Environmental Toxicology and Chemistry

Environmental Toxicology

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Environ Toxicol Chem., **Accepted Article** • DOI: 10.1002/etc.3791

Environmental Toxicology **Environmental Toxicology and Chemistry**
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Battery material core chemistry influences aquatic toxicology

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This article includes online-only Supplemental Data.

Submitted 18 October 2016; Returned for Revision 28 November 2016; Accepted 9 March 2017

Abstract: Lithium intercalation compounds such as lithium nickel manganese cobalt oxide (NMC) and lithium cobalt oxide (LCO) are used extensively in lithium batteries. Because there is currently little economic incentive for recycling, chances are greater that batteries will end up in landfills or waste in the environment. In addition, the toxicity of these battery materials has not been traditionally part of the design process. Therefore, to determine the environmental impact and the possibility of alternative battery materials, representative complex battery nanomaterials, LCO and NMC, were synthesized and toxicity was assessed in *Daphnia magna*. Toxicity was determined by assessing LCO and NMC at concentrations in the range of 0.1-25 mg/L. Acute studies (48-hours) showed no effect to daphnid survival at 25 mg/L whereas chronic studies (21-days) show significant impacts to daphnid reproduction and survival at concentrations of 0.25 mg/L for LCO and 1.0 mg/L for NMC. Dissolved metal exposures showed no effect at the amounts measured in suspension and supernatant controls could not reproduce the effects of the particles, indicating a nanomaterial-specific impact. Genes explored in the present study were actin, glutathione-s-transferase, catalase, 18s, metallothionein, heat shock protein and vitellogenin, Down regulation of genes important in metal detoxification, metabolism and cell maintenance was observed in a dose dependent manner. This study demonstrated battery material chemical composition could be altered to minimize environmental impacts.

Keywords: Nanotoxicology, Chronic, Daphnia, Gene expression

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INTRODUCTION

Batteries provide consumers with a portable and convenient form of stored chemical energy to fuel many everyday technologies. With increasing usage and expansion of technologies such as portable electronics and electric vehicles, complex metal oxides such as lithium intercalation materials are increasingly used as battery cathodes. Lithium intercalation materials based on layered transition metal oxides are common cathode materials that are able to perform high-speed reversible reactions with lithium and high voltage and energy densities[1]. Lithium cobalt oxide or "LCO" (Li_xCoO_2) is the most common cathode material in use today. However, a drive to reduce cost, improve performance, and improve stability is leading to a shift to more complex materials such as lithium nickel manganese cobalt oxide or "NMC" ($Li_xNi_yMn_zCo_{1-y-z}O_2$, $0 < x,y,z < 1$)[2],[3]. In NMC materials, replacing some of the Co with Ni and Mn can retain the same crystal structure, providing to opportunity to tune material composition to achieve specific performance characteristics.

NMC presently in use commercially typically consists of nanoparticles that are sintered into larger, micron-sized secondary particles. There is an industry-wide trend toward use of even smaller nanoscale materials (5 nm x 100 nm) in order to further enhance performance characteristics[4]. Examples include 1.) Faster charge and discharge rates; 2.) Enhanced conductivity attributed to increased surface area to volume ratio[5]; and 3.) Less material fracturing from expansion and contraction during cycling[6]. Even with today's micron-sized particles, mechanical stresses during charging and discharging induce fracturing in the primary nanoparticles and can even fracture the primary nanoparticles into smaller nanoparticles. Because NMC has a two-dimensional, layered crystal structure, nanoparticles produced by fragmentation preferentially adopt sheet-like morphologies[7] that we refer to as "nanosheets".

One impact of the lower cost of NMC materials is that there is also reduced economic incentive to implement infrastructure for recycling [7, 8] Consequently, reduced cost indirectly leads to an increased potential for NMC to end up landfills or waste in the environment, providing increased pathways for exposure.

Only a few studies have highlighted the toxicological implications and mode of action (MOA) of these nanoscale complex metal oxides. A prior study of LCO nanosheets and supported lipid bilayers showed that this material had the ability to alter synthetic bilayer compositional symmetry[9] and in bacteria toxicity was due to dissolution of nickel and cobalt ions[10]. The impact of nanoscale LCO and NMC on multicellular organisms is largely unknown. There have, however, been many studies that report the toxic effects of more simple metal oxide nanomaterials (NM). These studies show that, similar to the bacteria, effects are primarily due to metal dissolution or direct particle adherence[11-16]. Therefore designing battery NMs where the ions released are not toxic may reduce toxicity. In the past, the toxicity of these materials has not been part of the design process.

This study investigated the impact of NMC and LCO on survival and reproduction in the model multicellular aquatic organism, *Daphnia magna.* These materials have similar sheet-like morphology and size and only differ in chemical composition enabling us to evaluate how the metal composition of these materials impacts toxicity. *D. magna* are critical components of freshwater food webs, are sensitive to a wide range of environmental contaminants and are designated environmental model organisms by the United States Environmental Protection Agency (USEPA) and Organization for Economic Cooperation and Development (OECD).

In the present study, acute and chronic assays were conducted to determine both short and long term impacts of these materials to *D. magna*. Additionally, gene expression assays were

performed to determine organism molecular response upon exposure and determine whether the observed effects were from the NM exposure or the dissolved metals in solution. Metal ion control experiments accounted for metals leached from NMC and LCO particles and body burden assays were conducted to differentiate metals adsorbed to the daphnid carapace versus metals ingested and/or incorporated into tissues. This study seeks to assess the potential environmental hazard associated with these materials, provide molecular level information for mechanisms for toxicity and determine if materials could be redesigned to mitigate biological impacts.

METHODS

Synthesis of NMC and LCO nanosheets

Nanosheets of NMC and LCO were synthesized by adapting a previously reported method[9, 10, 17]. Briefly, to make NMC, a $LiNi_{1/3}Mn_{1/3}Co_{1/3}(OH)_2$ precursor was synthesized by adding an aqueous transition salt mixture containing 0.2 M cobalt(II) acetate, 0.2 M nickel(II) acetate, and 0.2 M manganese(II) acetate dropwise into 0.1 M aqueous LiOH under magnetic stirring. The resulting dark brown precursor was isolated by repeated cycles of centrifugation and resuspension in water (1X) and methanol (4X) followed by drying in a desiccator. The dried mixed metal hydroxide (0.250 g) was then added to a 10 g molten salt flux (6:4 molar ratio of $LiNO_3$:LiOH) at 205 °C with magnetic stirring. After 30 min, the reaction was quenched with water and the NMC was isolated via repeated cycles of centrifugation and resuspension in water (1X) and methanol (4X) followed by drying in a desiccator. To make LCO, the same method for making NMC was used, with the exception that the precursor was made using only 0.5 M cobalt(II) nitrate for the aqueous transition salt mixture. All centrifugation was completed using the Thermo Scientific Sorvall legend X1R Centrifuge with a Thermo TX-400 rotor at 4686 g. All

reagents were purchased from Sigma-Aldrich and ultrapure water (18 MΩ cm resistivity; Barnstead Nanopure) was used.

Characterization of NMC and LCO stoichiometry

A PerkinElmer Optima 2000 inductively coupled plasma-optical emission spectroscopy (ICP-OES) was used to determine the stoichiometry of samples. NMC and LCO were separately digested in freshly prepared *aqua regia* (3:1 v/v mixture of 37% v/v HCl and 70% v/v HNO_3) for 4 h and subsequently diluted in ultrapure water. Three replicate measurements were made of the ion concentrations. ICP-OES measurements for digested NMC yielded metal ratios of Li/Ni = (0.812 ± 0.015), Mn/Ni = (1.033 ± 0.011), and Co/Ni = (1.017 ± 0.006). These ratios indicate that the NMC material contains 1:1:1 of Ni:Mn:Co and lithiated to roughly 27% to give $Li_{0.27}Ni_{0.33}Mn_{0.33}Co_{0.33}O_2$. ICP-OES measurements for digested LCO yielded Li/Co = (1.002 ± 0.355), indicating that the material was lithiated to 100% to give $LiCoO_2$. Certified standards purchased from Fluka were used in all ICP-OES work.

Characterization of NMC and LCO crystal phase

A Bruker D8 Advance powder X-ray diffractometer (PXRD) was used to obtain diffraction patterns for synthesized NMC and LCO. The dry powder samples were deposited onto a zero-diffraction plate (SiO_2 from MTI corp) for analysis using a Cu Kα radiation source. The samples can be indexed to a R-3m space group previously reported for NMC and LCO[17-19].

