# AVIAN CONSUMPTION AND USE OF CONTAMINATED WATER SOURCES: TOXICOLOGICAL ASSESSMENTS OF EXPOSURE, EFFECTS AND SUSCEPTIBILITY.

**Final Report - Part I**

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By:

Dr. Michael Hooper, John Isanhart and Dr. Stephen Cox
The Institute of Environmental and Human Health
Texas Tech University
Box 41163
Lubbock, Texas 79409

Through:

Dr. Reynaldo Patiño
Texas Cooperative Fish and Wildlife Research Unit
U.S. Geological Survey, Cooperative Research Units Program
Texas Tech University
Box 42120
Lubbock, Texas 79409


Produced for:

Russell MacRae
United States Fish and Wildlife Service
New Mexico Ecological Services Field Office
2105 Osuna Road NE
Albuquerque, NM 87113

Dr. Kevin D. Reynolds
United States Fish and Wildlife Service
Arizona Ecological Services Field Office
2321 West Royal Palm Road, Suite 103
Phoenix, AZ 85021


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## Introduction

Availability of clean water sources is critical to the daily survival of wild bird species, while migratory species are dependent on water sources as they labor to reach their wintering or breeding grounds. Water availability in the western U.S. is a particularly important consideration in the life of local or migratory birds as its scarcity makes it a critical commodity. The occurrence of contaminated water sources in the arid or semi-arid areas of the western U.S. pose an important threat to local and migratory birds, as their need for water can often preclude their ability to choose between a variety of sources. Current data suggest that passerines and waterfowl are the species most at risk to injury from drinking acid mine tailings water (Stubblefield *et al*., 1997; Stratus, 2003). There is relatively little other data that exist on this topic, and what other information that does exist primarily addresses the toxicity of zinc, lead, and/or cyanide-rich water from mining sites and acidified water bodies to birds (Beyer *et al*., 2004; Henny *et al*., 1994; Rattner *et al*., 1987; Foster, 1999; Tyler and Omerod, 1992; Read and Pickering, 1999; Read, 1999; Sileo *et al.*, 2004). Poisoning of birds that use toxic tailings waters is of particular concern in arid Australia, with approximately 1000 birds dying annually in gold mine tailings dams (Read, 1999; Minerals Council of Australia, 1996). Examples of bird poisonings from water sources other than cyanide-contaminated water in the U.S. include incidents at the Berkeley Pit, Butte, MT (ENSR, 1996) and a petroleum refinery fly ash pond in Delaware (Rattner *et al*., 2006). Such anthropogenic landscape modifications pose compounding problems for nomadic or migratory species that are in search of food, water, and/or resting sites.

Birds require water for the maintenance of cellular homeostasis, tissue integrity, food digestion, waste excretion, hygiene, and numerous biochemical reactions (Koutsos *et al*., 2001). While many birds are able to obtain all of the water they require through succulent food, insects, or even metabolic water alone, most birds require drinking water as the primary source. Dehydrated birds often drink substantially more water than required to restore water homeostasis than their non-dehydrated counterparts (Takei *et al*., 1988). Dehydrated migratory and/or nomadic birds have been observed gorge drinking (Klaassen, 2004). In addition, drinking rates may increase with increasing osmolarity of the drinking water (Goldstein and Skadhauge, 2000). Migratory birds that use mine tailings waters, which often

contain elevated levels of toxic metals, for stopover sites may be at increased risk to injury or death as a result of gorge drinking and physiological responses to "salt" water.

The acidic and metal-contaminated water in the tailings, pregnant leach solution, stormwater, and process water storage ponds located at Phelps Dodge's Tyrone, Chino, and Morenci mines is of concern due to its potential toxicity and adverse effects in avian species. From September to November 2000, United States Fish and Wildlife Service (USFWS) biologists investigated a series of incidents involving 221 bird deaths at three mine sites located in New Mexico and Arizona (Stratus, 2003). These bird deaths were assumed to be linked to consumption and/or use of acid mine tailings water as all of the carcasses were found near pregnant leach solution ponds, tailings ponds and associated lakes or storm water retention basins (Stratus, 2003). Other highly decomposed bird remains were observed on or near metal-contaminated waters but were not collected and included in the total count. Many of of the carcasses (approximately 40%) were found near a 280 acre uncovered tailings pond containing high concentrations of copper, zinc, aluminum, magnesium, cadmium, manganese, cobalt, and iron in standing water (Table A.1). Our goal was to perform a series of studies to develop an understanding of how avian drinking behavior can potentially influence the occurrence of acid mine water (AMW) toxicity events and to characterize the behavioral, clinical, and pathological signs of acid metal-enriched water toxicosis that likely occurred in the birds that were found dead at the Phelps Dodge mines.

In drinking water assessments, palatability and the nature of acid metalliferous pond water toxicity in one avian species, the mallard duck (*Anas platyrhynchos*), were assessed. The mallard duck was selected because it is a standard test species for both pesticides and contaminants with data available for both acid and metals toxicity. In addition, mallards are representative of a highly impacted family on the mining sites (Stratus, 2003).

## Project Objectives

There are four primary objectives for this project, each represented by an individual study. The first evaluates effects in birds following consumption of synthetic acid metalliferous water (SAMW). The second, third and fourth objectives address the potential for altering SAMW toxicity based on availability of alternative water sources or chemical modification

of SAMW to reduce its toxicity. Exposure scenarios correspond to actual conditions measured at the Phelps Dodge mine sites where dead birds have been recovered. Mallards were food and water-fasted in all studies to simulate migratory physiological stressors of maintenance of water and energy balance in wild birds.

1. **Study A**. Assess the acute toxicity of acid SAMW under acute exposure scenarios simulating conditions at the Phelps Dodge mines in dehydrated mallard,

2. **Study B**. Assess the potential for alleviation of SAMW's avian toxic effects by neutralizing its acidic character,

3. **Study C**. Assess the potential role that clean water availability and consumption might play in protecting birds after a toxic exposure to SAMW, and

4. **Study D**. Assess the acute toxicity of SAMW to mallards after being diluted 10- and 100-fold.

Biomarkers of exposure and effect were the tools used to evaluate the toxicity of SAMW to mallards. These biomarkers include clinical chemistry, gross and histopathology, hematology, behavior, and mortality. Others have used or discuss the applicability of such endpoints to examine the toxic properties of metals to birds (Rattner *et al*., 2006; Fairbrother *et al*., 2004; Foster, 1999), and these methods have become common practice in toxicological studies. Clinical chemistry tests are useful tools that examine levels of important enzymes, electrolytes, minerals, and other proteins which help determine any physiological alteration that may be suggestive of disease, illness, or other dysfunction. Gross pathology is the recognition of disease or injury based on macroscopic examination of external and internal tissues at the time of autopsy. Histopathology is the diagnosis of disease or injury based on microscopic examination of tissues. Hematology is the science of blood, blood forming organs, and blood diseases. Complete blood counts are a common method used to diagnose blood diseases and assess immune function status. Behavioral endpoints identify the signs of toxicity associated with exposure to a particular chemical. Some behaviors can be used to

identify physiological dysfunctions. A table of clinical chemistry and hematology parameters and interpretations of alterations in parameters is provided below.

Table 1. Diseases/conditions associated with alterations in clinical chemistry parameters.

| Parameter | Diseases/Conditions Associated with Alteration in Parameter | |
|---|---|---|
| | Increase | Decrease |
| Total Serum Protein (g/dl) | dehydration, anemia, liver and kidney dysfunction | malabsorption, malnutrition, kidney and liver dysfunction, dehydration |
| Albumin (g/dl) | dehydration | GI malabsorption, malnutrition, kidney and liver dysfunction |
| Globulin (g/dl) | kidney and liver dysfunction, dehydration, anemia | kidney and liver dysfunction, anemia |
| Calcium (mg/dl) | excessive Vit A or D, excessive Ca intake, liver and kidney dysfunction, dehydration | hypoparathyroidism, elevated P, GI malabsorption, kidney dysfunction |
| Phosphorus (mg/dl) | renal dysfunction, cell or tissue injury | GI malabsorption, hypkalemia, hypercalcemia |
| Glucose (mg/dl) | acute stress, renal failure, pancreas dysfunction | liver dysfunction, elevated insulin, adrenal insufficiency |
| Alkaline Phosphatase (U/l) | intra- and extrahepatic biliary obstruction, bone damage | NA |
| Creatine Kinase (U/l) | heart or skeletal muscle damage | NA |
| Aspartate Aminotransferase (U/l) | liver dysfunction/damage, trauma, shock, myocardial or kidney infarction | NA |
| Uric Acid (mg/dl) | kidney dysfunction, GI bleeding | NA |
| Cholesterol (mg/dl) | increased dietary cholesterol, liver dysfunction | myocardial infaction, liver dysfunction, acute illness, malnutrition |
| Sodium (meq/l) | kidney dysfunction, dehydration, increased Na intake | heart, liver, or kidney dysfunction, vomiting, diarrhea, adrenal dysfunction |
| Potassium (meq/l) | kidney dysfunction, increased K intake | vomiting, diarrhea, malabsorption, renal tubular acidosis, increased aldosterone secretion |
| Chloride (meq/l) | similar causes as Na increase, metabolic acidosis, increased chloride intake | similar causes as Na decrease, metabolic alkalosis |
| Hemoglobin | dehydration, increase in RBC production, RBC lysis | excessive destruction of RBCs, excessive bleeding |

Source: Kaplan *et al.*, 2003 NA – Not Applicable

Table 2. Diseases/conditions associated with alterations in complete blood count parameters.

| Parameter | Diseases/Conditions Associated with Alteration in Parameter | |
|---|---|---|
| | Increase | Decrease |
| Total White Blood Cells | infection, recent inflammation, tissue damage | severe infection, presence of cytotoxic substance |
| Heterophils/Lymphocytes/Monocytes/ Eosinophils/Basophils | recent inflammation, infection, increased adrenal stress response | severe infection, bone marrow disorder |
| RBCs | fluid loss (dehydration and diarrhea) | anemia, hemolysis, hemorrhage |
| Polychromasia | anemia | NA |
| Hematocrit | dehydration | anemia, vitamin and mineral deficiencies, recent bleeding |
| Mean Cell Volume | macrocytic anemia | microcytic anemia |

Sources: Fairbrother *et al*., 2004; Jain, 1993

## Study A: Acute Toxicity of Synthetic Acid Mine Water to Mallards

### Introduction

Consumption of acid metalliferous water found on mining and processing sites is hazardous to birds. Our goal in Study A was to develop an understanding of how avian drinking behavior can potentially influence the occurrence of acid metalliferous toxicity events and to characterize the behavioral, clinical, and pathological signs of acid metal-enriched water toxicosis that likely occurred in the birds that were found dead at the New Mexico and Arizona mine sites. The main purpose of this study was to assess the acute toxicity of synthetic AMW that is representative of tailings pond or pregnant leach solution water found at the Tyrone Mine under an acute exposure scenario using water- and food-fasted mallard ducks.

### Methods

*Synthetic Acid Metalliferous Water Synthesis and Analysis*

Fifty gallons of SAMW was prepared according to water chemistry data obtained by the USFWS from a mine tailings pond where known bird mortalities have occurred (Stratus Consulting Inc. 2003; Table A.1). SAMW was formulated to match site water exactly in terms of both cation and anion concentrations. Metals and acid were added as chloride, nitrate, or sulfate salts to simulate site water ionic content. Reagent grade chemicals, deionized water, and 18 M trace-metal grade nitric acid were added to a 55 gallon drum and mixed using a reciprocating pump and electric mixer until most of the particulate material had dissolved. Any remaining undissolved chemical was removed with a GE Smart Water™ water filtration system that housed a 15 µm sediment filter. Nitric acid was used to adjust the pH to 2.0. The pH of the solution ranged between 2.01 and 2.03 from the time it was synthesized until the initiation of dosing (approx. 2 months). Concentrations of 15 elements in the solution were determined using flame and furnace atomic absorption spectroscopy and inductively-coupled plasma atomic emission spectroscopy (ICP-AES).

