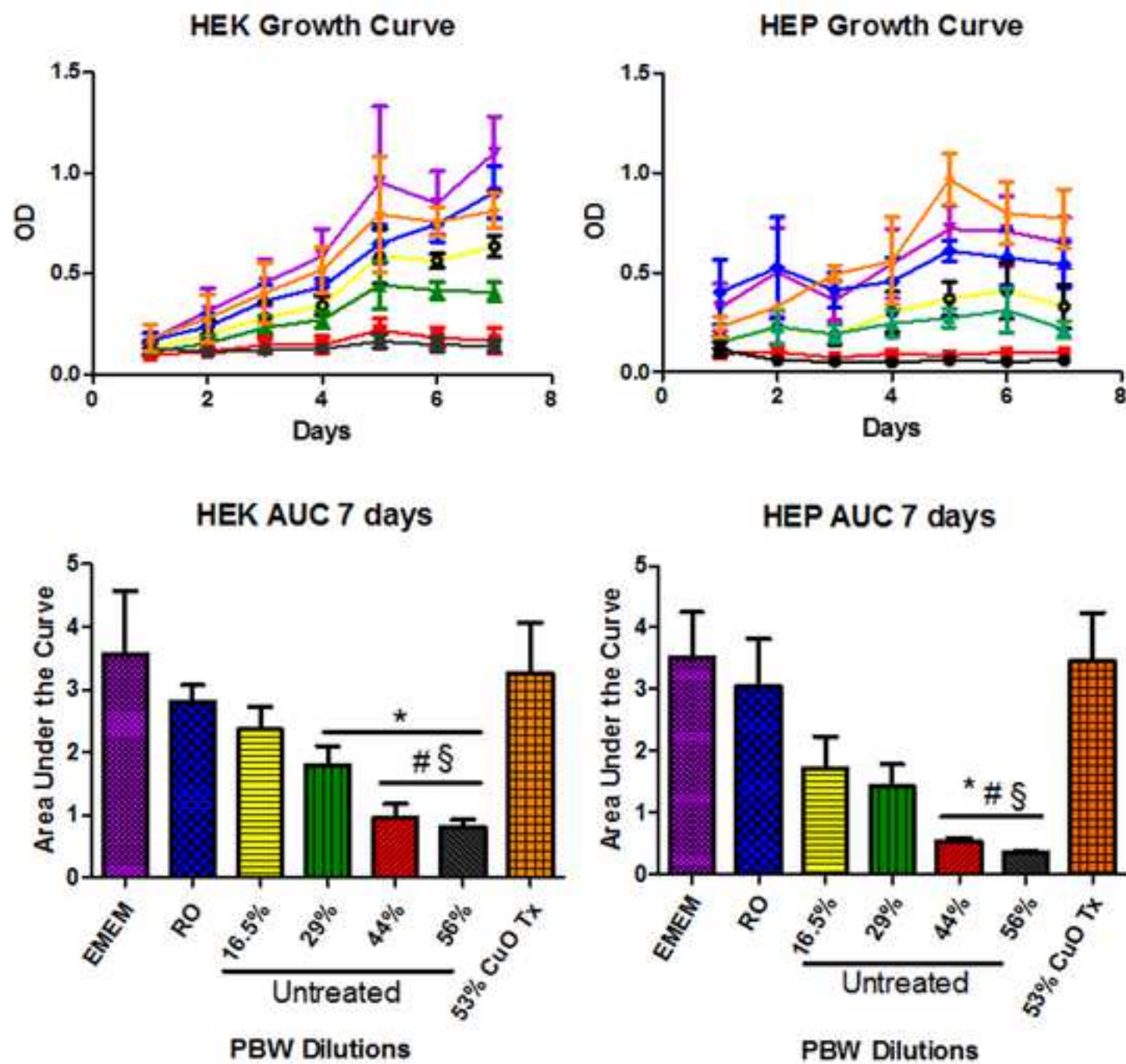
