Work Plan For Great Salt Lake Toxicity Tests

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Introduction:

Great Salt Lake (GSL) is a unique ecosystem, the fourth largest (largest in the western hemisphere) and least human-impacted hypersaline lake in the world[16]. Brine shrimp (*Artemia franciscana*) and brine fly larvae (2 species: *Ephydra hians* and *cinerea*) are the dominant grazers in the GSL food web, a relatively species poor ecosystem due to its high salinity. These invertebrates are very abundant and are the main source of food for many resident and migrating water birds, which have important ecological and conservation value. Some of these water bird species are threatened or endangered.

The purpose of this project is to determine toxic levels of mercury, copper, arsenic and lead, and possibly ammonia (when appropriate analytical chemistry methods have been identified[15]), to brine shrimp and brine fly larvae so that water quality criteria (WQC) can be developed for GSL. These potential pollutants were identified as priorities by the State of Utah Division of Water Quality (UDWQ) after public feedback and comments.

We will conduct acute and chronic toxicity tests of the above substances for brine shrimp and brine fly larvae (*E. cinerea*). American Society for Testing and Materials (ASTM) has not sanctioned standard methods for toxicity testing with either of these species. However, other ASTM-approved methods for invertebrates are established and will be used as a guide for the conduct of the toxicity testing proposed here.

In this work plan we present the protocols that lead to uniform rearing of the brine shrimp and brine fly larvae, as well as production of control and test (pollutant) solutions. We then focus on range-finding and acute testing, which must be completed before proceeding with the ecologically more relevant chronic testing (survival and reproduction with life-time exposure to the pollutant), which will be detailed in a future work plan. Range-finding establishes the range of concentrations for each pollutant that produces short term (96 hour) mortality for each species (producing mortality, but not necessarily 100% mortality). Once the range for each pollutant has been established, acute tests will be conducted to establish the concentration of each pollutant that will produce 50% mortality over 96 hours of exposure (LC50). This work will be conducted at North Carolina State University (NCSU) and University of Notre Dame (UND). Because UND has developed the organism rearing protocols and to ensure consistency in testing, a representative of the NCSU lab will visit UND to exchange information before conducting range-finding or acute testing.

Source animals and rearing conditions:

Given the uniqueness of the Great Salt Lake compared to other aquatic environments for which ecotoxicology studies have been developed, it is critical that our toxicology studies provide results that are applicable to the lake's environment. The environmental conditions found in the Great Salt Lake in April – October (the time when brine shrimp are present) over a 20 year period (1994 – 2013[16, 17]) are summarized in the table below.

PARAMETER	MEAN	STANDARD DEVIATION	RANGE
Salinity (ppt)	128.1	24.7	82.2 – 191.0
Temperature (^{o}C)	20.7	4.6	10.2 – 29.5
pH	7.9	0.4	7.1 – 8.7

Therefore, we will rear organisms and conduct toxicity tests under conditions that approximate average GSL conditions (salinity = 120 ppt, temperature = 20°C, pH = 7.9).

Consistency of test organisms employed by UND and NCSU labs must be ensured. For brine shrimp this will be achieved by using a single batch of brine shrimp cysts (resting eggs) that have been commercially harvested from GSL that will be divided between the labs. For brine fly larvae, all individuals will be obtained from a colony maintained at UND, where GSL was the source of individuals starting the colony and the colony has been in existence for approximately two years (6 – 8 generations).

Both species will be reared in environmental chambers that maintain temperature ($\pm$ 1°C) and a light:dark cycle (16:8, ~summer day using Gro-Lux® bulbs) using the following protocols:

> **Brine shrimp** will be hatched in 10 gallon aquaria at a salinity of 45 ppt, the optimum for hatching and hatchling survival. Twenty-four hours after hatching the salinity will be raised to 60 ppt over an hour; after another 24 hours, the salinity will be raised to 90 ppt over an hour; finally, after another 24 hours, the salinity will be raised to average lake salinity (120 ppt). During this time, individuals are fed ad libidum a high quality phytoplankton (*Dunaliella* sp.: 40 µg chl_a/L/2 days) from a colony established from GSL.
>
> **Brine fly larvae** will be reared in plastic containers (60 cm X 60 cm X 25 cm) that contain 12 cm of water that is maintained at average lake conditions. Gravel and GSL bioherm (approximately 30 cm X 15 cm X 15 cm) serve as a substrate for larvae and pupae, and an above water platform is provided to emerging adults for resting and mating. Larvae will be fed ad libidum (pupae and adults do not feed) a high quality food (*Coccochloris* sp.: 40 µg chl_a/L/2 days) from a colony established from the GSL.