*Study Design*

Eighteen 18-20 week old mallards (9 males, 9 females; NPIP certified disease free) were obtained (Dan and Imogene's Flying Mallard Ducks, Hartville, MO) and transported to the Texas Tech University Animal Care Resources Center. Mallards were 22-24 weeks of age at the time of dosing. Birds were banded and maintained individually in 2.5 $ft^3$ stainless steel rabbit cages at 20°C, 40-70% relative humidity, and 12h:12h light:dark photoperiod. All mallards acclimated for 1 month to acclimate to indoor, caged conditions prior to initiation of testing, with their body weight near or exceeding that recorded upon receipt into the animal facility. Ducks had *ad libitum* access to feed (Mazuri waterfowl maintenance diet in pellet form; PMI Nutritional, LLC, Brentwood, MO, USA) and reverse osmosis (RO) water during the acclimation period. All animal care was performed in accordance with the Texas Tech University ACRC and the Institutional Animal Care and Use Committee.

Dosing of each mallard was staggered at 2-min intervals to allow for changing of water bottle reservoirs and waste-collection bottles. SAMW consumption was adjusted for the density of the dosing solution (1.05 g/ml) and control consumption was based on 1 g/ml for RO water. Total dose (g SAMW/kg body mass) for each bird was calculated by dividing the mass of SAMW consumed (g) by the bird's body mass (kg). Total dose units were converted to mL SAMW/kg body mass by dividing by the density. Animals were observed continuously throughout the exposure period, with behavioral observations and water consumption data collected.

The water consumption measurement system consisted of multiple components that were located outside of the cage. Drinking water reservoirs were made from a 1-L plastic water bottle with an attached rubber stopper, straight tubing connector, approximately 8 inches of tubing, ratchet clamp, and quick-disconnect connector. The water reservoir was connected to a standard avian drink cup with a spring-loaded lever (GQF Manufacturing Company Inc., Savannah, GA, USA). A majority of spillage drained into waste-collection devices consisting of an inverted top-half of a 1-gal jug and funnel connected by tubing to a 2-L plastic water bottle waste reservoir. Small amounts of spillage were also collected using aluminum pans located below the immediate drinking area. Water consumption was measured by weighing water in source and waste water reservoirs. Water consumed was defined as the difference between:

A) the mass of water loss from in the source reservoir between the start and end of a defined time period, and
B) the total waste water recovered from
    1. the waste water / spillage reservoir under the drinking cup,
    2. the waste pan immediately under the drinking area inside the cage, and
    3. any remaining water in the drinking cup.

The study was performed on three separate days during a one-week time period, with three control and three treatment ducks being tested on each day. During each day, three control mallards were provided with RO water and three were provided with SAMW *ad libitum* in the morning following a 24-hr fast and dehydration period. Food was withheld during the dosing period for both control and treatment groups. Body mass measurements were collected for each mallard before the initiation of the dehydration and fasting period, at the initiation of dosing, and at the time of euthanasia or death. Body mass was collected with an electronic balance to the nearest 0.1 g.

*Animal Euthanasia and Sample Collection*

Birds were determined to be in moribund condition by visual signs of wing droop, immobility, lack of response to touch/visual/auditory stimuli, and/or inability to hold head erectly. All treatment ducks were observed until they were *in extremis* weighed, and euthanized via carbon dioxide asphyxiation. Control birds were euthanized as close as possible to the time treatment animals died. Due to the moribund condition and reduced blood flow of treatment ducks, we were unable to collect blood samples via jugular or brachial venipuncture and instead collected blood using cardiac puncture. Whole blood was collected with 1 mL syringes and placed in 5 mL serum separator tubes for serum clinical chemistry, 2 ml microcentrifuge tubes for metal residues, and microhematocrit tubes for determination of erythrocyte packed cell volume (PCV). Whole blood in serum separator tubes was allowed to clot at room temperature for 30 minutes and then centrifuged at 6,000 rpm for 10 minutes. Serum was decanted into 2-ml microcentrifuge tubes and frozen at -80°C until being shipped for analysis. All serum samples from treatment mortalities contained insufficient amounts of serum for clinical chemistry analyses, they were diluted with 18.0 mega-ohm water to a 1:3, 1:4, or 1:9 serum to water ratio. Serum samples were

analyzed with a Hitachi 911 Analyzer at the Texas Veterinary Medical Diagnostic Laboratory (TVMDL) for total serum protein, albumin, globulins, calcium, phosphorus, glucose, creatine kinase (CK), aspartate aminotransferase (AST), uric acid, cholesterol, alkaline phosphatase (ALP), and electrolytes. Additional dilutions of control mallard serum in the ratios of 1:3 (n=6) and 1:4 (n=2) were sent for analysis and compared to undiluted control mallard serum samples. Serum volumes from each control mallard were not sufficient for analysis of both dilutions.

Grossly observable lesions were documented, and tissues were collected for both metal residue and histopathological analyses for all birds. Bile was collected from gall bladders using 1-inch 23 ga needles and 1 mL syringes, placed in microcentrifuge tubes, and frozen at -20°C until analysis. A section of left lung, left kidney, brain, spleen, pancreas, left testis, and salt gland were collected, weighed, and frozen at -20°C for future metal analyses. Sections of right lung, right testis, right kidney, salt gland, spleen, pancreas, brain, tongue, esophagus, trachea, heart, proventriculus, ventriculus, duodenum, jejunum, ileum, ceca, and large intestine were fixed in 10% buffered formalin and stored in jars until being processed at the Colorado State University Veterinary Diagnostic Laboratory.

*Histopathology*

Tissue samples were embedded in paraffin and 5 µm sections of these tissues were histologically analyzed following routine hematoxylin and eosin staining. When dictated by histopathologic findings, specific tissue sections were also stained for the minerals, calcium and copper (VonKassa and Rhodanine methods, respectively). Primary histopathologic analysis was performed blindly without knowledge of treatment. Following analysis, observations in treated and control groups that were indistinguishable both qualitatively and quantitatively were considered to be background lesions and deemed unrelated to treatment.

*Tissue Metals Analysis*

Approximately 0.3 – 0.5 g of thawed tissue/fluid sample (liver, kidney, blood, or bile) was weighed in a 50 mL Teflon beaker. Samples were digested with trace-metal grade 18M nitric acid and 30% hydrogen peroxide. Digestion solutions were volumetrically diluted to 20 mL with milli-Q water, poured into 50 mL plastic centrifuge tubes, and stored at 4°C until

analysis. Any samples containing coagulated lipid were centrifuged at 3500 rpm for 10 minutes. Some digestion solutions containing excess lipid were filtered using filter paper. Aluminum was quantified by inductively coupled plasma-atomic emission spectroscopy using a background correction and all other elements (Cu, Zn, Mg, Mn, and Fe) by flame atomic absorption spectroscopy with a deuterium background correction. Values are reported on a wet weight (ww) basis. Standards for Cu, Zn, Mg, Mn, and Fe analyses were prepared in 3% nitric acid, while standards for Al analyses were prepared in an acid digested liver matrix. Spike returns for all 4 tissues were within ± 10% of total. Mean percent recoveries ± SD for Cu, Zn, Fe, Mn, and Al in a standard reference material (DOLT-2) were 96.9 ± 4.1 (n=3), 94.8 ± 1.4 (n=3), 83.1 ± 2.2 (n=3), 80.4 ± 0.8 (n=3), and 90.6 ± 83.0 (n=2), respectively. Mean recoveries of check standards throughout analyses for all elements and tissues were ± 10%. Data were not corrected for percent recoveries of spikes or reference material. Where data are listed as not quantified (NQ), tissue concentrations were below the lowest calibration standard, or below the method detection limit (MDL), whichever was the higher value. MDLs for metal combinations in water and tissue matrices were calculated according to USEPA test methods (USEPA, 1994; 40 CFR part 136, Appendix B). MDLs for all analytes in water were less than 1% of the analyte concentrations determined in SAMW. MDL determination details can be found in the report appendices under Study A.

*Statistical Methods*

Measures of central tendency were expressed as the mean ± standard deviation unless noted otherwise. All data analyzed using parametric methods were tested for normality and homogeneity of variances. When non-gaussian distribution, heterogeneity of variance, or unbalanced design were observed, nonparametric tests were chosen for subsequent analysis. The effect of SAMW treatment on hematocrit was tested using Wilcoxon Rank Sum Test. Differences between control and SAMW-treatment group tissue metal concentrations were analyzed using t-tests. Body mass dynamics were analyzed using t-tests. Water consumption rates during the first three hours of dosing was analyzed using a linear mixed-effects model with dose after log transformation as the fixed factor and time as the random factor, as well as the interaction between dose and time. Any differences in the model were further analyzed using t-tests to determine differences between treatment groups during water

consumption time periods. Water consumption over time within treatment differences were analyzed using analysis of variance after log transformation of water consumption data. Absolute water consumption data for the first three 20-min periods were summed for each treatment group. Following summation, analysis of variance was used to test for within group differences in absolute water consumption during the first three 1-hr periods. Pearson's product-moment correlation was used to assess the relationship between total AMW consumption or hematocrit and time to death, as well as the relationship between 20 min, 40 min, 60 min, 120 min, and 180 min AMW consumption and time to death. All statistical analyses were performed with R (version 2.2.0; R Foundation, Vienna, Austria). Results of statistical tests were considered to be significant at $p < 0.05$. Serum clinical chemistry endpoints were not analyzed with statistical methods due to the small serum sample numbers obtained from treatment mortality mallards. For these reasons, data from 5 AMW treatment mallard serum samples (3 mortalities and 2 survivors) were compared qualitatively to 8 control mallard samples.

## Results

*Water Consumption Totals and Rates*

Total doses of SAMW ranged from 52.4 - 270.1 mL SAMW/kg body mass for mortalities with a mean ± SD of 106.8 ± 75.0, while doses for the two survivor mallards were 25.6 and 40.0 mL SAMW/kg body mass(Table A.2). Total doses of control mallards ranged from 193.3 – 1,249.8 mL water/kg body mass with a mean ± SD of 543.2 ± 358.8.

There was a significant difference in water consumption rate (mL/kg/hr) due to the type of water ($p < 0.01$), as well as the drinking time period ($p < 0.001$) and the interaction between type of water and time period ($p < 0.05$). Control mallards drank significantly more water than AMW treatment mallards during the first three 20-min drinking periods and the subsequent two 1-hr drinking periods ($p < 0.05$ for all periods; Fig. A.1; Table A.3 and A.4). Control mallard water consumption rates were significantly higher during the first 20-minute drinking period in comparison to the third 20-min drinking period ($p < 0.05$) and the second and third 1-hr drinking periods ($p < 0.001$ for both periods). Drinking rates for SAMW treatment mallards were significantly higher during the first 20-min drinking period only in comparison to the third 1-hr drinking period ($p < 0.01$). Water consumption totals were

compared for the first three hours by totaling the initial three 20-minute intervals into a single hour value and comparing to the second and third hour data. Control totals were different from SAMW totals for all three hours of the assessment. Within controls, first hour consumption totals were significantly greater and double those of hours 2 and 3. Treatment totals for hour 1, over twice hour two and nearly 4 times hour 3 totals, were significantly higher than hour 3 values. Water consumption data after 3 hrs into the dosing study is not presented due to small sample sizes, deteriorating condition of treatment mallards, and unwillingness of mallards to continue to drink the dosing solution.

There were no significant relationships between time to death and total SAMW dose or any of the other consumption intervals. Although not significant, SAMW consumption following 60 min. appeared to be the best predictor of time to death ($r$=-0.57) when compared to all other time intervals. Relationships were not significant for any of the other time points most likely due to small sample sizes.

*Signs of Toxicity*

Common signs of toxicity, in general order of occurrence, among SAMW treatment mallards included lateral head shaking, nasal discharge or oral mucus production, signs of throat irritation, ataxia, signs of central nervous system (CNS) depression, increased breathing rate with shallow breaths, and death (Table A.5). Additional, less common signs of toxicity included regurgitation, subtle head and/or body shivering, coughing, and sneezing. Fecal material from treatment mallards was usually viscous, dark green, and lacking visible signs of urates (milky-white), while control mallards defecated clear and watery feces. None of these signs were observed in any of the control mallards. In most cases, vigorous lateral head shaking followed the first initial drinks. Oral mucus production was more common than nasal discharge. Mucus was usually clear and colorless; however, there were some instances of blue-green nasal discharge and/or oral mucus. Exaggerated swallowing behavior in the absence of drinking was suggestive of throat irritation and mucus production. Mallards that consumed enough SAMW to cause death showed all signs of toxicity through ataxia, and six of seven treatment mortalities showed signs of CNS depression. Signs of CNS depression included: reoccurring bouts of head dropping lasting 10-15 sec followed by recovery, lack of response to auditory/visual/touch stimuli, additional head droop and wing

droop, immobility, and/or closed eyelids. Seven of nine AMW treatment mallards died within 98-661 minutes following initiation of dosing. Two SAMW treatment mallards, one of each sex, survived exposure, presumably due to reduced consumption of SAMW. The two surviving mallards were euthanized and necropsied either 27 or 33 hours post-dose. No control mallards died prior to euthanasia at the termination of the study.