Characterization of nanomaterial morphology

Scanning electron microscopy (SEM). To determine average nanomaterial shape and size a Leo Supra55 VP scanning electron microscope with a 1 kV incident electron energy with a standard in-lens detector was used to obtain images of NMC and LCO. Samples for SEM

images were prepared by spin-coating (1000 rpm) a dilute methanolic solution of either NMC or $LiCoO_2$ onto a pristine boron-doped SiO_2 wafer. For transmission electron microscopy images of NMC and LCO, please refer to Dogangun et al. 2015 [9] and Hang et al. 2016, [10] respectively.

Characterization of NMC and LCO sedimentation behavior in culture media. To characterize how quickly NMC and LCO nanosheets sediment out of the aqueous phase, a Shimadzu UV-2401PC Research-Grade UV-vis spectrophotometer was used to analyze change in relative particle concentration the aqueous phase. Nanosheets of NMC or LCO were suspended into medium at 10 mg/L and UV-vis measurements were made periodically over 24 h. UV-vis measurements of the resulting suspensions in medium were referenced to a sample of medium to subtract out UV-vis signals from medium.

Characterization of metal release into culture media. To characterize metal release into the daphnid media, NMC or $LiCoO_2$ nanosheets were suspended into the medium at concentrations of 0.5 mg/L, 1 mg/L, 5 mg/L, and 25 mg/L for 72 h. The samples were then centrifuged at 4696 g for 10 min to remove the majority of particles in solution. The supernatant was subsequently ultracentrifuged for 2 h at 288,000g using a Beckman Coulter Optima Ultracentrifuge with a SW-41 Ti Rotor to ensure removal of remaining particles. The supernatant was analyzed for free Li, Ni, Mn, and Co content using a Thermo Scientific XSERIES 2 ICP-MS instrument with 3 sample replicates and 5 analytical replicates. Dynamic light scattering (Malvern Zetasizer Nano ZS) was used to evaluate effective removal of particles under the specified centrifugation conditions.

D. magna culture and exposures

Daphnids used in the present study were harvested from cultures maintained at the UW-Milwaukee School of Freshwater Sciences in the R. Klaper laboratory. Daphnid populations

were maintained in moderately hard reconstituted water (MHRW: 60 mg/L $CaSO_4$ and $MgSO_4$, 96 mg/L $NaHCO_3$, 4 mg/L KCl and .02 mL/L of a 330 mg/L $Na_2SeO_3*5H_2O$ solution)[20] incubated at 20 °C on a 16 : 8 light/dark cycle as described by EPA recommendations. Media was oxygenated for 48 hours with an oxygen stone prior to use. Daphnids were fed a combination of 20 mL freshwater algae (*Pseudokirchneriella subcapitata*) at an algal density of ~400,000 algal cells per mL and 10 mL of dissolved alfalfa (*Medicago sativa*) three times a week. Alfalfa stock was prepared by suspending 405 mg of alfalfa in 50 mL Milli-Q water after 20 min of agitation and 5 minutes of centrifugation at 5,000 rpm. Breeding populations were held at a concentration no greater than 1 adult daphnid per 50 mL of media in 1-liter glass beakers at a population density of 20 adult daphnids per liter. Neonates were only harvested from adults 14 - 28 d old, ensuring healthy daphnid neonates.

Acute exposures. Acute exposures are modified based on OECD 202 recommendations for *D. magna* acute immobilization test. Briefly, five daphnid neonates ≤ 24 h old were placed in 100 mL moderately hard reconstituted water (MHRW, control)[20], NMC or LCO nanosheets, supernatants or metal ions. For each treatment, a minimum of three replicates was performed and the percent of animals alive at the end of 48 hours was determined. Lithium nickel manganese cobalt oxide and LCO NMs were tested in acute exposures at nominal concentrations of 1.0 mg/L, 10 mg/L and 25 mg/L.

Chronic exposures. Chronic experiments were performed using five daphnid neonates < 24 h old exposed to control water, NMs or metal ion controls for 21 d in a static renewal exposure. Media was renewed three times a week and treatments were performed in quadruplicates. A total of 5 neonates were placed in 94 mL of MHRW, NMs, supernatants or metal ions. Daphnids were supplemented with 4 mL of algae (*Selenastrum capricornitum*) and 2

mL of alfalfa (*Medicago sativa*) at each media exchange to bring the total volume to 100 mL. Lithium nickel manganese cobalt oxide, LCO NMs and their respective supernatants were tested in chronic exposures at nominal concentrations of 0.25 mg/L, 0.5 mg/L, 1.0 mg/L, 5.0 mg/L and 25 mg/L and 0.05 mg/L, 0.1 mg/L, 0.25 mg/L, 0.5 mg/L, 1 mg/L, 5 mg/L, and 25 mg/L, respectively. Samples from chronic exposures were then flash frozen in liquid nitrogen at day 21 to determine daphnid gene expression. Samples were selected by determining treatments that caused equal impacts to daphnid survival and reproduction (one sub lethal concentration and one concentration that caused equal impacts to daphnid survival and reproduction). These concentrations were determined to be 0.10 mg/L and 0.25 mg/L for LCO and 0.25 mg/L and 1.0 mg/L for NMC.

Solution and suspension preparation

The highest concentration of NMC and LCO NMs and their respective supernatants tested was 25 mg/L. Five metal ion concentrations were tested for each metal. These concentrations overlapped with the concentrations of metals determined to leach from the NMs in daphnid media. The highest concentrations tested were 10 mg/L, 0.4 mg/L, and 0.2 mg/L for Li, Ni and Co, respectively, in both acute and chronic exposures. Mn was not assessed because it was not found in solution above the detection limit.

NMC and LCO stock suspensions were prepared by measuring out and mixing 100 mg of material with one-liter of ultra pure water in a one-liter vessel. Stocks were then sonicated for 20 min followed by an additional sonication time of 10 min prior to use. Suspensions were used for the entire 21-d exposure. NMC and LCO supernatants were prepared by sampling from the stocks on the day of the exposure media exchange after sonication. Samples were then placed

into 50 mL conical tubes and centrifuged for 10 min at 10,000 rpm. Care was taken to not disturb the pellet and minimize contamination of supernatant by particulate metals.

Metal salts were weighed and similarly placed in one-liter vessels and sonicated for 10 min to properly dissolve salt. The salts used in the present study to test free ion toxicity were cobalt chloride hexahydrate, lithium hydroxide and nickel (II) chloride. Molecular weight was accounted for to reach desired concentration of metal ion.

Body burden assay

The body burden assay was performed to determine the total amount of material adhered vs. ingested/adhered/internalized. Adult daphnids, 26- 27 days old, were exposed to either control water or NMC particles at 1 mg/L for 24 hours. Specifically, the exposure consisted of a total of five adult daphnids that were placed in 94 mL of MHRW or NMs and daphnids were supplemented with 4 mL of algae (*Selenastrum capricornitum*) and 2 mL of alfalfa (*Medicago sativa*) bringing the total volume to 100 mL. At the end of 24 hours the daphnids in the treated groups were pooled into six samples of 35 daphnids each, control groups were pooled into three samples of 15 daphnids each. For the treated samples, three of the six samples were treated with 4 mL of aqua regia for two minutes to dissolve adhered NMC particles. All six treated samples were rinsed with 30 mL of ultra pure water three times before ICP-OES analysis.

RNA extraction, reverse transcription and gene expression

RNA extraction. To determine the level of expression of genes involved with various cellular functions daphnids were harvested from chronic exposures at the end of the 21 d exposure period and immediately frozen with liquid nitrogen and stored at −80 °C until extraction. The surviving daphnids harvested from each replicate were pooled together to have a final total of 4 replicates per treatment (each with $n \leq 5$ daphnids per sample) for gene expression

analysis. The RNA from these samples was isolated using A Direct-zol™ RNA MiniPrep kit (Zymo Research). The isolation and purification of total RNA followed the manufacturers recommended protocol. A Thermo Scientific NanoDrop 8000 characterized total RNA immediately after elution using 1.5 μL total RNA to ensure quality and determine concentration. During this time the samples were stored on ice. The remaining sample was stored at −80 °C to wait further processing.

Reverse transcription. Total RNA was transcribed into cDNA from 250 μg of total RNA incubated in the presence of oligo$(dT)_{15}$ primer (Promega) and dNTPs at 65°C for 5 min. After a 4 min cooling period at 4°C, the samples were combined with a 5x buffer solution, SuperScript III reverse transcriptase, DTT, and RNaseOUT™ recombinant ribonuclease inhibitor (Life Technologies), mixed and incubated at 50 °C for 60 min. Lastly, the mixture was incubated at 70 °C for 15 min for primer extension and the final product was permanently stored at −20 °C immediately following synthesis.