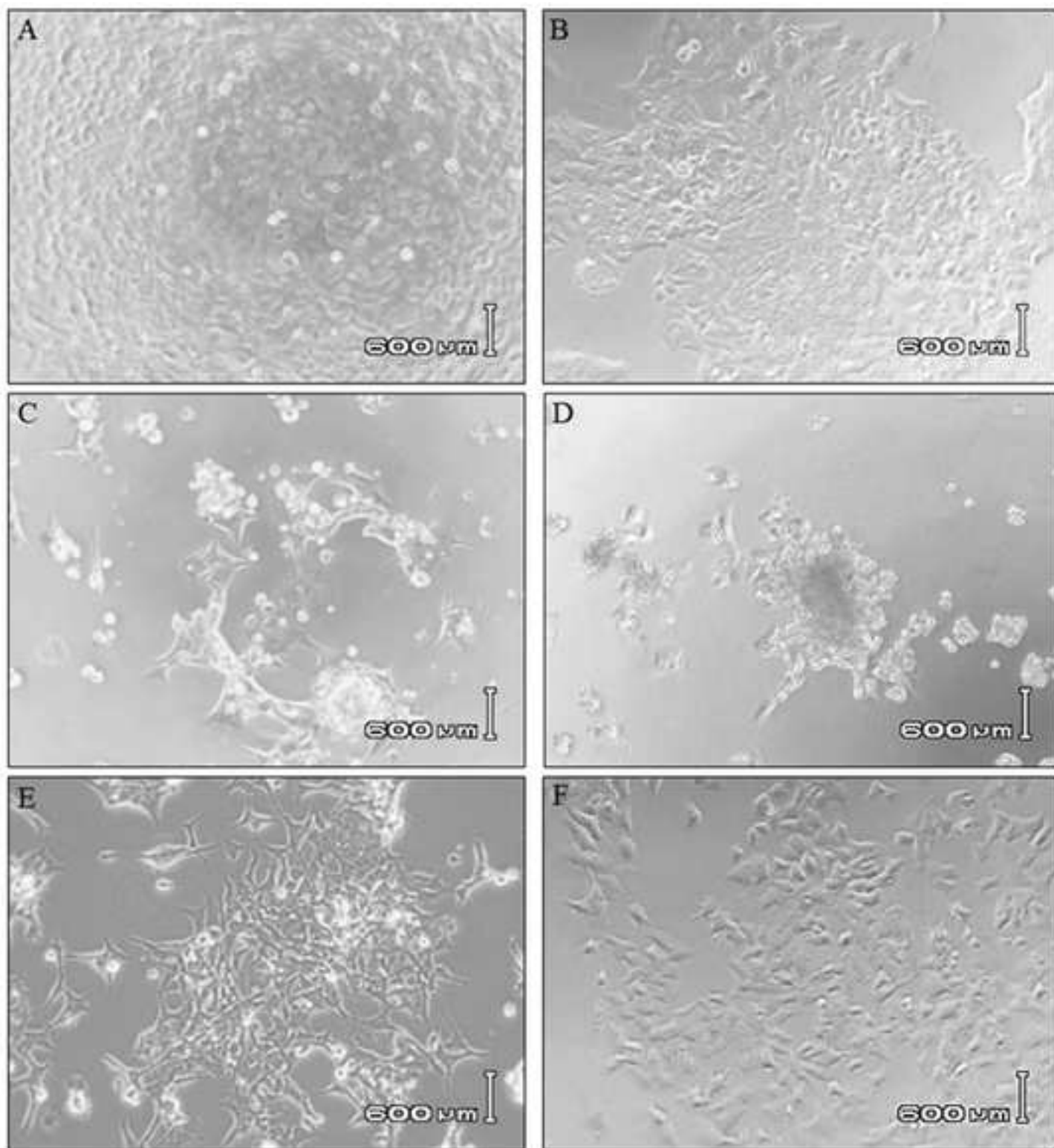