Mean percent body mass loss for control and treatment mallards following a 24-hr dehydration period prior to AMW exposure was 6.02 ± 1.3 and 6.66 ± 1.9, respectively (Table A.6). While a 24-hr dehydration period resulted in an approximately 6% loss of body mass for both groups, the period from initiation of dosing to death resulted in a mean percent body mass change of 0.68 ± 1.1 for controls and -6.15 ± 2.2 for treatment mallards. Overall mean percent body mass loss of control and SAMW treatment mallards from a hydrated condition to death was 5.38 ± 1.4 and 12.5 ± 2.3, respectively. Treatment period and overall study duration mass losses were significantly greater in SAMW treated birds.

*Clinical Chemistry and Hematocrit*

Mean percent recoveries of 1:3 serum sample dilutions ranged from 93.6% to 105% for all serum chemistry endpoints except for AST, which had a mean percent recovery of 115% (Table A.7). Mean percent recoveries of 1:4 serum sample dilutions ranged from 104% to 122% for all serum chemistry endpoints except for AST, which had a mean percent recovery of 146%. Overall mean percent recoveries, calculated as the mean of all individual percent recoveries from both 1:3 and 1:4 dilutions, ranged from 96.6% to 108% for all serum chemistry endpoints except for AST, which had an overall mean percent recovery of 123%.

All serum samples collected from SAMW treatment mallards were mildly to moderately hemolyzed, while control serum samples were not. Due to dilution-induced changes in some endpoints, changes in clinical chemistries were not considered notable until at least 30% elevated above or 20% depressed below control values. There was no apparent treatment-related effect on total serum protein, albumin, globulin, or cholesterol (Table A.8). SAMW-poisoned mallards had reduced mean serum levels of calcium, glucose, sodium, and chloride in comparison to control and treatment survivor mallards. Slight hypocalcaemia was observed in the 3 SAMW-poisoned mallards in comparison to both control and SAMW treatment survivor mallards. Mean glucose levels were nearly 10-fold lower in poisoned

mallards in comparison to survivor and control mallards, indicating severe hypoglycemia in mallards that died from SAMW. Mean sodium and chloride levels were only slightly lower (< 20%) in treatment mallards that died in comparison to both controls and treatment survivors.

Increased mean serum levels of phosphorus, ALP, CK, AST, uric acid, and potassium were observed in treatment mallards that died in comparison to both control and treatment survivor mallards. While mean potassium levels of SAMW-poisoned birds were only slightly elevated in comparison to controls, mean phosphorus, ALP, CK, AST, and uric acid levels ranged from approximately 2-15 times higher in mallards that died compared to controls. However, variability of these four endpoints for birds that died was relatively high and exceeded that of controls, which was likely influenced by reduced serum sample sizes of treatment mallards.

Mean hematocrit values from treatment mortalities were significantly higher than those of controls ($p < 0.001$). Mean hematocrits from the two SAMW treatment survivors were slightly elevated compared to controls, but lower than mean values from treatment mortalities. There was also a significant negative correlation between hematocrit and time to death ($p$=0.05; $r$=-0.75), with higher hematocrits coinciding with shorter times to death.

*Pathology*

Common grossly observable abnormalities in treated mallards included presence of increased clear or blue-green mucus and associated discoloration of the mucosa of esophagus, proventriculus, ventricular kaolin, and intestine. Clear or blue-green mucus was also present in the proximal trachea and nasopharynx of SAMW-treated mallards. Other abnormalities that were less common and more severe included petechial hemorrhages on the serosal surface of the duodenum and localized ulcerations of the ventriculus, mostly along the proventricular-ventricular junction, and duodenum. Reddening of the proventriculus, and erosion and reddening of the mucosa of the proximal duodenum, were noted in seven SAMW-treated birds. Similar lesions were absent in control birds.

Histopathologically, mild chronic portal hepatitis, mild to marked chronic heterophilic tracheitis were observed both in treated and control mallards and were considered background findings, unrelated to treatment. Minimal to mild splenic lymphoid necrosis

was noted in five SAMW-treated birds and in two control birds. Lymphoid necrosis is often a manifestation of stress in animals and is most likely a nonspecific effect. Increased incidence in treated birds as compared to controls is most likely an indicator of increased stress in treated birds.

Treatment-related histopathologic lesions were limited to esophagus, proventriculus, ventriculus, and duodenum (Table A.9). Esophagus in four of seven treated birds exhibited varying degrees of mucous gland ectasia with or without associated heterophilic inflammation. Occasionally glands were obliterated by heterophilic inflammation and necrosis. Condensed blue discoloration was often noted at the opening of the esophageal glands on the mucosa. This condensed material was negative for calcium and copper by special stains (VonKassa and Rhodanine methods, respectively). Its composition was uncertain but it could represent coagulated mucous and or other precipitate from SAMW.

Compared to control birds, the proventricular mucosa of treated birds (9/9) was variably eroded and denuded and covered by an amorphous layer of mucin and granular eosinophilic material (interpreted as fibrin). Often, along the eroded epithelium there was basophilic discoloration of the connective tissue scaffold suggesting mineralization. Additionally, there was marked congestion with or without heterophilic inflammation in the lamina propria and submucosa. The proventricular glands were within normal limits in treated and control animals. A male mallard survivor consuming the lowest dose of SAMW represented the most acute morphologic change in the proventricular mucosa. In this bird, individual or small clusters of mucosal epithelial cells were degenerative to necrotic (as indicated by cellular swelling, cytoplasmic eosinophilia and pyknosis) and in the process of being sloughed. In other areas there was complete loss of mucosal epithelium and the denuded connective tissue exhibited basophilic discoloration described above. In the two SAMW-treatment survivors, there was an apparent attempt at re-epithelization of focally extensive areas of the mucosa as suggested by lining of the mucosa by flattened epithelial cells as compared to columnar cells in the controls.

Changes in the ventriculus were noted in eight of nine SAMW-treated and one of nine control treated mallards (Table A.10). The changes in the control mallard included minimal infiltrate of heterophils in the submucosa. In contrast, the changes in the treated mallards were markedly more prominent and included a greater heterophilic response in the

submucosa with degenerate heterophils extending into the kaolin layer of some birds. Also, erosion or ulceration of the kaolin layer with subjacent congestion and hemorrhage were noted in most treated mallards (7/9). It is important to note that the changes in the ventriculus persisted while the proventriculus exhibited signs of repair in the SAMW-treated survivors.

Changes in the small intestine were noted in six of nine SAMW mallards. Changes in the small intestine included increase mucus and coagulated protein on the mucosal epithelial surface of the jejunum, small intestine congestion and hemorrhaging, and one case of coagulative necrosis in the duodenal lamina propria and denudation of the duodenal tips of villi.

Examples of macroscopic changes in internal tissues are provided in the appendix.

*Tissue Metal Residues*

In general, tissues from SAMW-treated mallards tended to have elevated concentrations of metals when compared to control mallards (Table A.11). Mean kidney copper, zinc, magnesium, iron, and manganese concentrations were significantly higher in SAMW-treatment mallards when compared to controls ($p<0.05$). Mean kidney copper concentration were approximately 5 times higher in treatment mallards compared to controls, and mean kidney manganese concentrations were approximately 3.5 times higher in treatment mallards compared to controls. Mean blood copper, zinc, magnesium and iron concentrations from SAMW mallards were significantly higher than control mallards, as well ($p<0.05$). Blood copper levels were approximately 18 times higher in SAMW-treatment birds when compared to controls, and SAMW mallard mean blood zinc levels were approximately twice the mean of controls. There were no significant differences in liver metal concentrations; however, mean liver copper concentrations were approximately 50% higher in treatment mallards than those of controls. Bile concentrations of Cu and Mn in SAMW-treated mallards were significantly higher than those from controls ($p<0.05$). In general, the highest mean concentrations of Cu, Zn, Mg, and Fe were found in liver samples, while bile samples contained the highest mean levels of Mn. Most of the tissue Al concentrations in digested solutions, control and SAMW-treatment, fell below the lowest analytical standard concentration of 0.05 mg/L (approximately 5ug/g tissue wt).

Discussion

Results of the present study indicate the synthetic version of the acid mine tailings pond water was highly toxic to mallards, with seven of nine mallards dying as quickly as 98 minutes following first exposure. Clinical, pathological, and tissue residue results from this study are consistent with literature pertaining to acute metal toxicosis, especially copper, in avian species. Time to death from other reports of copper or acid mine water-related waterfowl mortalities (Stubblefield *et al*. 1997; Henderson and Winterfield, 1975) have been similar to our findings, with birds being found *in extremis* or dead within 12-24 hrs following first *ad libitum* exposure.

Most of the clinical signs of toxicity, which were suggestive of renal dysfunction, liver damage, heart or muscle damage, potential biliary obstruction, dehydration, hemolysis, and/or shock (Table 1), are similar with previous reports of acute copper or acid mine water toxicosis in waterfowl (Stubblefield *et al*., 1997; ENSR, 1996). In addition to clinical signs of metals toxicosis, pathological signs of metal-induced damage, particularly copper, were observed in SAMW-treated mallards. Characteristic changes found in acute copper toxicosis are hemorrhage or necrosis of the liver and kidney, proventricular and ventricular necrosis, intestinal hemorrhage, elevated liver copper concentrations, and sometimes stomatitis (Henderson and Winterfield, 1975; Pullar, 1940; Jensen *et al*., 1991). Mallards from our studies demonstrated all of these changes with the exception of consistent liver and kidney damage and stomatitis. Reduced exposure duration and sudden mortality were likely responsible for the lack of stomatitis and kidney damage in our study. Based on the gross pathology and histopathology findings, we assume that the majority of the toxicity of the SAMW solution is related to the extremely high concentration of copper. Metal residues are also a commonly used and reliable index of exposure in acute/peracute metal toxicosis. While liver copper residues are commonly used as a reliable indicator of acute copper toxicosis, results from this study indicated that blood or kidney copper levels may be more sensitive indicators of acute copper toxicity in birds. All of the above signs of toxicity, with a particular emphasis on uric acid, liver enzymes, hematocrit, GI pathology, and tissue metal residues, are recommended for diagnosing AMW poisoning in birds.

While we recognize that the SAMW dosing solution used in this study had high concentrations of several potentially toxic metals, and several metals were at elevated levels

in more than one tissue in SAMW-treated mallards when compared to controls, we feel that the tissue residue data do not reflect acutely toxic levels for any of the metals except copper. For instance, mean liver and kidney Zn concentrations can range from 600-1100 µg/g dw and 1000-1700 µg/g dw, respectively, in zinc-poisoned mallards (Gasaway and Buss, 1972). Mean liver and kidney Zn concentrations from mallards in our study were 60.3 and 27.9 µg/g ww, or approximately 201 and 93 µg/g dw assuming 70% moisture, which are similar to reported control mallard liver and kidney Zn concentration (Gasaway and Buss, 1972). Conversely, a 970 mg dose of Zn shot in form of eight shot pellets containing 98% Zn resulted in mortality, incoordination, paralysis, anemia, macroscopic lesions in the cecum, intestine, gizzard, and liver, increased leukocyte counts, and alterations in liver enzyme, uric acid, phosphorus, glucose, calcium and total protein levels (Levengood *et al*., 2000). Another common sign of zinc-intoxication is pancreatitis (Sileo *et al*., 2004), which was not observed in Study A. While it is likely that zinc in the SAMW added to the overall toxicity, it is unlikely, based on the rapid progression to death and Zn tissue concentrations, that mallards in Study A were poisoned by Zn.

The lowest lethal dose of copper in mallards is noted to be 600 mg/kg (EXTOXNET, 1996). Another report indicates the dose required to produce mortality in adult mallards is 400 mg/kg (Pullar, 1940). As both of these cited doses are for copper sulfate, of which 40% is actually copper, their dose in copper alone is 240 and 160 mg/kg, respectively. Based on the geometric mean of these two values, 196 mg/kg, all seven of the lethally-treated AMW mallards consumed above the lowest lethal dose, as did one of the survivors (Table A.2). The average AMW consumption rate in the first 20 minutes was approximately 80 mL/kg/hr, which indicates there could be a high potential for acute mortality in a very short period of time in birds that are willing to drink AMW with such high concentrations of toxic metals.