Gene expression. Several genes were chosen to investigate the molecular impacts of NMC and LCO NMs. These genes included actin (*act*), which encodes for a protein important in cytoskeleton production and cell motility, glutathione-s-transferase (*gst*) and catalase (*cat*), important to xenobiotic detoxification and oxidative stress attenuation, metallothionein (*mtl1a*) and heat shock protein 70 kDa (*hsp70*), which respond to metal stressors, vitellogenin (*vtg1*) a gene that encodes for an egg yolk precursor protein and *18s* ribosomal RNA (*18s*), a gene important to cell function. Additional information on these genes may be found in Table 1. Primers for determining changes in gene expression were created using real-time Primer Quest Tool (Integrated DNA Technologies). Table 1 depicts the list of primers and their sequences tailored for this study.

Real-time quantitative PCR (qPCR) was performed on a StepOnePlus™ Real-Time PCR System (Life Technologies) using SYBR Green as the fluorescent intercalating dye (iTaq™ Universal SYBR® Green Supermix, Bio-Rad). Per reaction, cDNA and primers were mixed with SYBR Green following the recommended protocol. Starting with an initial 10 min denaturation at 95 °C, qPCR repeated 40 cycles of amplification, each cycle consisting of a 15 s at 95 °C period followed by 30 s at 62 °C period. Fluorescence of SYBR Green was detected at the end of each cycle. All qPCR experiments were done in two technical replicates. Miner and NORMA-Gene algorithm were used to analyze qPCR data, as previously reported.[21-23] All final relative fold change gene expression values obtained were $\log_2$ transformed before reporting.

Statistics

To determine the significance of the impacts of treatments compared to the controls, two statistical analyses (Mann Whitney U-test or One-Way ANOVA) were chosen depending on the distribution of the data and homogeneity of variances. The statistical analyses were performed using SPSS (IBM 2015). Impacts to daphnid survival were assessed using the nonparametric Mann Whitney U-test for two independent samples after the data failed normality and homogeneity of variances were determined. Outliers were identified using SPSS "Extreme values" identification process, which uses a step of 1.5 x the interquartile range. No outliers were found, and treatments were deemed significantly different than controls at $p < 0.05$. Impacts to daphnid reproduction were determined using One-Way ANOVA with Tukey HSD Post Hoc Test after the data was found to be normal and variances considered homogenous. No outliers were found, and treatments were deemed significantly different than controls at $p < 0.05$.

The relative gene expression data from *D. magna* chronic exposures were normalized to controls and $\log_2$ transformed to fit a normal distribution. Outliers were removed prior to

statistical analysis. Significant differences in relative expression were determined using either One-Way ANOVA with Tukey HSD Post Hoc Test or One-Way ANOVA with Dunnett's T3 Post Hoc Test after normality and homogeneity of variances were determined ($p < 0.05$) ($N > 3$).

RESULTS

Characterization of nanoscale NMC and LCO

SEM images of synthesized nanoscale NMC and LCO, demonstrate that both show sheet-like morphology (Supplemental Data, Figure S1). While the SEM resolution is limited by charging effects, the edge-on features can be approximated to be <5 nm in thickness (seen in Supplemental Data, Figure S1A and B). The nanosheets of NMC and LCO have a range of sizes in terms of their basal diameter (< 100 nm) because the sheet-like character of nanoscale NMC renders them fragile. Both synthesized NMC and LCO nanosheets were indexed to an $R\bar{3}m$ space group via powder X-ray diffraction measurements (Supplemental Data, Figure S2A and B).

Upon exposure to daphnid media, the electrophoretic mobilities of the NMC and LCO nanosheets were 0.19 ± 0.03 and 0.21 ± 0.06 μmcm/(Vs), respectively (Table 2). The colloidal stability of 10 mg/L NMC and LCO in daphnid medium was also assessed using UV-vis to analyze how the relative concentrations of NMC and LCO changed in the water column over time. Specifically, the sedimentation behavior of both LCO and NMC was analyzed by monitoring changes in optical absorbance over time, which is directly related to particle concentration[24-26]. Supplemental Data, Figure S3A shows that for NMC, an initial measurement at 0 h yielded a broad peak centering around 261.4 nm. At 3h, a sharper peak centered at 253.1 nm appeared with an absorbance reading of 0.127. Over 22.5 h, this peak at 253 nm decreased to an absorbance reading of 0.085, indicating an absorbance loss of 33%.

Supplemental Data, Figure S3B shows that for LCO, an initial measurement at 0 h yielded a peak at 275.5 nm with an absorbance reading of 0.229. Over 22.5 h, this peak shifted toward 269 nm with a final absorbance reading of 0.078; indicating a relative absorbance loss of 66%. The relative losses in absorbance show that LCO nanosheets sediment out of the water column faster than NMC over the course of 22.5 h.

Dissolution study

Lithium cobalt oxide and NMC leached measurable amounts of metal ions into solution when measured at 72 hours in daphnid media (Figure 1A and 1B). Most notably, for both materials, large quantities of Li were released into solution in a dose-dependent manner. For LCO, at 0.5 mg/L, 1.0 mg/L, 5.0 mg/L and 25 mg/L, Li was found at concentrations of 0.03 mg/L, 0.06 mg/L, 0.28 mg/L, and 1.32 mg/L in solution, respectively. For NMC, at 0.5 mg/L, 1.0 mg/L, 5.0 mg/L and 25 mg/L, Li was found at concentrations of 0.04 mg/L, 0.09 mg/L, 0.41 mg/L, and 2.04 mg/L in solution, respectively. Cobalt was found at lower concentrations, a maximum of 0.02 mg/L for LCO and 0.003 mg/L for NMC per 5 mg/L of material. Nickel was measured in solution at a maximum concentration of 0.08 mg/L for NMC at 5 mg/L. Manganese was found to be in solution below detection limits for NMC (< 0.001).

D. magna body burden study

Daphnids exposed to 1 mg/L NMC for 24 hours exhibited greater accumulation of NMC particles and or dissolved metals than daphnids with the same treatment plus an acid wash (Supplemental Data, Figure S4). Daphnids exposed to NMC particles accumulated 0.004 ± 0.00 μg/L, 0.323 ± 0.01 μg/L, 0.342 ± 0.01 μg/L and 0.386 ± 0.01 μg/L for Li, Ni, Mn and Co, respectively. Smaller amounts of particle and metal accumulation were measured for daphnids exposed to 1 mg/L NMC for 24 hours and treated with an acid wash. Daphnids with the acid

wash contained concentrations of Li, Ni, Mn and Co equal to 0.003 ± 0.00 μg/L, 0.190 ± 0.01 μg/L, 0.217 ± 0.01 μg/L and 0.273 ± 0.01 μg/L, respectively. In comparing NMC treated to NMC plus acid wash, the amount of each metal measured for Ni ($F = 0.023$, $df = 4$, $p < 0.05$), Mn ($F = 0.017$, $df = 4$, $p < 0.05$), and Co ($F = 0.068$, $df = 4$, $p < 0.05$) were found to be significantly different, except for Li ($p > 0.05$). Controls showed very little amounts of metal contamination. Nickel was found below the detection limit in control daphnids. The remaining three metals were measured at 0.004 μg/L, 0.004 μg/L, and 0.043 μg/L for Li, Mn and Co, respectively.

D. magna acute and chronic toxicity

Lithium nickel manganese cobalt oxide and LCO NMs had no impact on daphnid mortality at any of the tested concentrations ($p > 0.05$, 1-25 mg/L). However these materials were visible in the digestive tract and adhered to carapace *of D. magna*.

LCO nanoparticles and supernatant. Chronic exposures of LCO demonstrated significant negative impacts to daphnid survival, reproduction at concentrations greater than 0.25 mg/L (Figure 2A and 3A, respectively). The lowest concentration tested, 0.05 mg/L caused no impacts to measured endpoints. A concentration of 0.1 mg/L caused a 23.2% increased in daphnid reproduction compared to control but this was not found to be significant ($p < 0.05$). A concentration of 0.25 mg/L, decreased daphnid reproduction by 79.8% ($F = 32.942$, $df = 15$, $p < 0.05$) and daphnid survival by 40% at day 21 compared to control ($U = 0.0$, $p > 0.05$). Concentrations of LCO at 0.5, 1 and 5 mg/L caused 100% mortality by day 21 ($U = 0.0$, $p < 0.05$) and no reproduction was observed compared to control. No impacts to daphnid body size at any concentration were observed (Supplemental Data, Figure S5). Exposure to the LCO supernatant caused no impacts at similar concentrations where impacts were observed from LCO

nanomaterials (NM).