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Average, St. Dev. & Significance

Element (mg/L)	Before Treatment	After Treatment	
Arsenic	0.018 ± 0.001	0.002 ± 0.0	***
Selenium	1.8 ± 0.07	1.3 ± 0.05	**
Copper	0.0015 ± 0.001	0.93 ± 0.43	*
Calcium	102 ± 82	106 ± 15	
Strontium	3.3 ± 1.1	1.5 ± 0.4	*
Magnesium	44 ± 2.1	47 ± 1.7	
Sodium	610 ± 0.0	627 ± 27	
Uranium	0.98 ± 0.03	0.21 ± 0.03	***
Barium	0.037 ± 0.02	0.019 ± 0.01	
Potassium	12 ± 0.0	12 ± 0.8	
Silicon	12 ± 0.7	12 ± 0.5	
Vanadium	1.3 ± 0.07	0.1 ± 0.02	***
Phosphate	0.35 ± 0.07	0.05 ± 0.0	***
Sulfate	805 ± 21	807 ± 15	
Conductivity	3125 ± 143	3190 ± 62	
pH	7.31 ± 0.09	7.36 ± 0.05	

Components	% of total concentration	Species
Arsenic	58.7	HAsO_4^{-2}
	41.2	H_2AsO_4^-
Uranium	64.1	$\text{Ca}_2\text{UO}_2(\text{CO}_3)_3 \text{ (aq)}$
	32.2	$\text{CaUO}_2(\text{CO}_3)_3^{-2}$
	0.03	$\text{UO}_2(\text{CO}_3)_2^{-2}$
	3.5	$\text{UO}_2(\text{CO}_3)_3^{-4}$
	0.09	$\text{Ca}_2\text{UO}_2(\text{CO}_3)_3 \text{ (aq)}$
	0.02	$\text{CaUO}_2(\text{CO}_3)_3^{-2}$
Selenium	94.3	SeO_4^{-2}
	5.6	$\text{CaSeO}_4 \text{ (aq)}$
Vanadium	2.1	HVO_4^{-2}
	95.7	H_2VO^{-4}
	2.1	$\text{H}_2\text{V}_2\text{O}_7^{-2}$
	0.01	$\text{HV}_2\text{O}_7^{-3}$
	0.01	$\text{V}_4\text{O}_{12}^{-4}$

	Untreated		
	EMEM Control	PBW	PBW+media
Arsenic	0.003 ± 0.0	0.017 ± 0.0	0.010 ± 0.001
Copper	0.01 ± 0.0	0.0015 ± 0.001	0.018 ± 0.0
Selenium	0.013 ± 0.002	1.75 ± 0.07	1.15 ± 0.06
Uranium	0.00015 ± 0.0	0.975 ± 0.03	0.71 ± 0.01
Vanadium	0.0015 ± 0.0	1.25 ± 0.07	0.785 ± 0.007

	CuO NP-treated	
	PBW	PBW+media
Arsenic	0.0022 ± 0.001	0.0015 ± 0.0
Copper	0.926 ± 0.4	0.81 ± 0.0
Selenium	1.25 ± 0.05	0.855 ± 0.0.02
Uranium	0.208 ± 0.03	0.45 ± 0.01
Vanadium	0.102 ± 0.02	0.0795 ± 0.01

A Untreated PBW+Media		
Concentrations of Untreated PBW (log X)	% Viability (HEK cells)	% Viability (HEP cells)
EMEM	100	100
16.5% (1.217)	51.4	50.8
29% (1.462)	39	33.3
44% (1.643)	19.3	14.7
56% (1.748)	14.5	9.4
IC₅₀ Log [PBW]	1.264	1.243

B CuO-NP-treated PBW+Media		
Concentrations of CuO-NP- Treated PBW (log X)	% Viability (HEK cells)	% Viability (HEP cells)
EMEM	100	100
17% (1.230)	86.7	119.8
30% (1.477)	75.8	86.7
45% (1.653)	81	92.4
53% (1.724)	70.3	97.5
IC₅₀ Log [PBW]	2.744	5.327