Based on the findings from Study A, we conclude that acid metalliferous water bodies with similar chemical composition and acidity as SAMW pose a significant hazard to birds that come in contact with them. SAMW-treated mallards drink less water, on a time interval basis and overall, compared to control mallards. Despite reduced water consumption of SAMW-treated mallards, doses of SAMW were still sufficient to cause mortality and significant alterations in blood chemistry parameters and gastrointestinal pathology.

Table A.1. Concentrations of metals from mine-associated AMW, synthetic AMW dosing solution, and RO water analyzed by flame/furnace atomic absorption spectroscopy (AAS) and inductively-coupled plasma atomic emission spectroscopy (ICP-AES).

| Element | Data from USFWS AMW investigation (mg/L at pH = 2)* | Measured Concentrations from Synthetic AMW (mg/L at pH = 2) | RO water (mg/L) |
|---|---|---|---|
| Cu | 5840 | 5943 | 6.6 |
| Al | 3436 | 3718 | 3.5 |
| Zn | 2010 | 2071 | 2.3 |
| Mg | 1680 | 1596 | 1.8 |
| Fe | 1350 | 1351 | 1.2 |
| Mn | 738 | 746 | <0.5 |
| Ca | 400 | 493 | <0.1 |
| Cd | 21.9 | 22.2 | <0.5 |
| Co | 21.7 | 21.8 | <0.5 |
| Na | 12.4 | 17.3 | 0.1 |
| Ni | 10 | 10.8 | <0.5 |
| Cr | 4.2 | 4.8 | <0.02 |
| Se | 0.534 | 0.639 | <0.01 |
| V | 0.385 | 0.352 | <0.01 |
| As | 0.250 | 0.344 | <0.01 |

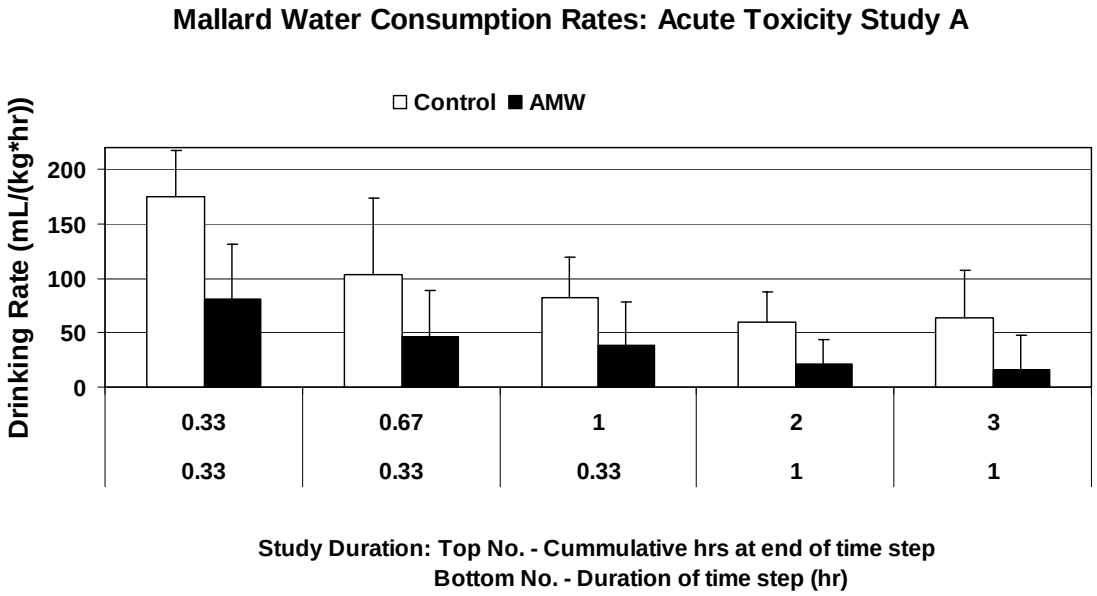
* Data from Stratus Consulting, Inc. 2003. AMW samples from mine site collected on Sept. 12, 2000.

Table A.2. Total synthetic acid metalliferous water and estimated metal component doses among SAMW treatment birds.

| | SAMW Consumption | Estimated Dose (mg/kg body mass) | | | | | |
|---|---|---|---|---|---|---|---|
| Mallard ID | (ml/kg body mass) | Cu | Al | Zn | Mg | Fe | Mn |
| M-3 | 270.1 | 1605 | 1004 | 559 | 431 | 365 | 202 |
| M-1 | 150.0 | 891 | 558 | 311 | 239 | 203 | 112 |
| F-1 | 139.8 | 831 | 520 | 290 | 223 | 189 | 104 |
| F-4 | 108.5 | 645 | 403 | 225 | 173 | 147 | 80.9 |
| M-5 | 75.9 | 451 | 282 | 157 | 121 | 103 | 56.6 |
| F-3 | 71.8 | 427 | 95.2 | 149 | 115 | 97.0 | 53.5 |
| M-2 | 52.4 | 311 | 195 | 109 | 83.6 | 70.8 | 39.1 |
| F-2[a] | 40.0 | 238 | 149 | 83 | 64 | 54 | 29.9 |
| M-4[a] | 25.6 | 152 | 95.2 | 53.0 | 40.9 | 34.6 | 19.1 |

[a] Survivor

**A**




**B**

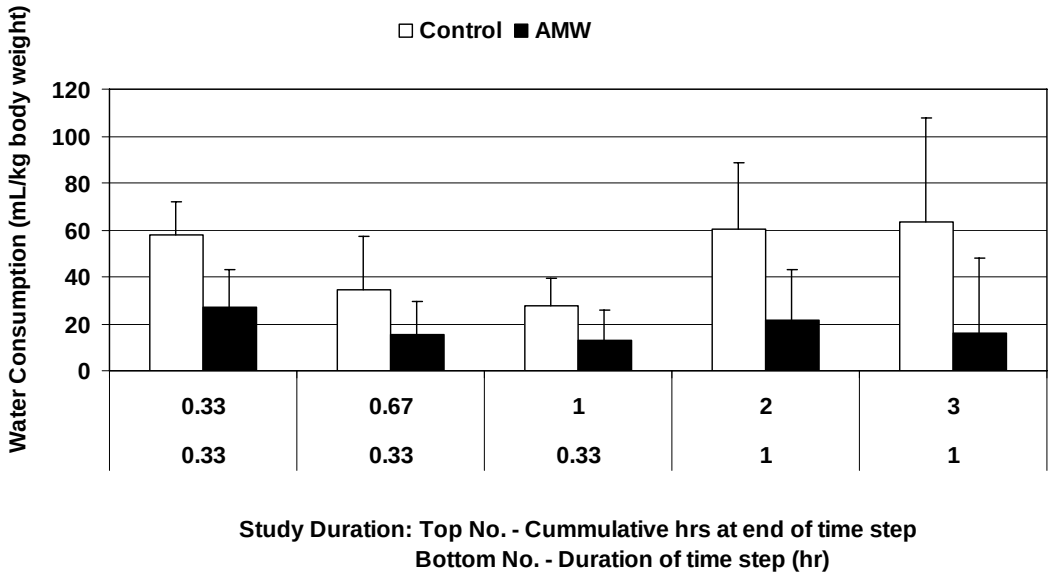



Figure A.1. Water consumption rates (A) and totals (B) for mallard ducks provided either clean water or synthetic AMW. Values are mean + SD for bars and error bars, respectively. N = 9 for each bar. In both graphs, control and treatment groups differed significantly ($p \leq 0.05$) at all time points from 0.33 through three hours. See Tables A.3 and A.4 for within-group comparisons.

Table A.3. Mallard duck water consumption rates. Water consumption rate data was collected after the 3rd hour, however SAMW mallard sample sizes decreased over time after the 3rd hour. These data correspond to those demonstrated in Figure A.1.A. N = 9 for each value. Letters indicate within group differences and asterisks indicate between group differences for each time step.

| Study Duration At End of Time Step (Hr) | Time Step Duration (Hr) | Water Consumption Rates [mL/(kg*hr)] | | | |
|---|---|---|---|---|---|
| | | Control | | SAMW | |
| | | Mean | SD | Mean | SD |
| 0.33 | 0.33 | 175.3 *[a] | 42.2 | 81.1[a] | 49.8 |
| 0.67 | 0.33 | 104.0 *[ab] | 70.2 | 45.9[ab] | 42.7 |
| 1 | 0.33 | 82.7 *[b] | 36.8 | 38.8[ab] | 39.0 |
| 2 | 1 | 59.8 *[b] | 28.1 | 21.2[ab] | 22.0 |
| 3 | 1 | 63.1 *[b] | 44.5 | 15.9[b] | 32.3 |

Table A.4. Mallard duck water consumption totals. Water consumption data was collected after the 3rd hour, however SAMW mallard sample sizes decreased over time after the 3rd hour. These data correspond to those demonstrated in Figure A.1.B. N = 9 for each value. Letters indicated within group differences and asterisks indicate between group differences for each time step.

| Study Duration At End of Time Step (Hr) | Time Step Duration (Hr) | Water Consumption Totals [mL/kg] | | | |
|---|---|---|---|---|---|
| | | Control | | AMW | |
| | | Mean | SD | Mean | SD |
| 0.33 | 0.33 | 58.1 * | 13.1 | 26.8 | 16.4 |
| 0.67 | 0.33 | 34.4 * | 21.8 | 15.2 | 14.1 |
| 1 | 0.33 | 27.3 * | 11.5 | 12.8 | 12.9 |
| 1st hr total | 1 | 120 *[a] | 42.8 | 57.5[a] | 38.4 |
| 2 | 1 | 60.1 *[b] | 26.8 | 21.2[ab] | 22.0 |
| 3 | 1 | 63.4 *[b] | 42.0 | 15.9[b] | 32.3 |

Table A.5. Signs of toxicity and associated means and ranges of times to signs of toxicity among SAMW-dosed mallards. Data are presented only for birds that demonstrated each specific sign.

| Statistical Measure | Time (min) to Signs of Toxicity Among SAMW-Exposed Mallards | | | | | | |
|---|---|---|---|---|---|---|---|
| | Head Shaking | Nasal Discharge/ Mucus | Throat Irritation | Ataxia | CNS Depression/ Dazed | Breathing Change | Death |
| Mean Time * (N) | 11 (9) | 45 (9) | 50 (9) | 117 (7) | 259 (6) | 183 (5) | 305 (7) |
| Range of Times (min) | 0 - 52 | 9 - 106 | 10 - 175 | 31 - 252 | 86 - 652 | 86 - 350 | 98 - 661 |

* Two of nine mallards survived exposure due to reduced AMW consumption and are not included in the calculation of mean time to death. Number of mallards showing signs is in parentheses.