*NMC nanoparticles and supernatant***.** Chronic exposures caused significant impacts to daphnid survival, reproduction and body size at concentrations greater than 0.25 mg/L, in a dose-dependent manor (Figure 2B, 3B and Supplemental Data, Figure S6, respectively). The lowest concentration tested, 0.25 mg/L caused a 21% increase in reproduction, though not significant, and no impacts to daphnid survival. However, 0.5 mg/L caused a significant 44% reduction in daphnid reproduction ($F = 28.740$, $df = 19$, $p < 0.05$) compared to control. No significant differences in body size or survival were found at this concentration. Daphnids exposed to 1.0 mg/L NMC particles exhibited significant impacts to body size, mortality and reproduction compared to control. This concentration caused an 87% decrease in daphnid reproduction ($F = 28.740$, $df = 19$, $p < 0.05$), a reduced body size by 15.5% ($p < 0.05$)(Supplemental Data, Figure S6), and a 60% reduced survival rate at day 21 ($U = 0.0$, $p < 0.05$) when compared to control. The two highest concentrations, 5.0 and 25 mg/L, elicited significant impacts to daphnid survival with 100% reduction in survival by day 21 ($U = 0$, $p < 0.05$). Exposure to the NMC supernatant caused no impacts at similar concentrations where impacts were observed from NMC NMs.

Nickel. Measured concentrations of Ni leached in the dissolution study did not cause impacts to daphnia survival or reproduction. However, chronic exposures to Ni caused significant impacts to daphnid survival and reproduction at 0.4 mg/L (Figure 4A and 5A, respectively). At this concentration, 100% daphnid mortality occurred at day 21 ($U = 0.0$, $p < 0.05$) and an 80.3 % reduction in daphnid reproduction was recorded ($F = 35.651$, $df = 18$, $p < 0.05$). At 0.1 and 0.2 mg/L Ni there was a 17.7 and 25.4% increase in daphnid reproduction. No impacts to daphnid body size were observed (Supplemental Data, Figure S7).

Cobalt. Co at concentrations leached from dissolution of particles did not cause

significant impacts to daphnid survival or reproduction. Exposure to cobalt controls did not cause a significant reduction to daphnid survival and reproduction until 0.1 mg/L (Figure 4B and 5B, respectively). At 0.1 mg/L Co, daphnids experienced an 89.6% reduction in reproduction ($F = 42.276$, $df = 15$, $p < 0.05$) and a 20% reduction in daphnid survival ($U = 0.0$, $p < 0.05$) when compared to controls. At 0.2 mg/L 100% reduction in daphnid survival was observed at day 21 ($U = 0.0$, $p < 0.05$). No impacts to daphnid body size were observed (Supplemental Data, Figure S8).

Lithium. Only the highest measured concentration of Li leached from 25 mg/L of NMC caused significant impairments to daphnid survival and to reproduction (Figure 4C and 5C, respectively), no other concentration of NMC or LCO measured Li leachate caused significant impacts. At 2.5 mg/L Li, daphnids exhibited a 97.8% reduction in reproduction ($F = 85.223$, $df = 12$, $p < 0.05$). At 2.5 mg/L, 5.0 mg/L and 10 mg/L 100% reduction in daphnid survival was observed at day 21 ($U = 0.0$, $p < 0.05$). No impacts to daphnid body size were observed (Supplemental Data, Figure S9).

Gene expression

Daphnids exposed to LCO and NMC for 21 d exhibited dose dependent down regulation of a number of genes explored in the present study (Figure 6A and 6B, respectively and Supplemental Data, Table S1). Of the NMs we tested, NMC appeared to down regulate genes to a greater extent than LCO.

Daphnids exposed to NMC and LCO supernatant controls exhibited a different response compared to the NMs themselves. Supernatent from ithium cobalt oxide suspensions caused an increase in the expression of *18s, vtg1* and *mtla*, but this was not significant (Figure 6, $p > 0.05$). However, LCO at 0.25 mg/L significantly decreased daphnid relative expression of *18s, cat,*

vtg1, gst, act, and hsp70 (Figure 6, $p < 0.05$). Only *vtg1* was significantly down-regulated with lithium cobalt oxide at 0.1 mg/L (Figure 6, $p < 0.05$). Dapnid gene expression upon exposure to the NMC supernatant showed no significant changes (Figure 6, $p < 0.05$). Daphnids exposed to concentrations of NMC at 1.0 mg/L showed significant down-regulation of genes 1*8s, cat, gst, act, mtla* and *hsp70* (Figure 6, $p < 0.05$). Daphnids exposed to a lower concentration of NMC, 0.25 mg/L, showed significant decreases in the relative expression of *cat, vtg1, gst* and *hsp70* (Figure 6, $p < 0.05$). For specific gene expression values and statistical information see table 1 in the supporting information (Supplemental Data, Table S1).

DISCUSSION

The results suggest that the effects elicited from both NMC and LCO particles were not due to the free ions alone, but rather are a specific effect caused by the ingestion and/or adhesion to the daphnid carapace of the NMs themselves (Figure 2 and 3). Lithium nickel manganese cobalt oxide (33% Ni, 33% Mn and 33% Co) and LCO (100% Co) NMs impacted daphnid survival, reproduction and body size in a dose dependent manor independent of the free ions present in suspension. In our study, the NMC and LCO particles visibly adhered to the daphnid carapace and accumulated inside the digestive tract of the organism. This was further supported by the body burden assay with NMC that demonstrated a significant amount of NMs both adhered and ingested/internalized in *D. magna*. Other studies report similar effects with metallic NMs. For example, metal and metal oxide nanoparticles have been shown to adhere to bacteria and algae and the authors concluded that it was nanoparticle adherence that caused toxicity due to localized delivery of toxic ionic metals[15, 16]. While free ions, Li, Ni, Mn, and Co in suspension do not explain the observed effects, NMC and LCO particulates could potentially be

delivering a localized high concentration of free ions on or inside of daphnid cells that were not accounted for by the free ion and supernatant control treatments.

Toxic metals have been shown to damage gut epithelial cells involved in nutrient uptake and sensitive organs such as the gills as these sites in particular have been shown to be the main sites of accumulation for water borne metals such as Ag[27] and Ni [28]. One study found that, upon exposure to sub-lethal concentrations of nickel and cobalt, degeneration of the hepatic caeca located in the digestive tract of *Daphnia magna* occurred.[29] In daphnids, the hepatic caeca (diverticula) are important to acid production[30], food digestion[31] and nutrient uptake.[32] In the presence of nickel, *D. magna* suffer from drastically reduced whole body glycogen content.[33] LCO and NMC nanomaterials may undergo chemical transformations such as redox-enabled dissolution of the material upon accumulation inside the digestive tract. Therefore, upon exposure to LCO and NMC materials, potential localized high concentrations of these metals in their bodies may cause daphnids to experience an impact to their energy budgets due to impaired nutrient assimilation. This might explain the decrease in reproduction, body size and increased mortality rate at low concentrations.

Chemical composition appeared to be important for determining toxicity and in particular the cobalt content of the nanomaterial indicating that choice of material may mitigate toxicity for these next-generation materials. Replacing toxic metals with those that are generally less toxic may be a way to improve sustainability. For example, CdSe/ZnS and InP/ZnS core/shell NMs were exposed to two cell lines, SH SYSY and A549 cells. CdSe/ZnS and InP/ZnS were well characterized with comparable physical and chemical properties only differing in chemical composition[34]. This study concluded that, substituting Cd and Se with In and P as the core material components in the core/shell NM mitigated all impacts to cellular viability at the tested

concentrations[34]. Lithium cobalt oxide was more toxic than NMC causing similar impacts to long term survival and reproduction at concentrations four times lower indicating chemical specific impacts. Of the three individual metals assessed in the present study, Co was the most toxic to *D. magna* (Figure 4B and 5B), impacting reproduction and survival at concentrations as low as 0.1 mg/L. Dissolution studies revealed higher amounts of Co leached from LCO NMs potentially explaining why this material was more toxic to daphnids (Figure 1). These results indicate that replacing toxic metals with less toxic metals (in their ionic form) may reduce the potential for impacts to environmental health.

A nanospecific effect may have also been caused by a physical impact as LCO and NMC were found to adhere to daphnids. Previous studies have shown that metal and clay particulates may cause physical insults to invertebrates at high concentrations due to constipation and feeding inhibition (e.g. disruption of collection and ingestion of algae) and that there may be unique impacts due to the particulate chemical composition[35]. Because LCO and NMC have the same morphology, similar density and aggregation but differ greatly in their impacts may indicate that physical effects were not an important factor at low concentrations. Our previous studies showed that 4 nm citrate coated gold nanoparticles adhered to daphnids (over the course of 48-hours) and physically impaired daphnid reproduction and body size in 21-d chronic studies[36]. These particles aggregated to sizes greater than a micron. However, the impacts to reproduction and growth were only observed at high concentrations (25 mg/L) and were thought to be due to the extended fused networks the agglomerated gold nanoparticles formed. Similar sized gold nanoparticles that did not form extended fused networks of particles; rather aggregated groups of individual nanoparticles and did not cause any effects at high concentrations (25 mg/L)[36]. This further supports our hypothesis that effects are related to chemical interactions with the organism

rather than physical, as LCO and NMC particles caused apical endpoint impacts at low concentrations (0.25 mg/L and 1 mg/L, respectively)(Figure 2 and 3).