Name of Material/ Equipment	Company	Catalog Number
CuCl2	Sigma	203149
Borosilicate glass balls	VWR	26396-639
Nitric Acid	Fisher	A509-P500
0.45 mm syringe filter	Fisher	SLHA 033S S
10X EMEM	Fisher	BW12-684F
Fetal Bovine Serum	ATCC	30-2020
L-glutamine	Fisher	BP379-100
NaHCO3	Sigma	S5761
Penicillin/Streptomycin	ATCC	30-2300
0.22 mm vacuum filter unit	Fisher	09-740-28C
HEK293	ATCC	CRL-1573
HEPG2	ATCC	HB-8065
Trypsin	Sigma	SV3003101
MTT	Sigma	M2128
D-penicillamine	Fisher	ICN15180680
96-well plates	Fisher	07-200-92
DMSO	Fisher	D12814
Spectra Max 190	Molecular Devices	
Visual MINTEQ version 3.0	KTH Royal Institute of Technology	
ICP-MS	Agilent	
IC DIONEX DX 500	Dionex	
VWR Incubator	VWR	

Comments/Description

6 mm

Trace metal grade

Details of instruments, models and detection limits were published in Reddy et al., 2013.

Details of instruments, models and detection limits were published in Reddy et al., 2013.



1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
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Title of Article:

REMOVAL OF TRACE ELEMENTS BY COPPER OXIDE NANOPARTICLES FROM INSITU RECOVERY BLEED WATER AND ITS EFFECT ON CELL VIABILITY

Author(s):

Jodi Behilz, K.J Reddy, Sreejayan Nair, Thomas Johnson, Ronald Tjalkens, Kemp Krueger, and Suzanne Clark

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CORRESPONDING AUTHOR:

Name: K J Reddy
Department: Ecosystem Science and Management
Institution: University of Wyoming
Article Title: Removal of trace elements by carbon oxide nanoparticles from in situ recovery bleed water and its effects on cell viability
Signature: [Signature] Date: 09/24/14

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This piece of the submission is being sent via mail.

Editorial comments:

1. Please state the goal of your protocol in the Short abstract. Please ensure not remain within the 10-50 words limit.

The goal of this protocol was to integrate the native environmental sample into a cell culture format assessing the changes in toxicity due to CuO-NP treatment.

2. Please re-write steps of your protocol section in imperative tense, as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.). For instance, in step 1.1, "Collect two types of water samples.." and mention PBW is wastewater..." as a NOTE below the step.

We have rewritten the protocol section in the imperative tense. Step 1.1.1 added PBW is wastewater..." as a NOTE below the step

3. Please try to avoid usage of phrases such as "should be", "could be", "would be" and write in the active/imperative style. For instance in step 1.2.

All uses of the phrases "should, could or would be" have been removed from the protocol section. They still exist in some instances in the text. If you would like me to rephrase those sections please let me know.

4. Please make sure the entire protocol section is written in imperative tense. "Place the solution..", "Allow the solution to cool...". "Allow the pellets to dry..", "Scrape the pellets out..." etc.

Hopefully I have corrected this.

5. Please mention the centrifugation speed and time in step 3.2. and where needed.

Step 2.4 added speed and time, Step 3.2 added speed and time, more explanation added to step 7.3 including centrifugation speed and time

6. Please replace step 5.2 as a note. Additionally please mention how to obtain Visual MINTEQ.

5.2 has been replaced with note 5.1.2, The link for downloading visual MINTEQ has been added.

7. Please revise the text to avoid the use of any pronouns (i.e. "we", "you", "your", "our" etc.). If you feel it is very important to give a personal example, you may use the royal "we" sparingly and only as a "NOTE:" after the relevant protocol step. Please use the Ctrl+F function to find and replace the pronouns.

We have left only two instances of our in the text, there are none within the protocol. If you believe we need to change these pronouns I will rewrite those statements as well.

8. References are not in correct JoVE format. Please make sure that your references comply with JoVE instructions for authors. In-text formatting: corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text of the manuscript. Citation formatting should appear as follows: (For 6 authors or less list all authors. For more than 6 authors, list only the first author then *et al.*): [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source*. **Volume** (Issue), FirstPage – LastPage, doi:DOI, (YEAR).]

We believe the in-text formatting of the reference numbers to be correct. If this is still incorrect please let us know.

We apologize, we downloaded the Zotero JoVE format and didn't double check that it formatted correctly. Apparently the format listed on the site is incorrect and we don't know how to download the csl file from the link you have listed on the JoVE website. We have manually corrected the following:

#16, 21, 30, 32, 37, and 39 had two names listed plus et al for more than 6 references. We have removed the second name.