Table A.6. Mean (SD) percent body mass change of control and SAMW treatment mallards from study A. N = 9 for both groups except when noted otherwise.

| | Percent Body Mass Change [Mean (SD)] | |
|---|---|---|
| Time Period | Control | SAMW |
| Hydrated Condition To 24-Hr Into Dehydration (-24 hr to 0 hr) | -6.02 (1.3) | -6.66 (1.9) |
| Initiation Of Dosing To Death | 0.68 (1.1)** | -6.15 (2.2)* |
| Hydrated Condition To Death | -5.38 (1.4)** | -12.5 (2.3)* |

* N = 7

** Significantly different than paired treatment mean by t-test at $p < 0.001$.

Table A.7. Comparison of serum clinical chemistry results from undiluted, 1:3, and 1:4 diluted serum samples from control mallards.

| Parameter | Mean conc. ± 2 SD | | | Mean conc. ± 2 SD | | |
|---|---|---|---|---|---|---|
| | Undiluted (n = 8) | 1:3 dilution (n = 6) | Mean Recovery | 1:4 dilution (n = 2) | Mean Recovery | Overall Mean Recovery* |
| Total Serum Protein (g/dl) | 4.24 ± 0.64 | 4.14 ± 0.90 | 98.06% | 4.89 ± 0.87 | 114.0% | 102.1% |
| Albumin (g/dl) | 2.22 ± 0.31 | 2.25 ± 0.42 | 100.9% | 2.63 ± 0.49 | 119.5% | 105.5% |
| Globulin (g/dl) | 2.02 ± 0.41 | 1.87 ± 0.41 | 93.61% | 2.25 ± 0.71 | 107.0% | 96.95% |
| Calcium (mg/dl) | 12.2 ± 0.98 | 11.9 ± 1.52 | 97.19% | 13.3 ± 0.71 | 110.3% | 100.5% |
| Phosphorus (mg/dl) | 7.53 ± 4.70 | 8.04 ± 4.99 | 99.54% | 7.28 ± 4.03 | 122.1% | 105.2% |
| Glucose (mg/dl) | 243 ± 145 | 262 ± 179 | 102.1% | 244 ± 143 | 117.3% | 105.9% |
| Alkaline Phosphatase (U/l) | 112 ± 157 | 120 ± 152 | 101.6% | 76.0 ± 49.5 | 108.8% | 103.4% |
| Creatine Kinase (U/l) | 1080 ± 1751 | 1283 ± 2259 | 104.7% | 896 ± 245 | 118.0% | 108.0% |
| Aspartate Aminotransferase (U/l) | 36.2 ± 25.2 | 45.3 ± 34.6 | 115.4% | 40.0 ± 0.00 | 145.8% | 123.0% |
| Uric Acid (mg/dl) | 5.42 ± 2.54 | 5.29 ± 2.68 | 94.11% | 5.30 ± 5.37 | 104.3% | 96.64% |
| Cholesterol (mg/dl) | 308 ± 94.7 | 310 ± 105 | 99.19% | 337 ± 77.8 | 114.2% | 103.0% |
| Sodium (meq/l) | 152 ± 14.3 | 156 ± 4.97 | 102.2% | 177 ± 8.49 | 118.8% | 106.4% |
| Potassium (meq/l) | 7.32 ± 8.32 | 7.45 ± 9.31 | 96.29% | 6.90 ± 6.08 | 110.9% | 99.94% |
| Chloride (meq/l) | 103 ± 4.89 | 99.8 ± 5.25 | 95.85% | 113 ± 2.83 | 110.0% | 99.39% |

Note: Serum volumes from each mallard were not sufficient for analysis of both dilutions.
* Calculated as the mean of all individual percent recoveries from both 1:3 and 1:4 dilutions.

Table A.8. Mean ± SD serum clinical chemistry results from SAMW treatment and control mallards.

| Parameter (units) | Parameter Mean ± SD | | |
|---|---|---|---|
| | SAMW Treatment Mortalities (n = 3 of 7)[a] | SAMW Treatment Survivors (n = 2)[b] | Control (n = 8 of 9)[c] |
| Total Serum Protein (g/dl) | 3.78 ± 1.25 | 3.00 ± 0.08 | 4.33 ± 0.54 |
| Albumin (g/dl) | 2.14 ± 0.68 | 1.56 ± 0.12 | 2.34 ± 0.27 |
| Globulin (g/dl) | 1.67 ± 0.58 | 1.45 ± 0.21 | 1.96 ± 0.28 |
| Calcium (mg/dl) | 10.8 ± 0.51 | 12.2 ± 0.62 | 12.2 ± 0.92 |
| Phosphorus (mg/dl) | 14.3 ± 5.79 | 8.59 ± 1.43 | 7.85 ± 2.27 |
| Glucose (mg/dl) | 29.6 ± 20.9 | 241 ± 40.5 | 258 ± 81.0 |
| Alkaline Phosphatase (U/l) | 192 ± 96.1 | 108 ± 53.3 | 109 ± 68.2 |
| Creatine Kinase (U/l) | 6579 ± 4797 | 902 ± 886 | 1186 ± 972 |
| Aspartate Aminotransferase (U/l) | 393 ± 100 | 73.0 ± 66.5 | 44 ± 14.8 |
| Uric Acid (mg/dl) | 79.7 ± 22.7 | 27.0 ± 15.8 | 5.29 ± 1.52 |
| Cholesterol (mg/dl) | 370 ± 162 | 261 ± 4.45 | 317 ± 48.6 |
| Sodium (meq/l) | 135 ± 23.5 | 166 ± 10.7 | 161 ± 9.96 |
| Potassium (meq/l) | 10.0 ± 4.64 | 4.52 ± 1.42 | 7.32 ± 4.11 |
| Chloride (meq/l) | 91.4 ± 5.79 | 107 ± 9.12 | 103 ± 6.52 |
| Hematocrit | 75 ± 6[de] | 63 ± 7.8 | 50 ± 5[d] |

[a] Serum samples from treatment mallards were diluted either 1:4, 1:3, or 1:9 with 18.0 mega-ohm water. All parameter values have been corrected for dilution factors, but not for recoveries noted in Table A.7. Serum sample quantities from all other treatment mortalities were not sufficient for analysis.

[b] Survived exposure to SAMW. These birds were euthanized either 27 or 33 hours post-dose.

[c] Mean calculated from 8 diluted control samples; two of 1:4 dilution and six of 1:3 dilution.

[d] n = 7 for SAMW-exposed mallards (mortalities only); n = 9 for control mallards.

[e] Mean is significantly different than paired control mean by Wilcoxon Rank Sum Test at $p < 0.001$.

Table A.9. Occurrence of histopathological changes in tissues following acute SAMW and control water treatments in mallard ducks.

| Treatment | Esophagus | Proventriculus | Ventriculus | Duodenum |
|---|---|---|---|---|
| AMW-Treated (all birds) | 4/7 | 9/9 | 8/9 | 6/9 |
| AMW-Treated Survivor | 1/2 | 2/2 | 2/2 | 0/2 |
| Control | 0/6 | 0/9 | 1/9 | 0/9 |

Table A.10. Nature of histopathological changes in the ventriculus following acute SAMW and control water treatments in mallard ducks.

| Treatment | Erosion or ulceration of kaolin | Heterophilic inflammation | Congestion and hemorrhage |
|---|---|---|---|
| AMW-Treated | 7/9 | 7/9 | 7/9 |
| AMW-Treated Survivor | 2/2 | 2/2 | 2/2 |
| Control | 0/1 | 1/1 | 0/1 |

Table A.11. Mean ± SD concentrations of elements detected in livers, kidneys, blood, and bile from SAMW treatment and control mallards. N = 9 for each tissue/metal combination unless otherwise noted. NQ = Not Quantifiable.

| | Concentrations of Elements (µg/g wet weight ± SD) | | | | |
|---|---|---|---|---|---|
| Tissue | Cu | Zn | Mg | Fe | Mn |
| Liver (SAMW) | 236 ± 111 | 60.3 ± 13.7 | 284 ± 37.6 | 1039 ± 508 | 13.5 ± 3.1 |
| Liver (Control) | 156 ± 142 | 59.2 ± 8.2 | 300 ± 20.7 | 1389 ± 589 | 5.3 ± 0.8 |
| Kidney (SAMW) | 38.1 ± 14.3$^{***}$ | 27.9 ± 5.2$^{**}$ | 292 ± 48.0$^{*}$ | 195 ± 43.3$^{*}$ | 13.2 ± 4.7$^{***}$ |
| Kidney (Control) | 7.5 ± 1.4 | 21.8 ± 2.0 | 248 ± 29.3 | 148 ± 23.6 | 3.9 ± 1.2 |
| Blood (SAMW) | 36.5 ± 23.9$^{***}$ | 12.8 ± 6.1$^{**}$ | 139 ± 18.2$^{***}$ | 617 ± 67.3$^{***}$ | NQ |
| Blood (Control) | 2.0 ± 0.8 | 5.6 ± 3.4 | 99.2 ± 19.7 | 424 ± 19.7 | NQ |
| Bile (SAMW) | 81.2 ± 47.5$^{*}$ | NQ | 203 ± 67.2 | 9.2 ± 3.6 | 37.9 ± 47.3 |
| Bile (control) | 39.4 ± 17.0 | NQ | 192 ± 38.0 | 8.1 ± 7.8 | NQ |

Treatment values differ from control values, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$
[a] n = 7

## Study B: Acute Toxicity of Neutralized Synthetic Acid Mine Water to Mallards

### Introduction

One of the remediation techniques used on acid metalliferous water sources, such as tailings ponds, is the addition of lime to neutralize the acidic character and reduce dissolved metal content. Such a remediation technique may reduce the toxicity of AMW to avian species. Our goal in Study B was to assess the potential for alleviation of AMW's avian toxic effects by neutralizing its acidic character with lime.

### Methods

*Neutralized AMW Dosing Solution Preparation*

Reagents for the preparation of the dosing solution included 95% CaOH (lime) and AMW that was synthesized in study A. Two 21-L batches of neutralized AMW (NAMW) were formulated and then mixed together into one 42-L batch. The first batch was prepared by adding 540.2 g of lime to 21-L of AMW in a large carboy. The solution was stirred periodically with a mixer while adding lime to the AMW solution. The pH of the mixed solution was 6.97 three days after being formulated. The second batch was prepared in the same manner, however 504.9 g of lime was added to the AMW solution. Both NAMW batches were combined to formulate approximately 42 L of the dosing solution. The NAMW solution contained a light green precipitate layer and a clear colorless aqueous layer when unstirred. A total of 1098 g of lime was added to the 42 liters of NAMW solution to adjust it to a pH of 7.21 on the first dosing day. The pH of the dosing solution gradually increased over an eight-day period (time from beginning of study to end of study) from 7.21 to 7.38 (Table B.1). Concentrations of elements in the aqueous layer of NAMW were measured by ICP-AES (Table B.2).

*Study Design*

Eighteen 22-24 week-old mallards of mixed sex were commercially obtained (see Study A) and transported to the Texas Tech University Animal Care Resources Center. Birds were banded and maintained in 2.5 $ft^3$ stainless steel rabbit cages at 20°C, 40-70% relative humidity, and 12h:12h light:dark photoperiod. Ducks had *ad libitum* access to feed (Mazuri

waterfowl maintenance diet in pellet form; PMI Nutritional, LLC, Brentwood, MO, USA) and tap water. All mallards were allowed 2 weeks to acclimate to indoor, caged conditions prior to initiation of testing, with their body weight near or exceeding that recorded upon receipt into the animal facility. All animal care was performed in accordance with the Texas Tech University ACRC and the Institutional Animal Care and Use Committee.

The study was performed during a 48-hr test period with nine control and nine NAMW treatment mallards. Three groups of six mallards were used in this study in order to facilitate reasonable timing of necropsies. A total of three females and three males were used in each 48-hr test. The first group was tested from November 12 – 14, 2005, the second group from November 13 – 15, 2005, and the third group from November 19 – 21, 2005. All mallards were fasted and dehydrated for 24 hrs prior to initiation of the dosing study. The NAMW dosing solution and tap water were presented to treatment and control mallards, respectively, in semicircular plastic bowls with an approximate volume of 350 mL. Bowls were used in this study instead of bottles to simulate field conditions in which birds would likely agitate metal precipitates in the sediment, and therefore receive an oral dose of AMW reflective of both the precipitate in addition to the aqueous solution. The drinking apparatus used in study A would not allow this type of drinking scenario. Control mallards received tap water instead of RO water to serve as a clean water source that is more representative of a natural clean water source for wild birds. Tap water was provided to control mallards for the remaining studies as well. Bowls were filled with approximately 300 mL of either NAMW or tap water at the initiation of dosing as well as when bowls needed to be re-filled. NAMW and sediments were mixed with an electric mixer during transfer from the stock solution container into individual plastic bowls. All mallards were allowed *ad libitum* access to NAMW, and water consumption data were not collected during the study, as the plastic bowls were not amenable to water wastage data collection. Animals were observed periodically throughout exposure duration and behavioral observations were collected. Food was withheld during the entire study period for all control and treatment mallards.

*Animal Euthanasia and Sample Collection*

Treatment and control birds were euthanized following 48 hrs access to either NAMW or tap water. All birds were allowed access to their designated water type until time of death.

Birds were euthanized by carbon dioxide asphyxiation. Blood was collected using cardiac puncture with 1 mL syringes. Whole blood was placed in 5 mL serum separator tubes for serum clinical chemistry, 2 ml microcentrifuge tubes for metal residues, and microhematocrit tubes for determination of erythrocyte PCV. Whole blood in serum separator tubes was allowed to clot at room temperature for 30 minutes and then centrifuged at 6,000 rpm for 10 minutes. Serum was decanted into 2-ml microcentrifuge tubes and frozen at -80°C until being shipped for analysis. Serum samples were analyzed with a Hitachi 911 Analyzer at TVMDL for total serum protein, albumin, globulins, calcium, phosphorus, glucose, CK, AST, uric acid, cholesterol, ALP, and electrolytes.

Gross pathological lesions were documented, and tissues were collected for both metal and histopathological analyses for all birds. Sections of right lung, right testis or ovaries, right kidney, salt gland, spleen, liver, pancreas, brain, tongue, esophagus, trachea, heart, proventriculus, ventriculus, duodenum, jejunum, ileum, ceca, and large intestine were fixed in 10% buffered formalin. Histopathology samples have been stored for possible analysis at a future date.