Gene expression results further supported a unique nano-specific effect as the impacts could not be explained by the supernatant controls that accounted for background dissolved metals in suspension (Figure 6). Another study exploring different metal oxides found that the metal oxide and their respective soluble metal had a distinct molecular fingerprint[12] and the dissolved metals could not replicate the molecular level response upon exposure to their respective NM. Lithium nickel manganese cobalt oxide and LCO caused a significant down regulation of genes involved in oxidative stress, heavy metal metabolism and toxicity, reproduction and cell maintenance related pathways whereas controls did not. Other studies report that upon exposure to metal oxide NMs have trigger the down regulation of a wide range of genes, more so than up regulation[12]. The observed down regulation of 18s rRNA may potentially indicate decreased translation and cellular response to the exposure.

CONCLUSION

In the present study, we found that NMC and LCO NMs leached measurable amounts of lithium, nickel and cobalt into solution; however, controls that accounted for free ion and supernatant toxicity could not explain the observed effects when NMC and LCO NMs were present in suspension. Furthermore, particles were ingested by daphnids and found to adhere to their carapace leading us to believe that the effects we observed are NM specific. While we cannot rule out a metal ion-induced mechanism for toxicity, we hypothesize that the observed impacts are caused by the physical adherence of the NM to biological membranes and a localized delivery of a high concentration of toxic metals. In addition, our study demonstrated that the toxicity of the materials in question is dependent upon chemical composition with the

replacement of cobalt by nickel and manganese leading to increased daphnid survival and reproduction. Therefore, replacing toxic metals with less toxic metals may increase the environmental compatibility and sustainability of next generation battery materials. To our knowledge this is the first study assessing the toxicity of a multicomponent complex metal oxide to *Daphnia magna*.

With the growth in lithium ion based energy storage technologies industry seeks to develop new materials with enhanced qualities, such as increased intercalation rates, energy storage capacity and resistance to degradation. First generation materials such as LCO may be replaced by nanomaterials such as NMC as they enable industry to achieve their performance standards. The most important quality of battery material design should be its environmental biocompatibility, however, the toxicity of these materials has not been part of the design process. In order to develop sustainable cathode NMs we must get ahead of the production cycle. Two important considerations are the impacts of using NM versions of these materials as well as the consequences of replacing nickel and manganese for cobalt as we shift from LCO to NMC.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

Acknowledgment—This work was funded by the National Science Foundation Center for Chemical Innovations grant CHE-1503408: Center for Sustainable Nanotechnology.

Data Availability—The Center for Sustainable Nanotechnology keeps a repository of all data associated with projects that can be made available upon publication and request by others.

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Figure 1. ICPMS results for metal dissolution in daphnid media at 72 hours. Particle dissolution for (A) lithium cobalt oxide (LCO) nanoparticles and supernatant and (B) lithium nickel manganese cobalt oxide (NMC) nanoparticles and supernatant. Error bars represent standard deviation, $n = 3$.

Figure 2. Average chronic daphnid survival rate over 21 days compared to controls. Survival rate for (A) lithium cobalt oxide (LCO) nanoparticles and supernatant and (B) lithium nickel manganese cobalt oxide (NMC) nanoparticles and supernatant. Error bars represent standard error of the mean, $n \geq 4$. Asterisks indicate significance compared to control ($p < 0.05$).

Figure 3. Average chronic daphnid reproduction over 21 days compared to controls. Percent control reproduction per individual (A) for lithium cobalt oxide (LCO) nanoparticles and (B) for lithium nickel manganese cobalt oxide (NMC) nanoparticles. Error bars represent standard error of the mean, $n \geq 4$. Asterisks indicate significance compared to control ($p < 0.05$).

Figure 4. Average chronic daphnid survival rate over 21 days compared to controls. Average survival rate for (A) nickel, (B) cobalt and (C) lithium. Error bars represent standard error of the mean, $n = 3$. Asterisks indicate significance compared to control ($p < 0.05$).

Figure 5. Average chronic daphnid reproduction over 21 days compared to controls. Percent control reproduction per individual for (A) nickel, (B) for cobalt and (C) lithium. Error bars represent standard error of the mean, $n = 3$. Asterisks indicate significance compared to control ($p < 0.05$).

Figure 6. Average daphnid relative fold change gene expression (Log_2) at day 21 compared to controls. Average gene expression for (A) lithium cobalt oxide (LCO) nanoparticles and supernatant and (B) lithium nickel manganese cobalt oxide (NMC) nanoparticles and

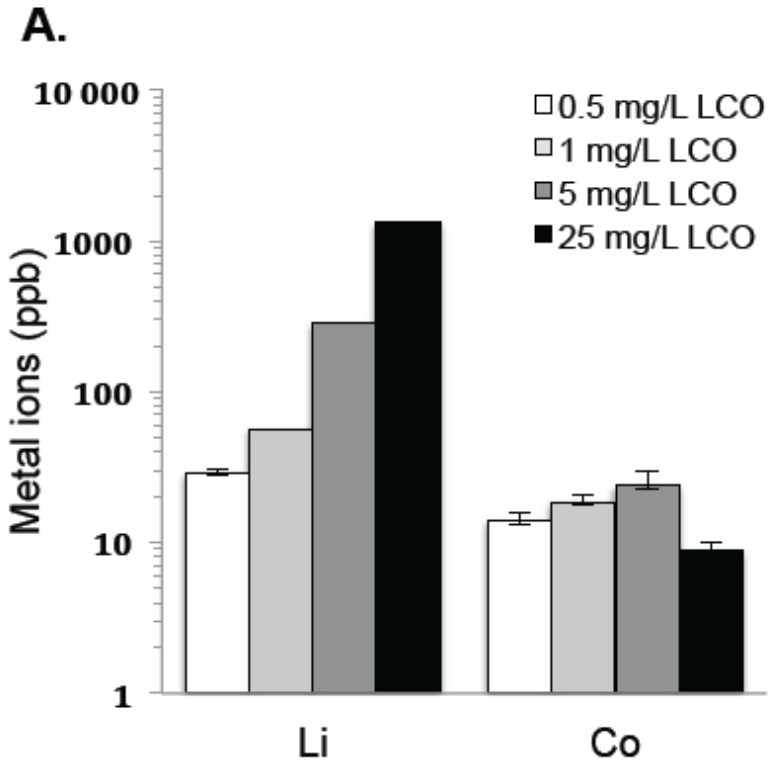
supernatant. Error bars represent standard error of the mean, $n \geq 4$. Asterisks indicate significance compared to control ($p < 0.05$).

Table 1. Nanomaterial characterization in daphnid media

Milli-Q water	ζ-Potential (mV)	Electrophoretic Mobility (μmcm/Vs)
LCO	-6.25 ± 0.45	-0.49 ± 0.03
NMC	-7.08 ± 1.75	-0.55 ± 0.33
Daphnia media		
LCO	2.73 ± 0.70	0.21 ± 0.05
NMC	2.50 ± 0.40	0.20 ± 0.02

Table 2. Target genes

Target Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Function	Accession #
Glutathione S-transferase (*gst*)	CAA CGC GTA TGG CAA AGA TG	CTA GAC CGA AAC GGT GGT AAA	Xenobiotic detoxification	AF448500.1
Catalase (*cat*)	CAG GAT CAT CGG CAG TTA GTT	CTG AAG GCA AAC CTG TCT ACT	Oxidative stress attenuation	GQ389639.1
Metallothionein (*mtla*)	TTG CCA AAA CAA TTG CTC AT	CAC CTC CAG TGG CAC AAA	Metal detoxification	KF561474.1
Vitellogenin (*vtg1*)	CTG TTC CTC GCT CTG TCT TG	CCA GAG AAG GAA GCG TTG TAG	Reproduction, sexual maturation and general stress	AB252737.1
18s (*18s*)	CGC TCT GAA TCA AGG GTG TT	TGT CCG ACC GTG AAG AGA	Protein synthesis	AM490278.1
Heat shock protein 70 (*hsp70)*	CCT TAG TCA TGG CTC GTT CTC	TCA AGC GGA ACA CCA CTA TC	Response to heat; protein folding	EU514494.1
β-Actin (*act*)	CCA CAC TGT CCC CAT TTA TGA A	CGC GAC CAG CCA AAT CC	Cytoskeleton production, cell maintenance	AJ292554.1