Stock solutions (controls):

Stock solutions with a salinity of 120 ppt for laboratory culture of brine shrimp and brine fly larvae will be created by dissolving Instant Ocean® synthetic sea salt (~800 ml measured volumetrically) and Morton® Solar Salt Water Softening Crystals from GSL (~1200 ml measured volumetrically) in 15 L of deionized water (hydrometer reading = 1.082). The mixing

of Instant Ocean® and water softener salts is the working protocol for studying brine shrimp and brine fly larvae at UND (Belovsky unpubl.). pH will be maintained at 7.9 ± 0.2 by adding 0.1N nitric acid to decrease the pH or 0.1N sodium hydroxide to raise the pH as needed. Solutions will be prepared in 20L Nalgene carboys. Filtered triplicate samples will be collected from each batch to verify that the salt concentrations and pH are within acceptable ranges.

Test solutions (treatments):

The pollutants chosen for these studies were selected by UDWQ after soliciting public comment. American Chemical Society (ACS) reagent grade chemicals will be employed. To maximize comparability of test results with published ecotoxicology studies[2-14] that have been conducted with other aquatic invertebrates, the following chemicals will be used to produce test solutions:

- As (arsenic) as sodium arsenate;
- Cu (copper) as copper chloride;
- Hg (mercury) as mercury chloride;
- Pb (lead) as lead nitrate.

Because the addition of the above chemicals will alter salinity and pH, preliminary tests of salinity and pH will be conducted and the base water will be modified to maintain average GSL conditions (120 ppt salinity and a 7.9 pH) for each pollutant concentration. Based on UND protocols, the following mixtures (dry volume) of Instant Ocean® and water softener salts produce the following salinities:

Salinities	Morton's Water Salt	Instant Ocean	Hydrometer
(1.5%) 15 ppt	≅ 85 ml/15 L	≅ 55 ml/15 L	1.009
(2.5%) 25ppt	≅ 190 ml/15 L	≅ 125 ml/ 15 L	1.016
(3.0%) 30 ppt	≅ 270 ml/15 L	≅ 170 ml/15 L	1.02
(4.5%) 45ppt	≅ 400 ml/15 L	≅ 250 ml/15 L	1.03
(6.0%) 60 ppt	≅ 540 ml/15 L	≅ 350 ml/15 L	1.04
(9.0%) 90 ppt	≅ 810 ml/15 L	≅ 510 ml/15 L	1.06
(12.0%) 120 ppt	≅ 1200 ml/15 L	≅ 800 ml/15 L	1.082
(15.0%) 150 ppt	≅ 1500 ml/15 L	≅ 980 ml/15 L	1.104

Range finding tests:
For each pollutant to be tested, a preliminary range finding test will be conducted to establish concentrations to be used in the acute tests so that well resolved concentration-response (mortality or immobility) curves result. Range finding will be conducted with 10-fold increases in dissolved concentrations (e.g., 0 (controls), 10, 100, 1,000, 10,000, 100,000 µg of the pollutant/L). Exposure to each concentration will be tested on three replicates for each organism over a 96 hour period on a static renewal basis (100% water changes occurring at 24, 48 and 72 hours) in the absence of food (EPA standard procedure for acute toxicity testing). Each replicate for a pollutant concentration will be conducted as follows:

Brine shrimp – nauplii will be moved from the stock culture (see above) to a 1 L container at the same salinity, pH and temperature where they are starved for 24 hours. Twenty individuals will then be held in a 50 ml HDPE beaker containing 30 ml of the test solution.

Nauplii will be used, because we assume that they are most sensitive to pollutants given their small size and soft exoskeleton.

Brine fly larvae – 3rd instar larvae will be moved from the stock colony to a 1 L container at the same salinity, pH and temperature where they are starved for 24 hours. Ten individuals will then be held in a 250 ml HDPE beaker containing 150 ml of test solution. A small piece of Teflon mesh will be provided as an attachment substrate. Third instar larvae are used, because we assume that they are more sensitive to pollutants given their small size, and while smaller larvae may be more sensitive, they are too difficult to handle.