*Statistical Methods*

Measures of central tendency are expressed as the mean ± standard deviation. All data analyzed using parametric methods were tested for normality and homogeneity of variances. When the assumptions of parametric tests were not met, data were reanalyzed after log transformation or analyzed using non-parametric methods. Body mass dynamics were analyzed using t-tests. Serum clinical chemistry endpoints and hematocrit were analyzed using either t-tests or Wilcoxon Rank Sum tests. All statistical analyses were performed with R (version 2.2.0 ; R Foundation, Vienna, Austria). Results of statistical tests were considered to be significant at $p < 0.05$.

## Results

*Signs of Toxicity*

There were no mortality events during the study. Sublethal effects included mild dehydration, body mass loss, lethargy, and subtle shivering in NAMW mallards. Other common signs of toxicity in NAMW mallards included mild to vigorous lateral head shaking,

production of oral mucus, and gular fluttering. Less common signs of toxicity included subtle shivering and regurgitation. These signs of toxicity were absent in control birds. Nonetheless, all birds appeared to be in relatively good health at the time of euthanasia.

Both treatment groups lost approximately 5% body mass from the period 24-hrs prior to dose to the initiation of dose (Table B.3). NAMW mallards lost significantly more mass than control mallards from the periods from initiation of dosing to death (0 hr to 48 hr), as well as the period from hydrated condition to death (-24 hrs to 48 hrs). NAMW treatment mallards lost approximately 17% of body mass from the time of initiation of dose to completion of study, nearly twice that amount lost by controls.

*Clinical Chemistry and Hematocrit*

NAMW treatment mallards had significantly lower mean levels of serum total protein, albumin, and globulin in comparison to controls ($p < 0.05$; Table B.4). NAMW treatment mallards had elevated mean serum levels of phosphorus ($p < 0.05$), glucose ($p < 0.001$), alkaline phosphatase ($p < 0.05$), and uric acid ($p < 0.01$) when compared to control mallards. Electrolytes (sodium, potassium, and chloride) were also significantly elevated when compared to control mallards ($p < 0.001$). Mean serum uric acid levels and alkaline phosphatase levels were nearly 3.5 and 2 times higher, respectively, in NAMW treatment mallards when compared to control mallards.

Mean hematocrit levels from NAMW mallards were significantly higher than control hematocrit levels ($p < 0.001$). Mean hematocrits from NAMW mallards were approximately 23% higher than mean control hematocrit values.

*Gross Pathology*

Gross abnormalities in the oral cavity and esophagus of NAMW mallards included discoloration of the mucosal surface of the esophagus (light brown) and the presence of small to large amounts of yellow-green NAMW precipitate. There was a single case of NAMW precipitate in the proximal trachea of a NAMW treatment mallard. Proventricular lesions ranged from mild to severe reddening of the mucosal surface and inflammation. The most severe lesions were present in the ventriculus with abnormalities including discoloration (blue-green vs. yellow-green of controls) and ulcerations or mild to moderate erosion of the

kaolin. Duodenal and lower gastrointestinal contents of some NAMW birds were light green and similar in color and viscosity to the NAMW dosing solution, however no abnormalities were noted on the mucosal surfaces of these tissues.

Examples of macroscopic changes in internal tissues are provided in the appendix.

## Discussion

The synthetic NAMW dosing solution tested in this study was mildly toxic to mallards. Gross lesions were generally mild to moderate and limited to the proventriculus and ventriculus in NAMW treated mallards following a 48-hr exposure period. Alterations in serum biochemistry parameters indicate that the NAMW has an effect on liver and kidney function, regulation of protein homeostasis, and electrolyte balance. Mallards were less averse to NAMW than SAMW and were observed using typical waterfowl filter-feeding behavior. Mallards do not only ingest water from the aqueous layer of NAMW, but also sift through the entire mixture and ingest precipitated metals.

Liming the acid metalliferous water source effectively reduced dissolved metal levels, however, the process ultimately increased precipitated levels of metals. Such practices on metal mining and processing sites may ultimately decrease the toxicity of acid metalliferous water to wildlife, but likely do not eliminate risk of injury. This study demonstrated that birds can accumulate metal sediments in their upper gastrointestinal tract. These metals can ultimately damage tissues and alter biochemical processes.

Table B.1. pH values of NAMW over an 8-day period during mallard study B. Treatment studies occurred from November 12 – 21, 2005.

| Date | pH of NAMW |
|---|---|
| November 12, 2005 | 7.21 |
| November 13, 2005 | 7.28 |
| November 14, 2005 | 7.33 |
| November 15, 2005 | 7.36 |
| November 19, 2005 | 7.38 |

Table B.2. Concentrations of metals from the aqueous layer of synthetic neutralized AMW and tap water analyzed by inductively-coupled plasma atomic emission spectroscopy and flame AA. Analysis results for sodium were unreliable based on variability of check standard results.

| Element | Measured Concentrations from NAMW Aqueous Layer (mg/L) | Measured Concentrations from SAMW (mg/L) | NAMW as % of SAMW | Measured Concentrations from Tap (mg/L) |
|---|---|---|---|---|
| Cu | 0.715 | 5943 | 0.01 | 0.053 |
| Al | 1.325 | 3718 | 0.04 | 0.319 |
| Zn | 4.102 | 2071 | 0.20 | 0.024 |
| Mg | 1056 | 1596 | 66.17 | 37.62 |
| Fe | 0.266 | 1351 | 0.02 | <0.01 |
| Mn | 368.7 | 746 | 49.42 | <0.01 |
| Ca | 753.9 | 493 | 152.92 | 61.76 |
| Cd | 3.629 | 22.2 | 16.35 | <0.01 |
| Co | 0.119 | 21.8 | 0.55 | <0.01 |
| Na | na | 17.3 | na | 249.05 |
| Ni | <0.05 | 10.8 | <0.46 | <0.01 |
| Cr | <0.05 | 4.8 | <1.04 | <0.01 |
| Se | 0.180 | 0.639 | 28.17 | <0.01 |
| V | <0.05 | 0.352 | <14.20 | 0.01 |
| As | <0.05 | 0.344 | <14.53 | 0.005 |

Table B.3. Mean ± SD body mass changes of control and NAMW treatment group mallards in study B. N = 9 for each group.

| | Percent Body Mass Change [Mean ± SD] | |
|---|---|---|
| Time Period | Control | NAMW |
| Hydrated Condition To Initiation of Dosing (After 24-Hr Dehydration - -24 hr to 0 hr) | -5.0 ± 3.0 | -5.6 ± 1.3 |
| Initiation Of Dosing To Death (0 hr to 48 hr) | -3.3 ± 0.8* | -12.1 ± 3.1 |
| Hydrated Condition To Death (-24 hr to 48 hr) | -8.2 ± 2.9* | -17.0 ± 3.7 |

* $p < 0.001$

Table B.4. Serum clinical chemistry and hematocrit results (mean ± SD) from control and treatment mallards following a 48-hr acute *ad libitum* exposure to synthetic neutralized acid mine water.

| Parameter (units) | Parameter Mean ± SD | |
|---|---|---|
| | Control (n = 9) | NAMW Treatment (n = 7)[a] |
| Total Serum Protein (g/dl) | 4.13 ± 0.39 | 3.47 ± 0.48[b] |
| Albumin (g/dl) | 2.25 ± 0.17 | 1.94 ± 0.27[b] |
| Globulin (g/dl) | 1.87 ± 0.38 | 1.51 ± 0.23[b] |
| A/G Ratio | 1.24 ± 0.24 | 1.27 ± 0.10 |
| Calcium (mg/dl) | 12.8 ± 1.10 | 12.62 ± 1.12 |
| Phosphorus (mg/dl) | 5.72 ± 0.87 | 7.07 ± 1.07[b] |
| Glucose (mg/dl) | 179 ± 14.0 | 284 ± 83.8[d] |
| Alkaline Phosphatase (U/l) | 65.5 ± 21.6 | 110 ± 43.7[b] |
| Creatine Kinase (U/l) | 574 ± 293 | 1813 ± 3495 |
| Aspartate Aminotransferase (U/l) | 82.4 ± 44.3 | 95.1 ± 77.7 |
| Uric Acid (mg/dl) | 5.12 ± 2.14 | 18.1 ± 12.9[c] |
| Cholesterol (mg/dl) | 315 ± 52.9 | 324 ± 106 |
| Sodium (meq/l) | 160 ± 3.02 | 169 ± 1.62[d] |
| Potassium (meq/l) | 4.22 ± 0.37 | 5.23 ± 0.77[b] |
| Chloride (meq/l) | 107 ± 2.67 | 121 ± 7.29[d] |
| Hematocrit | 51.8 ± 4.52 | 63.9 ± 5.23[e] |

[a] Serum sample quantities were insufficient for 2 of 9 NAMW treatment mallards.
[b] Mean is significantly different than paired mean by Wilcoxon Rank Sum Test at $p < 0.05$.
[c] Mean is significantly different than paired mean by Wilcoxon Rank Sum Test at $p < 0.01$.
[d] Mean is significantly different than paired mean by Wilcoxon Rank Sum Test at $p < 0.001$.
[e] N=9; Mean is significantly different than paired mean by t-test at $p < 0.001$.

## Study C: Synthetic Acid Mine Water Time Course Study with Mallards

### Introduction

Short-term exposure to acid metalliferous water followed by ingestion of clean water can potentially be survived by exposed birds. Therefore, clean water availability is an important requirement for survival of exposed birds in the field. The purpose of study C was to assess the potential role that clean water availability and consumption might play in protecting birds after a toxic exposure to SAMW.

### Methods

*Treatment Water Source*

Treatment mallards were dosed with the same SAMW used in Study A. The pH of SAMW was measured at 2.05 and the density of SAMW was consistent with study A (1.05 g/mL).

*Study Design*

Thirty 8-week old mallards (15 males, 15 females) were obtained from a commercial gamebird grower (see study A), raised at The Institute of Environmental and Human Health aviary facility, and later transported to the Texas Tech University Animal Care Resources Center when the birds had reached approximately 22-weeks of age. Mallards were approximately 25-29 weeks of age at the time of dosing. Birds were banded and maintained individually in 2.5 $ft^3$ stainless steel rabbit cages at 20°C, 40-70% relative humidity, and 12h:12h light:dark photoperiod. All mallards were allowed approximately three weeks to acclimate to indoor, caged conditions prior to initiation of testing, with their body weight near or exceeding that recorded upon receipt into the animal facility. Ducks had *ad libitum* access to feed (Mazuri waterfowl maintenance diet in pellet form; PMI Nutritional, LLC, Brentwood, MO, USA) and tap water during the acclimation period. All animal care was performed in accordance with the Texas Tech University ACRC and the Institutional Animal Care and Use Committee.

The study was performed during two separate weeks, with 4 weeks in between each dosing trial. Sixteen mallards (8 males and 8 females) were tested during the first week and

fourteen mallards (7 males and 7 females) were tested during the second week. All mallards were fasted and dehydrated for a 24-hr period prior to the dosing period. During each trial, eight mallards were provided *ad libitum* access to SAMW for a maximum of 55-min or a maximum dose of 40 mL/kg, whichever event occurred first. The exposure duration and maximum dose were based on results from study A, which indicated that the lowest lethal dose was above 40 mL/kg and most birds did not consume that dose in less than one hour. Immediately following the SAMW exposure period, the water source was changed to tap water and SAMW treatment mallards were allowed *ad libitum* access. Control mallards were allowed free access to tap water at the same time SAMW treatment birds were provided tap water. Control mallards were pair-fed with SAMW treatment mallards. An individual mallard was assigned to be pair-fed with an individual of the same sex from the SAMW group. SAMW treatment mallards were allowed *ad libitum* access to food. Food was provided approximately three to four hours after birds first received access to water. Body mass measurements were collected for each mallard before the initiation of the dehydration and fasting period, at the initiation of dosing, and at the time of euthanasia. Body mass was collected with an electronic balance to the nearest 0.1 g.

Dosing of each mallard was staggered at 2-min intervals to allow for changing of water bottle reservoirs and waste-collection bottles. SAMW consumption was adjusted for the density of the dosing solution (1.05 g/ml) and control consumption was based on 1 g/ml for tap water. Animals were observed continuously throughout the exposure period, with behavioral observations and water consumption data collected. Water consumption measurement methods were the same as those used in Study A.