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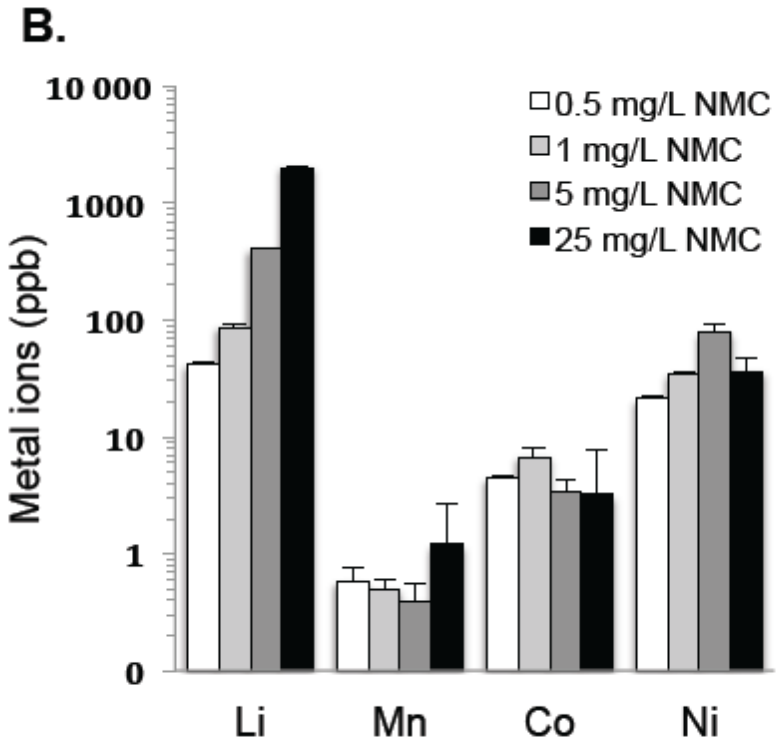

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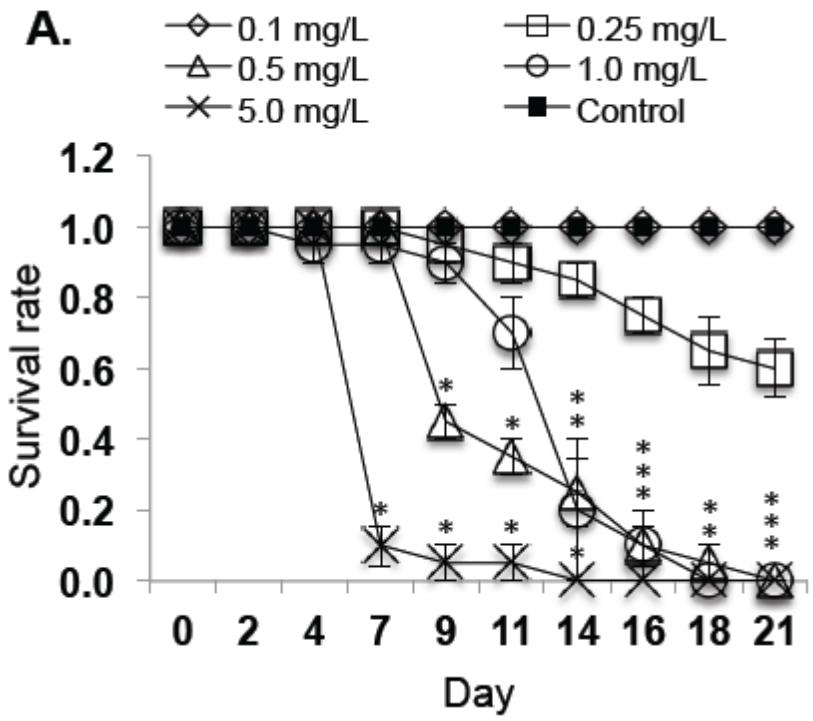

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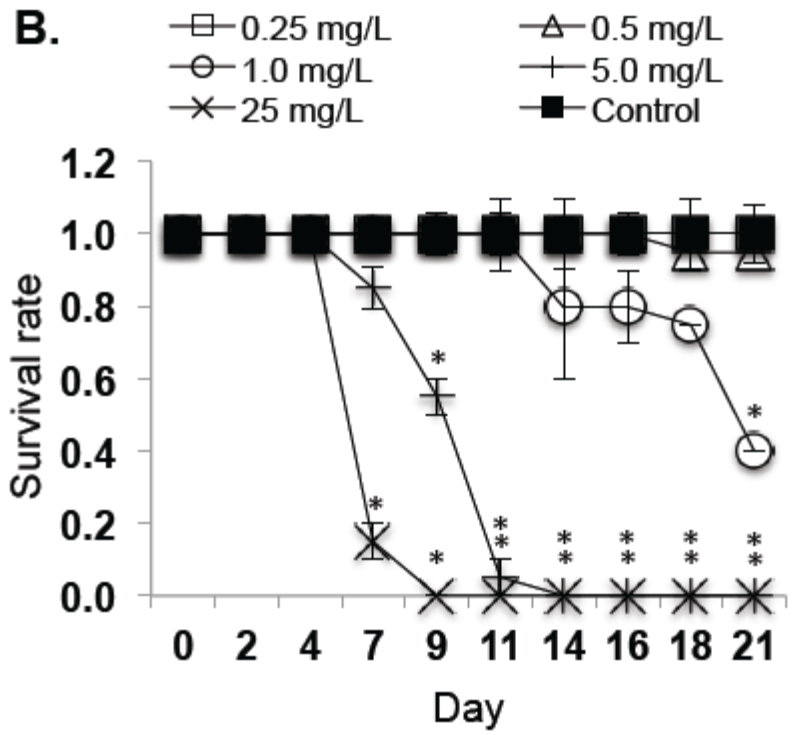

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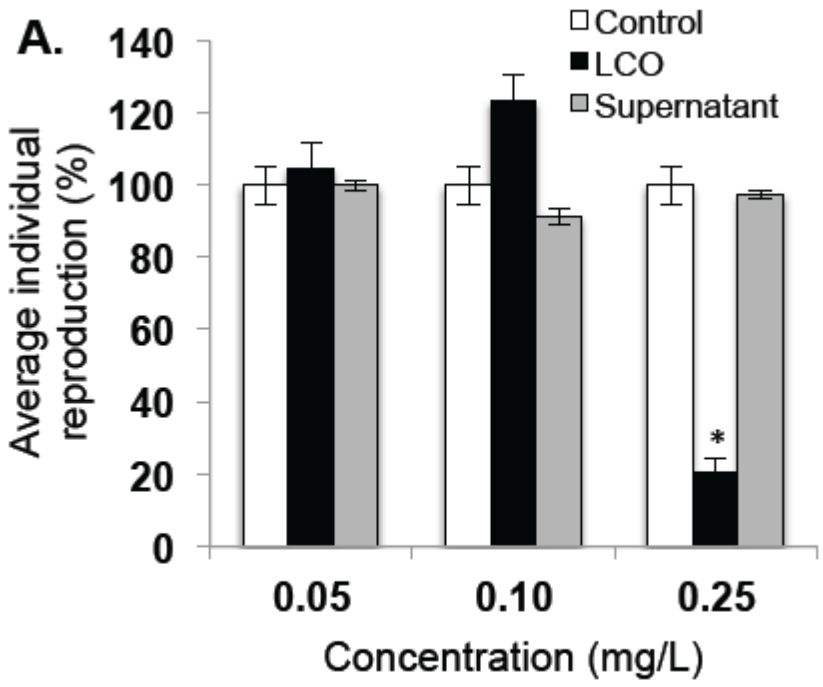

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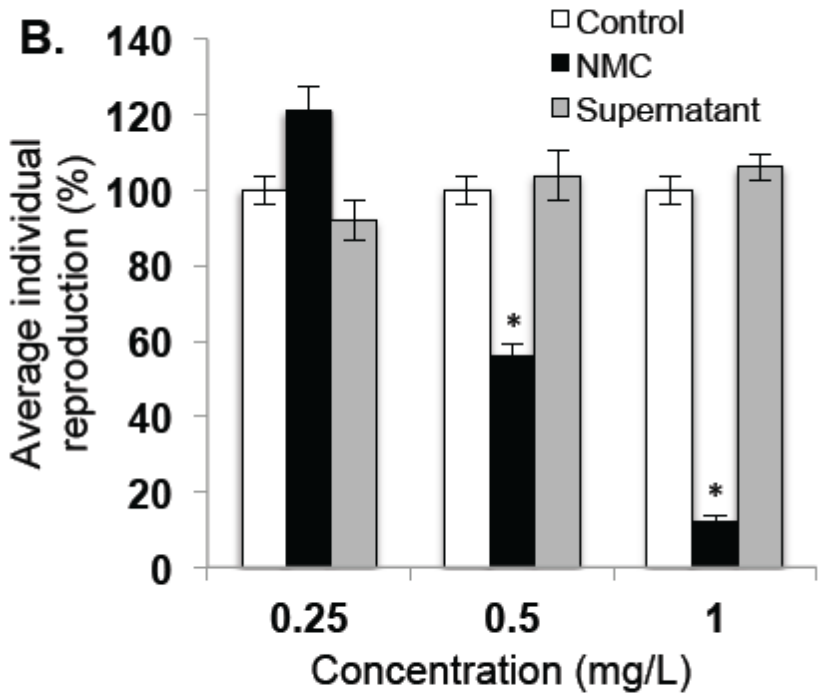