#20, 26, 34, 48 had no DOI #

We have removed the second initial from all of the reference

We have double checked that all references have page numbers.

We do not know how you would like websites references formatted. Please let us know how you would like references 1-9 formatted.

Thank you for your time and help,

Jodi Schilz

KJ Reddy

Editorial comments:

1. Thank you so much for submitting your revised manuscript. All of your previous revisions have been incorporated into the most recent version of the manuscript.
2. Please download this version of the Microsoft word document (File name: 52715) for any subsequent changes
3. Step 4.2 please provide a reference “Analyze... spectroscopy” or please describe in details how these steps are carried out.

We have added a reference to step 4.2 and 4.3 as well as adding a statement in the NOTE after 4.3 stating that there is a description of the IC and ICPMS procedure in that paper.

4. Please make sure that the protocol is in continuity. Section 5 included in the protocol is not in continuation with previous and later steps. Please add more details as to how these analysis are complementing the following steps.

The geochemical modeling was performed with the information obtained from the elemental analysis and was performed in parallel with all other tests. We have moved it down to step #7 in line with the other analysis. We arranged the steps differently and emphasized that our mass spectroscopy data is inputted into MINTEQ in order to model the ion activities in this specific water.

5. Please provide a reference for NOTE under step 7.2 OK

6. In step 7.3. you mention “..decant PBW”. Please specify when is this added from the time of the culture and how much is used. Please provide more details.

We have changed “decant PBW” to “decant the PBS used to rinse the cells”. This should read PBS not PBW.

7. In step 7.6 you mention “Recover cells..” please specify how are the cells recovered?

We have changed this statement to read: Incubate cells for 24-hours at 37 °C allowing them to recover (form tight adhesions to the plate) before performing baseline MTT readings of cell density.

8. Section 9 is not in correct tense. Make sure all the protocol steps are in imperative tense.

OK, also step 9.4 was changed to a NOTE.

9. After you have made all of the recommended changes to your protocol (listed above), please

re-evaluate the length of your protocol section. There is no page limit for the protocol text, but there is a 3 pages limit for filmable content. If your protocol is longer than 3 pages, please highlight (in yellow) 2.75 pages (or less) of text to identify which portions of the protocol are most important to include in the video; i.e. which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVEs instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

OK, We have highlighted the steps in section 2.

10. Ensure that each figure or data table legend includes a short title, followed by a short description of each panel and/or a general description. All figures showing data must include measurement definitions and error bars (if applicable). Please include the figure legends as part of the manuscript text (not part of the figure file) directly below the representative results text.

The figure and data table legends have been edited to include a general description.

11. To reference hyperlinks, please follow the format for “Homepages” as mentioned in this link. Please copy paste and follow the instructions.

<http://www.ncbi.nlm.nih.gov/pmc/pmcdoc/tagging-guidelines/citations/v2/citationtags.html#N40122B>

OK We have edited all web pages to follow this format.

Editorial comments:

1. The Long Abstract reads like an original research article; it should focus more on the methods. – ***We have tried to alter the long abstract to include more about the abstract and incorporate major concerns from viewer number 3.***
2. Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.
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Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript is an outstanding contribution from the authors in the area of CuO-NPs treatment to effectively remove contaminants from PBW and reduce mammalian cytotoxicity.

Major Concerns:

Cell starvation questions has been successfully assessed by measuring growth factor concentrations in the media periodically during treatment. This technique has future scope of research as well.-- ***Added a comment on line 684 including cell starvation as possible future work.***

Minor Concerns:

Disposal of wastes can be addressed better by the technique. – ***Disposal of used CuO-NPs is addressed through the regeneration process of CuO-NPs by other studies (Reddy et al., 2013), which is referenced in the paper. Regeneration of CuO-NPs is addressed in lines 127-130 and 683.***

Reviewer #2:

Manuscript Summary:

This is a well-written paper that reports important results on the use of copper oxide nanoparticles to remove contaminants from uranium mine production bleed water and the effect on mammalian cells. While I believe it could be published in its current form, I believe it would be improved with minor revision and have recommended a few items for the authors to consider in their final revision.