Tests will occur under conditions that approximate average GSL conditions (120 ppt salinity, 20^oC temperature, and a 7.9 pH). Tests with As, Cu and Pb will be conducted in programmable environmental chambers (temperature $\pm$ 1.0^oC and 16:8 light:dark cycle using Gro-Lux® bulbs). Mercury tests will be performed in a fume hood at NCSU, where temperature and light cycle will be similarly controlled respectively with a programmable water bath and light cycle with a full spectrum light source controlled by a timer.

Beakers will be acid washed (5% HNO_3) prior to use. Each beaker will be covered with parafilm® to reduce evaporative water loss and associated changes in salinity and pollutant concentration. The resulting headspace in each beaker will provide ample gas exchange (O_2 and CO_2) on a 24 hour basis for the animals between daily treatment solution changes. Individuals will be randomly assigned to treatment groups, and the placement of beakers in the incubators will be randomized as well. pH, DO, and conductivity in each beaker will be checked daily, as well as in a "monitoring replicate" (no test organisms present) for each concentration.

Dead and immobile individuals will be recorded daily. Dead individuals are those that are immobile and unresponsive to stimuli (touched with pipette), while immobile individuals are not observed to move until touched with the pipette. Dead individuals are removed by pipette daily at the time of treatment solution change. Analytical chemistry of the treatment solutions will not be conducted in the range testing work, because it is only necessary to establish which treatment solutions produce mortality or immobility and to reduce analytical costs.

The test protocols used in the range-testing and acute toxicity tests are summarized below.

	Brine shrimp	**Brine flies**
Test type	96- hour static renewal	96-hour static renewal
pH/temperature/salinity	pH 7.9±0.1 / 20 ± 1°C / 120 ppt	pH 7.9±0.1 / 20 ± 1°C / 120 ppt
Photoperiod	16 h light / 8 hours dark	16 h light / 8 hours dark
Replicates per concentration	3 in range-test; 5 in acute test	3 in range test; 5 in acute test
Organisms per chamber	20	10
Chamber size/ solution volume	50ml / 30ml	250ml / 150ml
Test acceptability	$\geq$ 90 control survivorship	$\geq$ 90 control survivorship

<u>Acute Toxicity Assays</u>:

Concentration ranges for acute assay treatment solutions – With range finding results for each pollutant, the concentration range for each pollutant's acute test will be selected using a 40% dilution series composed of 5 test solution concentrations (start with a concentration likely to produce 100% mortality and dilute this concentration by 40% for 4 additional treatment concentrations). The range of concentrations employed in the acute tests for a pollutant will be the values spanning 50% mortality and should provide well resolved concentration-response (mortality or immobility) curves. A control (no pollutant) will monitor background mortality and immobility of the organisms.

If we observe, as suspected, that mercury concentrations leading to mortality in brine shrimp and brine fly larvae are greater than those reported as harmful to birds, mercury will be dropped from acute testing. Regardless, mercury will be examined in chronic tests, as it accumulates in the food web and may chronically affect brine shrimp and brine fly survival and reproduction.

Acute assay protocols – Acute assays will span a much narrower range of concentrations for each pollutant than examined during range-finding and will follow the same protocols with the following differences:

1. <u>Replication</u> for each treatment solution concentration will be increased from 3 to 5 beakers for each organism. This should produce a more definitive measure of concentration-response (mortality or immobility) curves.

2. <u>Analytical chemistry</u> will be conducted to verify the pollutant's concentration in each treatment solution and its change over 24 hours. To save on analytical chemistry costs, acute tests for a given pollutant will be conducted simultaneously for brine shrimp and brine fly larvae. Given that the two organisms may respond differently to a pollutant's concentration, we suggest that 7 concentrations (5, with 3 in common, for each organism) can be employed to establish well resolved concentration-response (mortality or immobility) curves for each organism. Each day, a freshly-made treatment solution will be used to replace the solution in the beakers and a sample will be saved for chemical analysis (4 initial samples + a duplicate on day 1 to verify analytical consistency). The solution removed from the beakers after day 1 will be combined into a sample to assess changes in the pollutant's concentration over 24 hours (1 final sample + a duplicate to verify analytical consistency). Therefore, 49 samples will be chemically analyzed for each pollutant (7 concentrations X [4 initials + 1 duplicate initial + 1 final + 1 duplicate final]).

Treatment solution samples will be filtered through acid washed (5% HNO_3) 0.22 µm syringe filters that have had 3 volumes of sample water passed through them prior to retaining the sample in the appropriate acid washed (5% HNO_3) sample tubes (500 ml). Teflon sample tubes will be used for As, Cu and Pb samples, whereas glass tubes will be used for Hg samples. Samples will be preserved and kept at 4^oC in the dark for shipping to Brooks-Rand (Seattle, WA) for analysis.