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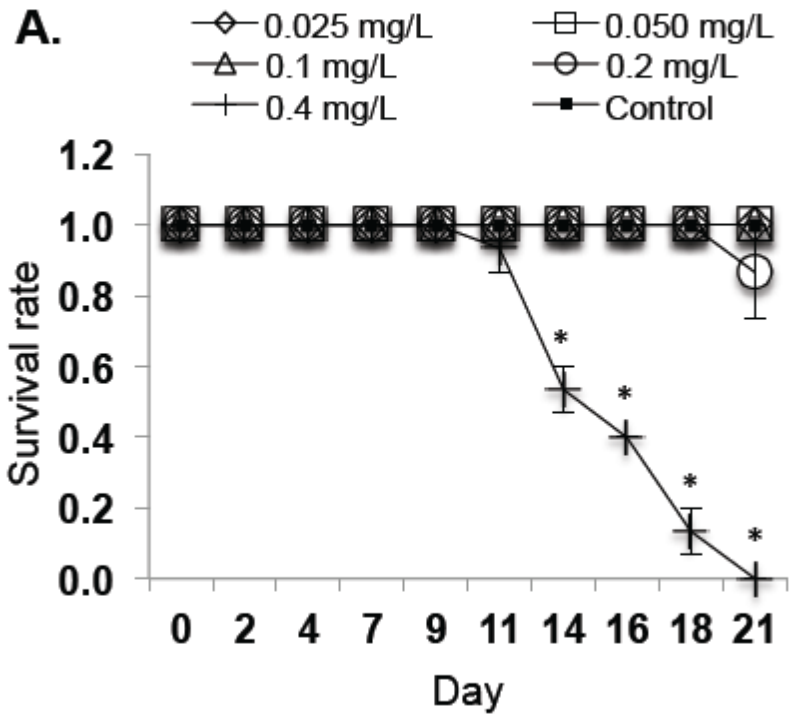

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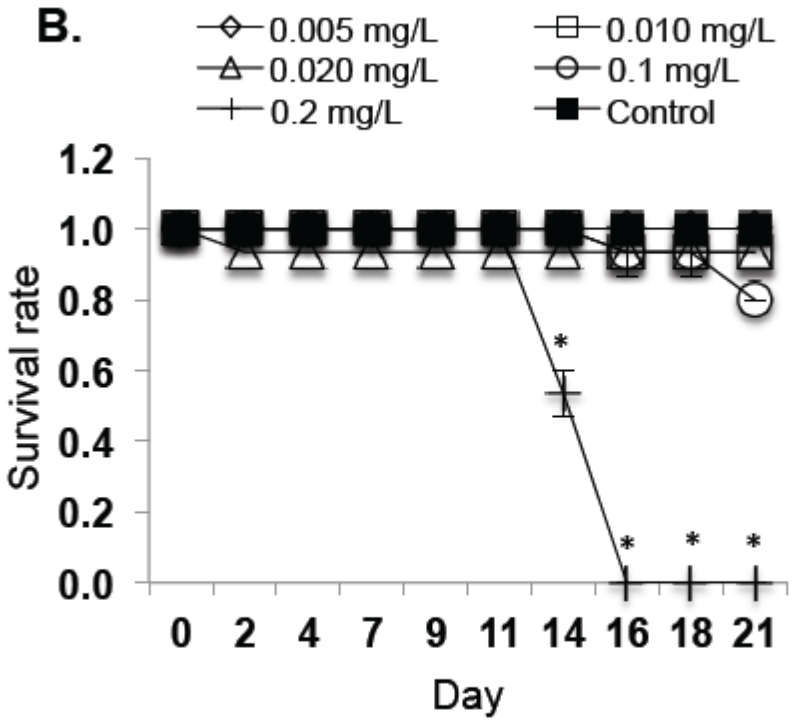

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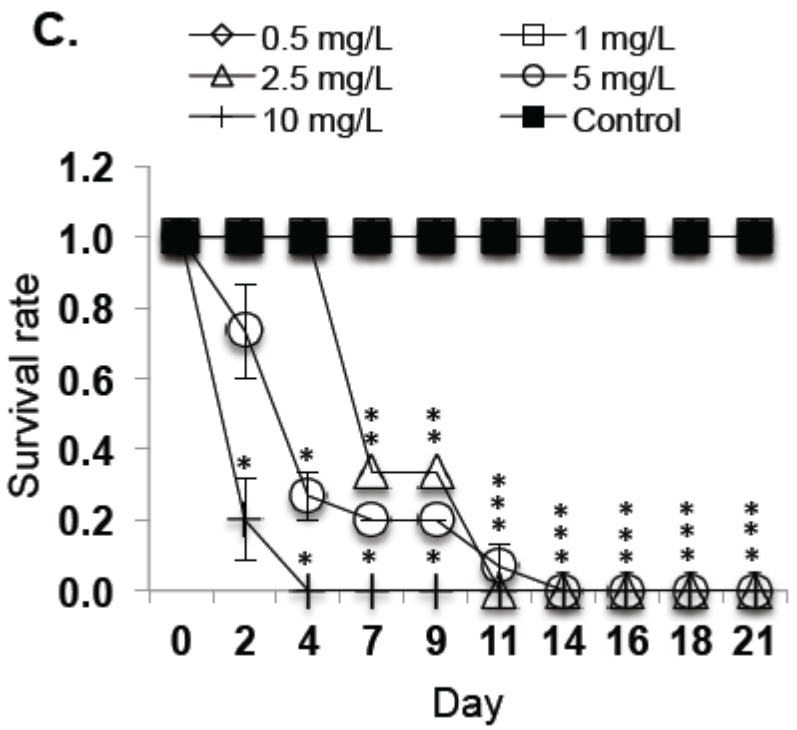

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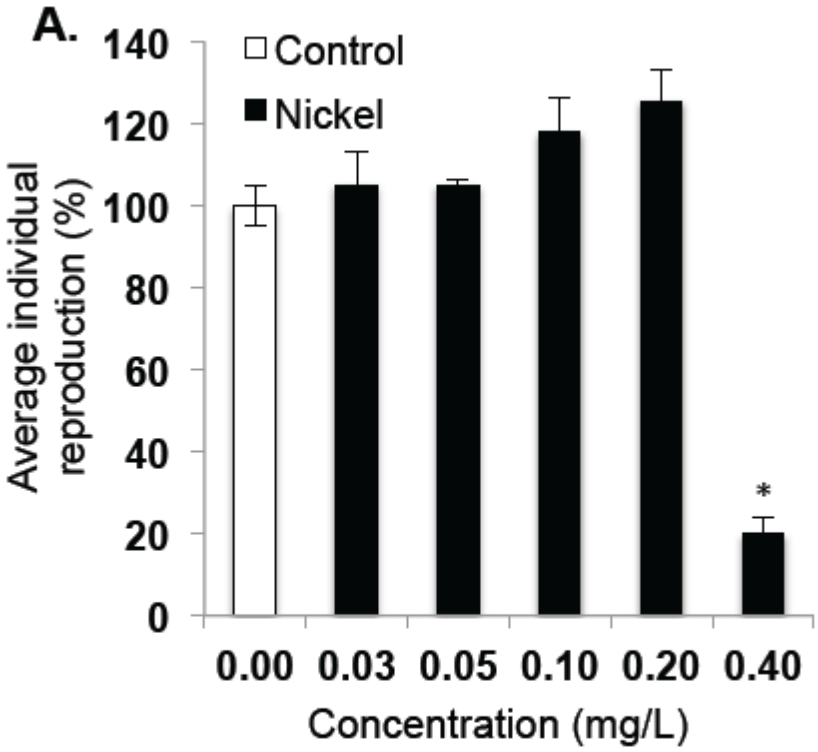