Major Concerns:

No major concerns.

Minor Concerns:

No minor concerns.

Additional Comments to Authors:

On line 115, the authors use the term "CuO-NPs" without definition (although it is in the abstract, it should be given here as well to allow the paper to stand alone). The definition is provided on line 146, but that should be moved to line 115. —***CuO-NPs was defined on line 115***

In line 169, the authors use the term "10x EMEM". What is this? Later, this is identified as a cell growth or culture media, but this should be identified at the first use. Since this is not a cellular biology journal, I recommend that a few words of explanation be added at the first use of specialized terms such as this to make the paper/videos more accessible to the broader audience targeted by this journal. — ***Additional descriptions of EMEM and how it is used have been added.*** On line 289 (and used again in 294, 296, and 300), the acronym "PBS" is introduced without definition. (Could this be misspelled and actually represent fetal bovine serum, FBS? My technical definition of PBS is polybutadiene-styrene plastic, but as it is later indicated to be a liquid, I suspect that this is not the authors' intended meaning.) If it is not misidentified, please define PBS and add it to the list of chemicals in the Table of Materials/Equipment. — ***PBS is phosphate buffered saline and has been defined on line 306 and has been added to the Table of Materials/Equipment.*** Similarly for DMSO on line 316: while I am confident that this is dimethyl sulfoxide, the authors should not leave their readers/viewers guessing. — ***DMSO is now defined as dimethyl sulfoxide on line 334. Please note lines have shifted due to edits in previous sections.***

"Pellets" are referenced in lines 189, 192, and 194-195. Are these the precipitated CuO particles? — ***The term pellets has been replaced with CuO-NPs.***

In line 530, "charged" is misspelled as "charge". — ***This has been corrected***

The upper graph in Figure 1A contains a black line, which appears to correspond to the 16.5% yellow bar in the lower graph. Should this line be yellow, like the one in the upper graph of Figure 1B? — ***The line in figure 1A has been changed to yellow to match the line in Figure 1B.***

In Tables 1 and 2, up to 5 significant figures are given. I suspect that the accuracy of the analyses does not justify this level of reported precision. I recommend that the table be edited to include the appropriate number of significant figures (perhaps 3?) for each reported value. -- ***Significant figures were adjusted.***

Reviewer #3:

Major Concerns:

No major concerns. In general a well developed experimental protocol that melds aspects of geochemistry and ecotoxicity together to produce an engaging study of the application of CuO nanoparticles. I would have liked to see more prominent in the text (and also mentioned in the Abstract) that this in vitro study is limited in its wider context due to dilution effects and changes in pH from traditionally alkaline leach of the PBW. This is just to better place this study in context. –***We have tried to adjust the abstract to reflect these concerns as well as the editorial concerns that it doesn't focus on the methods.***

Some more information on the nature of the CuO-NPs would have also been very useful. This is possibly contained in some other related publications but information such as the particle size distribution, presence or not of aggregates and some SEM images of the particles would have provided the reader with some more (and essential) basic information on the nature of the absorbent medium. – ***This study did not measure the attributes of the CuO-NPs directly so we only refer to those numbers from Martinson and Reddy, 2009.***

Minor Concerns:

Sometimes abbreviations are used and other times not. Use abbreviation after the first mention and thereafter. –***We corrected this issue.***

I still do not know what EMEM is after reading the manuscript twice. – – ***Additional descriptions of EMEM and how it is used have been added*** Check significant figures in tables. – ***Significant figures in tables have been reduced.***

Figures are inanimate so cannot "show" something or be used at the beginning of a sentence. This is commonplace in manuscripts but still is incorrect irrespective of how common an error that it is. – ***These have been corrected in the text.***

Could have been a more general summary of U and contaminant removal techniques in the introduction. Just a few more lines and key references to round things out a little more. – ***More was not added about Uranium and the removal techniques in order to expand upon the methods and results. If addition of this information is recommended by the editors it can be added.***

Please see the attachment – ***Comments in text have been addressed with track changes in text.***

Additional Comments to Authors:

None other than specific comments marked in the text. – ***Comments in text have been addressed with track changes in text.***