*Animal Euthanasia and Sample Collection*

Control and treatment birds were euthanized via carbon dioxide asphyxiation following either 1, 3, or 7 days post-dose. Whole blood was collected with 1 mL syringes and placed in 5 mL serum separator tubes for serum clinical chemistry, 2 ml microcentrifuge tubes for metal residues, and microhematocrit tubes for determination of erythrocyte PCV. Whole blood in serum separator tubes was allowed to clot at room temperature for 30 minutes and centrifuged at 6,000 rpm for 10 minutes. Serum was decanted into 2-ml microcentrifuge tubes and frozen at -80°C until shipped for analysis. Serum samples were analyzed with a

Hitachi 911 Analyzer at the TVMDL for total serum protein, albumin, globulins, calcium, phosphorus, glucose, CK, AST, uric acid, cholesterol, ALP, and electrolytes.

Gross pathological lesions were documented, and tissues were collected for histopathological analyses for all birds. Sections of right lung, testes or ovaries, right kidney, liver, spleen, pancreas, brain, tongue, esophagus, trachea, heart, proventriculus, ventriculus, duodenum, jejunum, ileum, ceca, and large intestine were fixed in 10% buffered formalin and stored in jars until processed.

*Histopathology*

Histopathological analyses were performed at Colorado State University's College of Veterinary Medicine and Biomedical Sciences. Tissue samples were embedded in paraffin and 5 µm sections of these tissues were histologically analyzed following routine hematoxylin and eosin staining. When dictated by histopathologic findings, specific tissue sections were also stained for minerals. Primary histopathologic analysis was performed blindly without knowledge of treatment. Following analysis, observations in treated and control groups that were indistinguishable both qualitatively and quantitatively were considered to be background lesions and deemed unrelated to treatment.

*Statistical Methods*

Measures of central tendency were expressed as the mean ± standard deviation. All data analyzed using parametric methods were tested for normality and homogeneity of variances. When the assumptions of parametric tests were not met, data were reanalyzed after log transformation or analyzed using non-parametric methods. To control for experimental-wise error, we used a linear mixed-effects model to analyze for a treatment-related effect on water consumption (mL/kg). Dose was used as the fixed factor and time as the random factor, as well as the interaction between dose and time. Any differences in the model were further analyzed using t-tests or Wilcoxon Rank-Sum Test to determine differences between treatment groups during consumption time periods. Among day SAMW total doses were analyzed using analysis of variance. SAMW treatment mortalities and their paired controls were not included in water consumption or total dose analyses. Serum clinical chemistry endpoints were analyzed using either t-tests or Wilcoxon Rank Sum tests. Differences in

body mass between treatment groups were analyzed using a linear mixed-effects model to determine differences in body mass change between treatment groups for all three bird collection time points. All statistical analyses were performed with R version 2.2.0 (R Foundation, Vienna, Austria). Results of statistical tests were considered to be significant at $p < 0.05$.

## Results

### *Total Doses and Water Consumption*

Total doses of SAMW in treated mallards ranged from 15.0 to 36.7 mL/kg, not including one female mallard that was accidentally allowed to consume 46.1 mL/kg SAMW and subsequently died. Total SAMW doses were not significantly different among the three different AMW-treated groups (Figure C.1, Tables C.1, C.2, and C.3).

On average, AMW treatment mallards tended to drink less tap water than controls during the day of SAMW treatment on a time interval basis. This was not the case in SAMW-treated mallards from the second to seventh day however. During these days, although not statistically significant in most cases, SAMW-treated mallards on average tended to consume more clean water than controls.

### *Food Consumption and Body Mass Dynamics*

In general, SAMW-treated mallards consumed very little, if any food at all on the same day as treatment (Table C.4). Whereas the SAMW-treated mallards that did eat food were generally hesitant to consume it, pair-fed controls were very eager to eat and tended to devour all available food immediately after it was presented. Three of 13 mallards did not eat on the same day they were exposed to SAMW. Four of nine mallards were not eating 1 day after exposure, and three of nine mallards were still not eating 2 days after exposure. Normal *ad libitum* food consumption was determined to be approximately 66 ± 16 g/kg/day in our laboratory-maintained mallards. Whereas day 3 mallards were eating normal mean *ad libitum* food levels by 1 day after exposure, day 7 birds were not eating normal mean levels of food until 3 days after SAMW treatment.

There were no significant differences in body mass change between treatment groups for all three mallard collection time points (Table C.5) due to the paired feeding strategy.

*Clinical Chemistry*

There were no significant differences in clinical chemistry parameters between treatment groups for all three collection time points except for total serum protein and albumin levels (Table C.6). Total serum protein levels from SAMW mallards from day 1 were significantly reduced in comparison to controls ($p < 0.01$). Albumin levels from AMW mallards from all three collection time points were significantly reduced in comparison to controls ($p < 0.05$). Although not statistically different, mean levels of ALP, CK, AST, uric acid, and potassium were higher in day 1 and day 3 AMW mallards in comparison to controls, while mean sodium and chloride levels were lower in SAMW mallards in comparison to controls. Such trends were not observed in day 7 SAMW mallards.

*Survival and Pathology*

Two SAMW-treated female mallards died within the first 24 hrs of the study. One mallard consumed 31.0 mL/kg SAMW, while the other mallard consumed 46.1 mL/kg SAMW, a dose greater than our estimated lethal limit. Gross lesions in these birds were consistent with birds poisoned in Study A. There were no control group mortality events prior to euthanasia at the end of each study period.

Gross lesions in SAMW-treated mallards euthanized one day post-treatment included mild to severe ventricular and proventricular ulcerations and hemorrhaging, erosion of the kaolin, and presence of yellow-green oral mucus. Some ventricular ulcers were atypically black in color in comparison to brownish-yellow ulcers observed in mallards acutely poisoned by SAMW. Gross lesions in SAMW-treated mallards euthanized three days post-treatment were observed in similar tissues, however they were less severe in nature. Mild ventricular lesions were the most commonly observed abnormality. Gross treatment-related lesions in SAMW-treated mallards euthanized seven days post-treatment were relatively mild in nature and limited strictly to the ventriculus. Lesions were few in number and were only slightly raised in nature and/or colored brownish-green or brownish-orange. Bilateral testicular necrosis was noted in one SAMW-treated bird euthanized 7 days post-treatment. Since similar lesions were not observed in other treated male birds, it is likely that this was an incidental finding in this bird unrelated to treatment. The necrosis observed in the testis

was coagulative and would be consistent with infarction which could be related to previous kaolin ulceration and a transient septicemia.

As observed in previous studies, the histologic lesions were restricted to the anterior gastrointestinal tract, including esophagus, proventriculus, and ventriculus (Table C.7). Changes noted in the liver and trachea of many treated and control birds were determined to be background and unrelated to treatment.

Definitive treatment-related changes in the proventriculus and esophagus are transient and were not observed after 1 day post treatment. They were most likely related to local irritation caused by ingestion of SAMW. The only observed esophageal lesion (dilated glands filled with mucous) was consistent with abundant mucus seen in the upper GI and respiratory tract of these birds. Changes (degeneration, ulceration and heterophilic inflammation) in the ventriculus lasted longer, until at least 3 days post-treatment, but were not seen in birds euthanized 7 days post treatment. These decreases in lesion severity over time were likely correlated with concurrent increases in food intake.

Examples of macroscopic changes in internal tissues are provided in the appendix.

Discussion

Nearly all mallards consuming typically sublethal doses of SAMW were able to survive exposure if allowed access to clean water and/or food. A time course evaluation of the healing process of upper gastrointestinal tract lesions in mallards treated with SAMW indicated most of the lesions can heal if a bird can obtain clean water and food. However, the healing process may be different in terms of duration and nature in wild birds that are exposed to more physiologically-stressed conditions than our laboratory mallards. The most significant finding from this study is the occurrence of prolonged anorexia and decreased food consumption in birds with mild to severe ventricular pathology. Whereas large birds, such as some waterfowl, may have enough fat stores to survive a period of several days without food, small birds, with higher bioenergetic demands and similar ventricular lesions, may be less likely to survive without food for a similar time period.

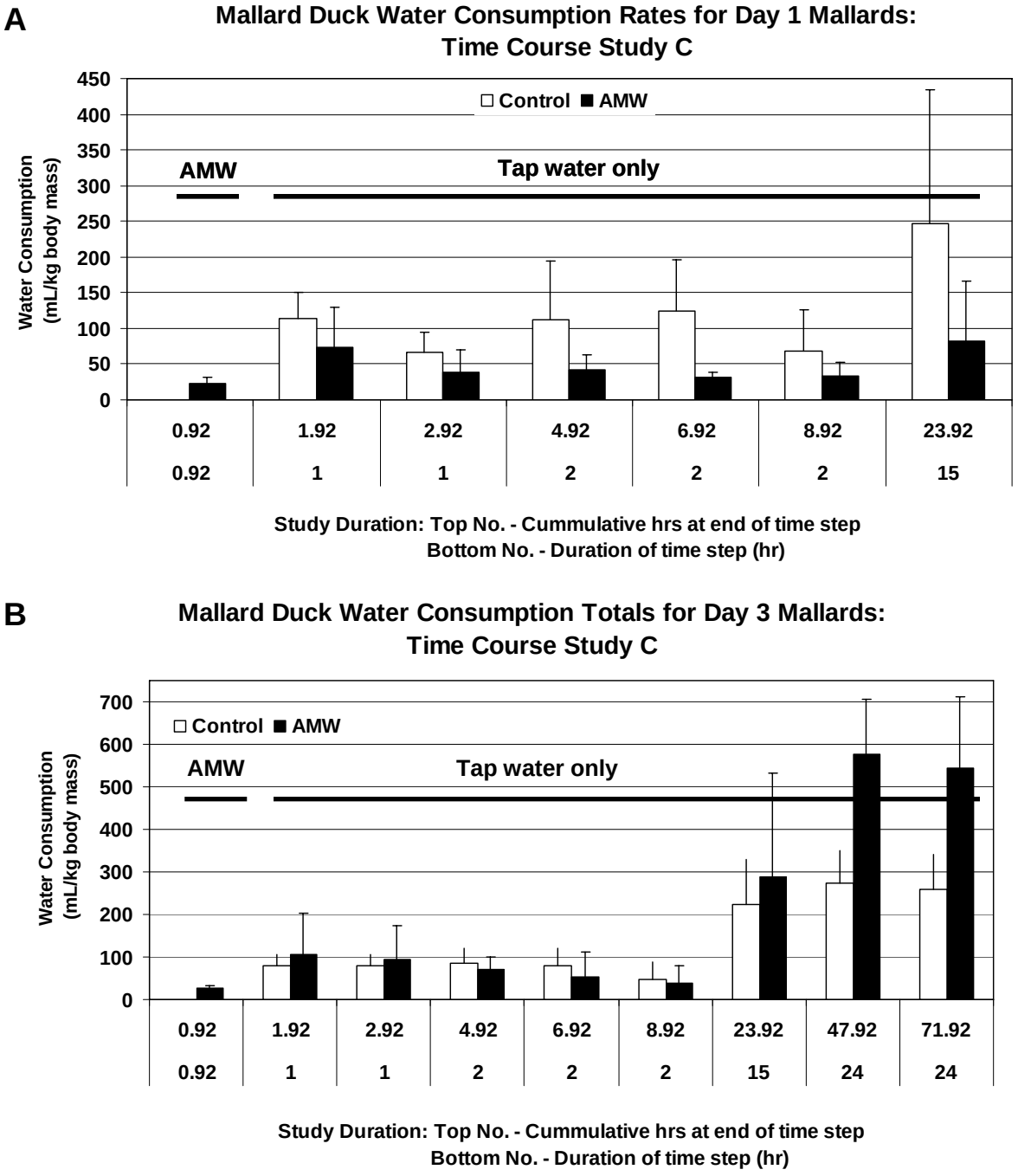



Figure C.1. Water consumption totals for mallards euthanized 1 day after SAMW exposure (A), 3 days after SAMW exposure (B), and 7 days after exposure (C, next page). Values are mean + SD for bars and error bars, respectively. N values and statistical assessments are presented in Tables C.1, C.2, and C.3.