The following protocols will be employed for preservation before shipping and by Brooks-Rand for metal analyses[1]:

Pollutant	EPA Method
Arsenic (As)	Preserved in 0.1% HNO_3 & reductive precipitation using modified EPA Method 1640
Copper (Cu)	Preserved in 0.1% HNO_3 & column chelation using modified EPA Method 1640
Lead (Pb)	Preserved in 0.1% HNO_3 & column chelation using modified EPA Method 1640
Mercury (Hg)	Preserved in 0.2% H_2SO_4 & EPA Method 1631E.

Acute assay data analyses – two responses to toxicity will be measured: mortality and immobility. Records of daily deaths and immobility will be recorded, but analyses will be conducted on overall mortality and immobility over the entire 96 hour assay period for a given pollutant concentration. Measures of mortality and immobility for a given pollutant concentration will be presented relative to the respective values observed in the simultaneous controls (no pollutant). For an assay to be considered successful, $\geq$ 90% of individuals in the control must survive.

With the above measures, the concentration-response (mortality or immobility) curves will be developed for a given pollutant and organism. These curves will be calculated via standard analytical procedures with diagnostic checks for homogeneity of variances using standard statistical packages (GraphPad Prism 6.0 or SYSTAT 13.0). With the concentration-response curves, a number of toxicity effects for a pollutant can be estimated:

1) LC50 and EC50 is computed as the concentration eliciting 50% mortality (LC50) and 50% immobility (EC50) relative to the organism's control values.

2) Lowest concentration (LOEC) affecting mortality and immobility is defined as the first test concentration to produce a statistically significant increase in mortality or immobility relative to control values.

3) No effect concentrations (NOEC) is the next lowest concentration tested relative to the LOEC.

Data archiving -- all water chemistry, QA/QC data, and toxicity (mortality and immobility) data will be archived and made available to any interested parties per State of Utah practices.

Chronic Toxicity Assays:

Details for developing chronic toxicity assays await the results from the acute toxicity assays, as pollutant concentrations lower than those observed to produce mortality and immobility in the acute assays must be used to assess long term effects. In addition, chronic toxicity assays examine not only the effect of the pollutant on long term mortality, but also the organism's

growth and reproduction. Finally, because chronic assays are long term from birth through reproduction (life time), the organism must be fed, which can lead to results varying whether the water only contains the pollutant or the organism's food is reared in water containing the pollutant. Therefore, chronic toxicity assays are much more interesting ecologically, but they are also more difficult to properly design.

We anticipate that chronic toxicity assays will require weeks (4 to 8) versus days (4) for acute assays. The chronic tests will be conducted at average GSL conditions (120 ppt salinity, 20^oC temperature, and a 7.9 pH) with brine shrimp fed ad libidum the high quality phytoplankton, *Dunaliella* sp. (40 µg chl_a/L/2 days) and for brine fly larvae (pupae and adults do not feed) fed ad libitum the high quality food, *Coccochloris* sp. (40 µg chl_a/L/2 days). These are the rearing conditions described earlier.

The acute toxicity assay was designed to be done in the least time and at the least expense to enable us to focus more fully on the chronic assays.

Work Timeline and Budget:

Completion of range-finding and acute assays should take ~3 months out of the first year and cost ~$61,800 (see attached budget) of the first year's $150,000 budget. Therefore, 9 months and ~$88,200 will be available to initiate chronic assays. We presume that chronic assays will entail less analytical chemistry, because they are not subject to the same rigorous Water Quality Criteria required by EPA, which will lead to substantially reduced cost. Therefore, we believe that the remaining 21 months and ~$238,000 (2 years at $150,000/year) will allow us to conduct chronic assays and add ammonia tests, if appropriate analytical chemistry method is identified[15].

Budget for Range-finding and Acute Assays (3 months):

	UND	**NCSU**	**TOTAL**
Technician	$ 7,500	$ 9,000	$16,500
Undergraduates	1,250		1,250
Benefits	3,693	1,530	5,223
Materials & supplies	5,225	3,000	8,225
Travel		3,467	3,467
Analytical chemistry	11,090	7,568	18,658
Direct costs	28,758	24,565	53,323
Indirect costs	4,782	3,685	8,467
Total	$33,540	$28,250	$61,790

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