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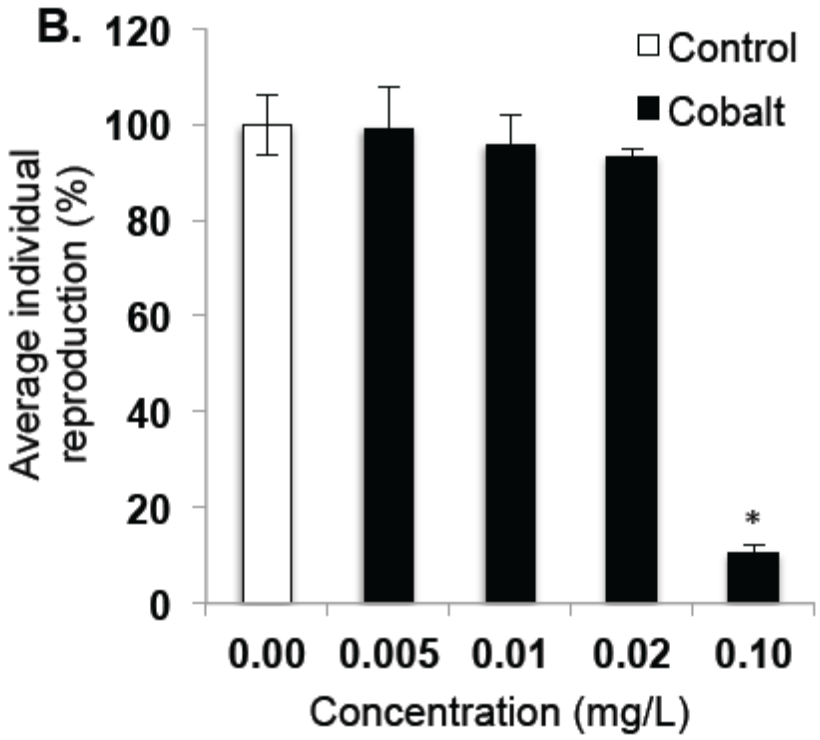
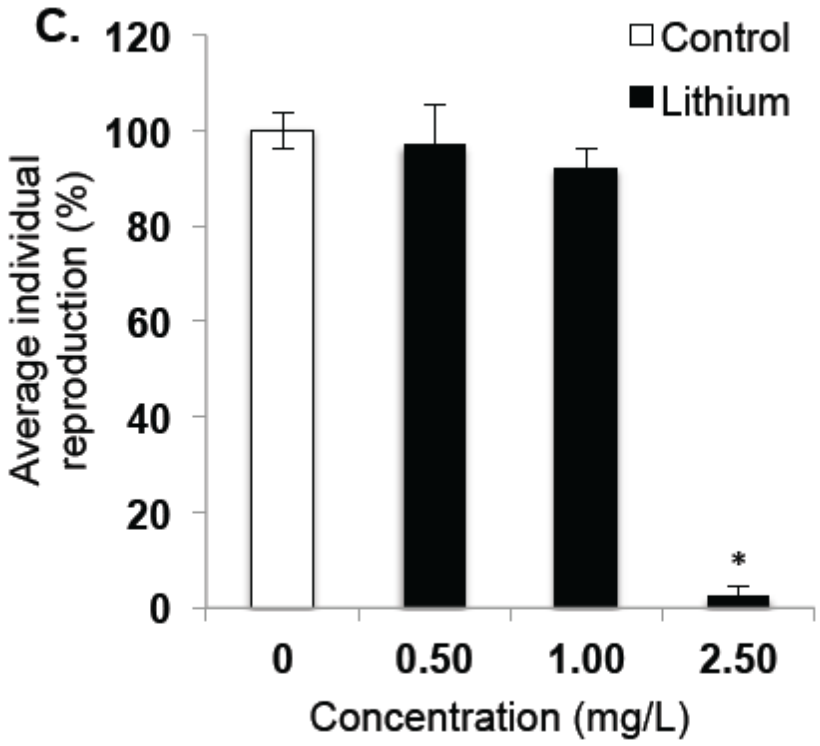


Figure 5B



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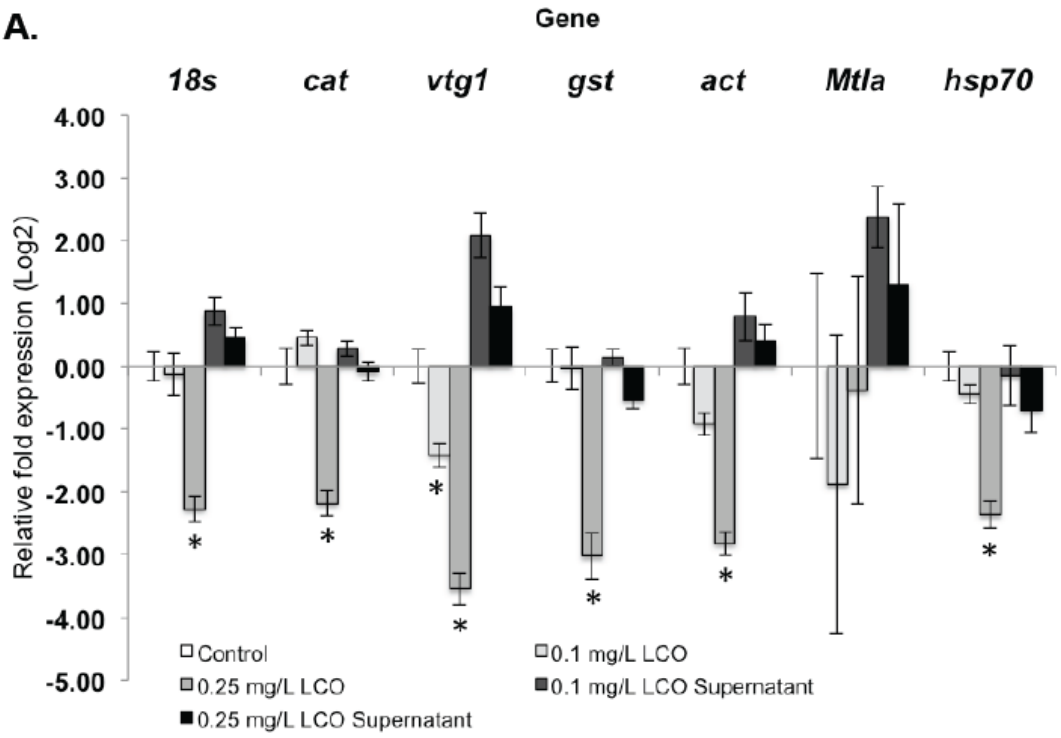

Figure 6A

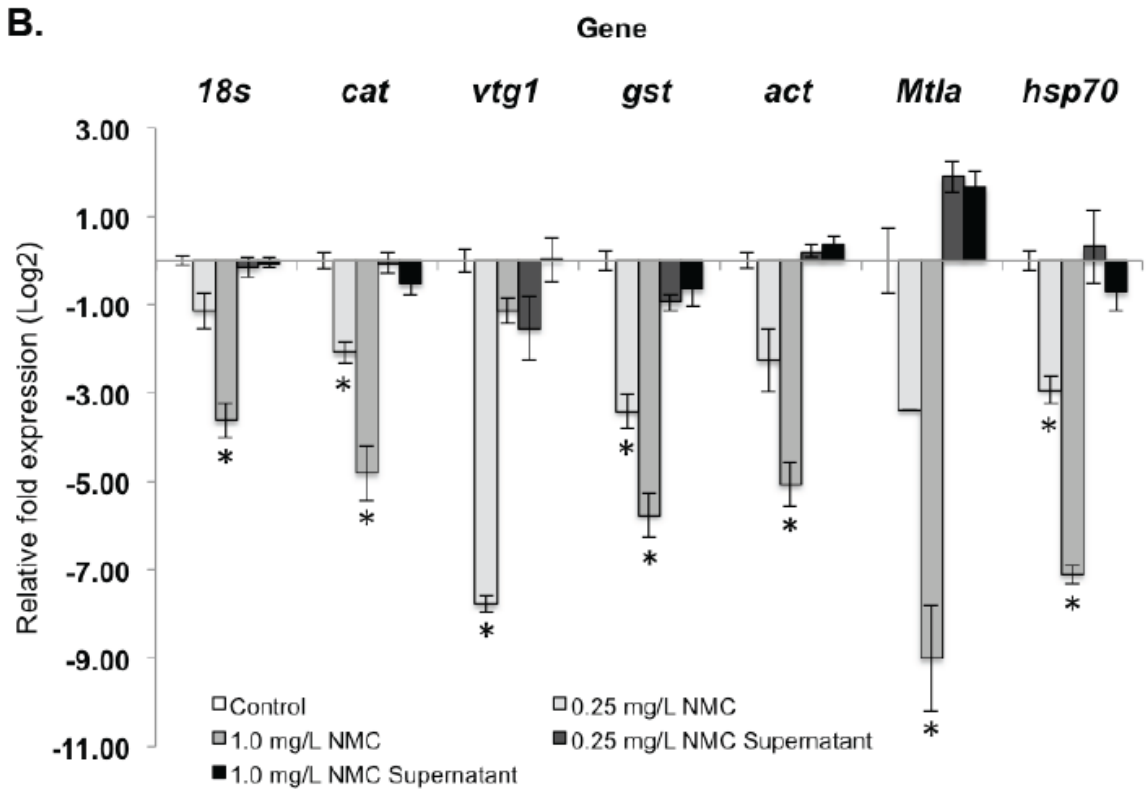

Figure 6B