Fig. C.1 continued on next page

**C**

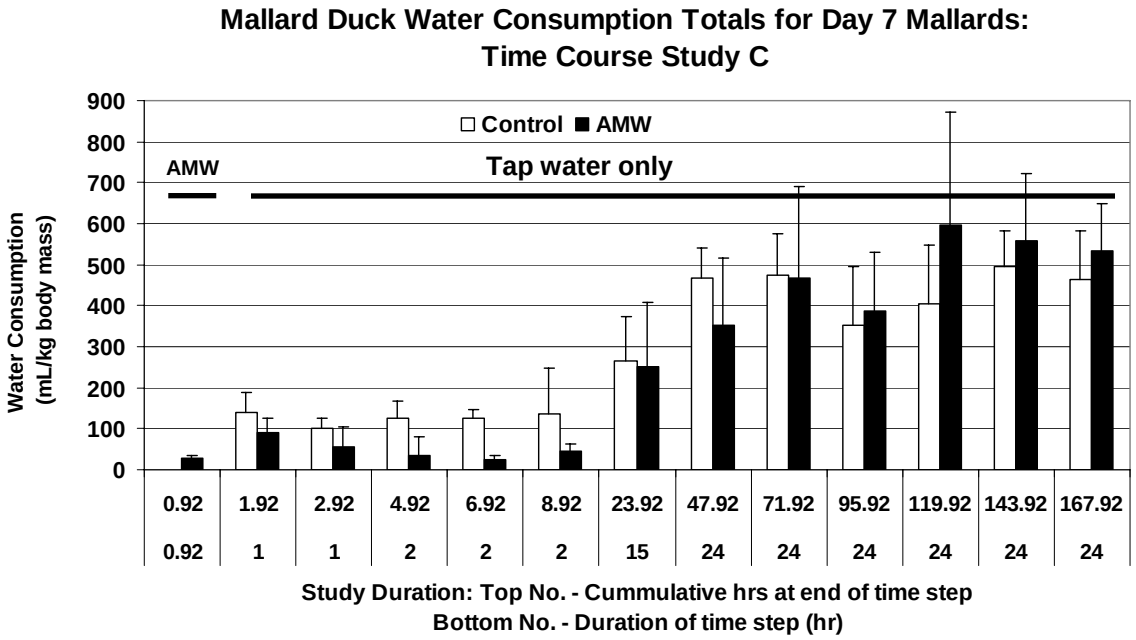



Figure C.1. Continued

Table C.1. Water consumption totals (mean ± SD) of mallards receiving tap water or SAMW. These data correspond to those demonstrated in Figure C.1.A. Treatment and control mallards were euthanized 1 day after treatment mallards were exposed to SAMW. SAMW mallards received SAMW only during the first 0.92 hr time step and received tap water for the remaining of the study. Values with asterisks differ significantly ($p < 0.05$). Time periods with no letter designation did not differ significantly between groups. N = 5 for both groups.

| Study Duration at End of Time Step (hrs) | Time Step Duration (hrs) | Water Consumption (ml/kg body mass) | | | |
|---|---|---|---|---|---|
| | | Control | | SAMW | |
| | | Mean | SD | Mean | SD |
| 0.92 | 0.92 | na | na | 23.4 | 7.5 |
| 1.92 | 1 | 113 | 36.9 | 74.0 | 55.1 |
| 2.92 | 1 | 67.3 | 27.5 | 38.2 | 32.3 |
| 4.92 | 2 | 111 | 82.6 | 42.2 | 20.1 |
| 6.92 | 2 | 124* | 72.7 | 31.1 | 7.9 |
| 8.92 | 2 | 68.0 | 58.1 | 33.4 | 19.2 |
| 23.92 | 15 | 248 | 187 | 82.3 | 84.7 |

Table C.2. Water consumption totals (mean ± SD) of mallards receiving tap water or SAMW. These data correspond to those demonstrated in Figure C.1.B. Treatment and control mallards were euthanized 3 days after treatment mallards were exposed to SAMW. SAMW mallards received SAMW only during the first 0.92 hr time step and received tap water for the remaining of the study. Values with asterisks differ significantly ($p < 0.05$). Time periods with no letter designation did not differ significantly between groups. N = 4 for both groups.

| Study Duration at End of Time Step (hrs) | Time Step Duration (hrs) | Water Consumption (ml/kg body mass) | | | |
|---|---|---|---|---|---|
| | | Control | | SAMW | |
| | | Mean | SD | Mean | SD |
| 0.92 | 0.92 | na | na | 26.3 | 5.9 |
| 1.92 | 1 | 79.1 | 28.0 | 104.6 | 98.2 |
| 2.92 | 1 | 78.7 | 26.9 | 95.0 | 77.9 |
| 4.92 | 2 | 85.1 | 34.3 | 70.4 | 29.0 |
| 6.92 | 2 | 80.7 | 39.7 | 53.8 | 57.7 |
| 8.92 | 2 | 47.2 | 42.2 | 37.1 | 42.1 |
| 23.92 | 15 | 224 | 106 | 288 | 243 |
| 47.92 | 24 | 273* | 75.7 | 578 | 129 |
| 71.92 | 24 | 260* | 82.4 | 545 | 167 |

Table C.3. Water consumption totals (mean ± SD) of mallards receiving tap water or SAMW. These data correspond to those demonstrated in Figure C.1.C. Treatment and control mallards were euthanized 7 days after treatment mallards were exposed to SAMW. SAMW mallards received SAMW only during the first 0.92 hr time step and received tap water for the remaining of the study. Values with asterisks differ significantly ($p<0.05$). Time periods with no letter designation did not differ significantly between groups. N = 5 for both groups.

| Study Duration at End of Time Step (hrs) | Time Step Duration (hrs) | Water Consumption (ml/kg body mass) | | | |
|---|---|---|---|---|---|
| | | Control | | SAMW | |
| | | Mean | SD | Mean | SD |
| 0.92 | 0.92 | na | na | 29.1 | 5.5 |
| 1.92 | 1 | 141 | 46.8 | 90.5 | 34.8 |
| 2.92 | 1 | 99.8 | 24.7 | 56.7 | 49.7 |
| 4.92 | 2 | 127* | 42.0 | 34.8 | 46.9 |
| 6.92 | 2 | 126* | 19.0 | 23.1 | 10.1 |
| 8.92 | 2 | 138 | 109 | 45.1 | 18.8 |
| 23.92 | 15 | 265 | 107 | 252 | 155 |
| 47.92 | 24 | 469 | 72.7 | 353 | 162 |
| 71.92 | 24 | 474 | 100 | 466 | 224 |
| 95.92 | 24 | 354 | 142 | 389 | 143 |
| 119.92 | 24 | 404 | 143 | 598 | 274 |
| 143.92 | 24 | 496 | 87.4 | 557 | 164 |
| 167.92 | 24 | 463 | 119 | 532 | 116 |

Table C.4. Total food consumption (g/kg/day) of SAMW treated mallards. Mallards were euthanized either 1, 3, or 7 days after exposure to SAMW for a maximum 55-min period. Controls mallards were pair-fed with SAMW treatment mallards. Values with different letters are significantly different.

| Day | Food Consumption in g/kg/day [Mean ± SD] (# of birds eating) |
|---|---|
| 0 | 9.9 ± 11.7 (10/13) |
| 1 | 36.3 ± 37.7 (5/9) |
| 2 | 34.5 ± 39.2 (6/9) |
| 3 | 53.1 ± 57.8 (4/5) |
| 4 | 77.7 ± 58.6 (5/5) |
| 5 | 79.8 ± 42.5 (5/5) |
| 6 | 49.3 ± 25.5 (5/5) |

Table C.5. Mean ± SD body mass changes of control and SAMW treatment group mallards in study C. N = 4 for Day 1 and Day 3 mallards for both treatment groups. N = 5 for Day 7 mallards for both treatment groups.

| | Control (mean ± SD) | | | SAMW Treatment (mean ± SD) | | |
|---|---|---|---|---|---|---|
| Time Period | Day 1 | Day 3 | Day 7 | Day 1 | Day 3 | Day 7 |
| % body mass change from hydrated condition to initiation of dosing | -8.3 ± 4.4 | -6.8 ± 2.7 | -8.8 ± 4.0 | -8.1 ± 2.8 | -9.4 ± 3.8 | -7.8 ± 2.4 |
| % body mass change from initiation of dosing to death | 1.4 ± 2.1 | 1.1 ± 2.5 | 4.2 ± 7.7 | -0.5 ± 4.3 | 0.1 ± 6.8 | 1.2 ± 6.02 |
| % body mass change from hydrated condition to death | -7.1 ± 4.4 | -5.8 ± 2.3 | -5.1 ± 4.4 | -8.7 ± 2.9 | -9.5 ± 3.0 | -6.8 ± 3.8 |

Table C.6. Serum clinical chemistry results (mean ± SD) from Day 1, 3, and 7 control and treatment mallards from Study C.

| Parameter (units) | Parameter Mean ± SD | | | | | |
|---|---|---|---|---|---|---|
| | Day 1 Birds | | Day 3 Birds | | Day 7 Birds | |
| | Control (n = 4) | SAMW (n = 4) | Control (n = 4) | SAMW (n = 4) | Control (n = 5) | SAMW (n = 5) |
| Total Serum Protein (g/dl) | 4.1 ± 0.3** | 2.7 ± 0.7 | 4.4 ± 0.7 | 3.2 ± 0.9 | 4.2 ± 0.8 | 3.5 ± 0.6 |
| Albumin (g/dl) | 2.17 ± 0.4** | 1.3 ± 0.2 | 2.1 ± 0.3* | 1.4 ± 0.3 | 2.1 ± 0.1* | 1.7 ± 0.3 |
| Globulin (g/dl) | 1.9 ± 0.3 | 1.4 ± 0.4 | 2.3 ± 0.7 | 1.7 ± 0.6 | 2.1 ± 0.8 | 1.8 ± 0.4 |
| A/G Ratio | 1.2 ± 0.3 | 0.9 ± 0.1 | 1.0 ± 0.4 | 0.9 ± 0.2 | 1.1 ± 0.3 | 1.0 ± 0.2 |
| Calcium (mg/dl) | 14.0 ± 2.9 | 14.4 ± 5.2 | 16.6 ± 6.0 | 15.8 ± 5.7 | 19.6 ± 11.9 | 15.4 ± 6.2 |
| Phosphorus (mg/dl) | 5.0 ± 0.8 | 5.3 ± 1.4 | 6.2 ± 2.2 | 4.9 ± 1.0 | 7.9 ± 5.2 | 6.3 ± 2.6 |
| Glucose (mg/dl) | 208.2 ± 28.7 | 336 ± 257 | 185 ± 20.8 | 204 ± 48.4 | 219 ± 45.8 | 201 ± 23.6 |
| Alkaline Phosphatase (U/l) | 1137 ± 2099 | 1210 ± 1821 | 230 ± 239 | 508 ± 625 | 274 ± 402 | 248 ± 251 |
| Creatine Kinase (U/l) | 503 ± 397 | 610 ± 285 | 301 ± 109 | 437 ± 76.4 | 407 ± 113 | 284 ± 105 |
| Aspartate Aminotransferase (U/l) | 36.2 ± 13.2 | 59.5 ± 38.0 | 27.2 ± 14.9 | 43.2 ± 22.8 | 61.0 ± 46.4 | 34.0 ± 15.3 |

Continued

Table C.6. Continued.

| Parameter (units) | Parameter Mean ± SD | | | | | |
|---|---|---|---|---|---|---|
| | Day 1 Birds | | Day 3 Birds | | Day 7 Birds | |
| | Control (n = 4) | SAMW (n = 4) | Control (n =4 ) | SAMW (n = 4) | Control (n = 5) | SAMW (n = 5) |
| Uric Acid (mg/dl) | 2.5 ± 0.6 | 8.8 ± 5.9 | 2.7 ± 0.5 | 3.9 ± 2.1 | 4.0 ± 1.8 | 2.6 ± 1.2 |
| Cholesterol (mg/dl) | 292 ± 95.2 | 167 ± 66.4 | 337 ± 175 | 186 ± 100 | 208 ± 92.8 | 208 ± 86.0 |
| Sodium (meq/l) | 159 ± 6.6 | 143 ± 18.6 | 160 ± 1.9 | 153 ± 2.7 | 154 ± 9.2 | 157 ± 4.9 |
| Potassium (meq/l) | 4.4 ± 0.4 | 8.8 ± 7.4 | 4.4 ± 1.0 | 4.8 ± 1.2 | 7.4 ± 6.2 | 5.3 ± 1.9 |
| Chloride (meq/l) | 111 ± 3.9 | 102 ± 8.5 | 111 ± 2.8 | 108 ± 8.4 | 109 ± 3.8 | 111 ± 1.7 |

* Mean is significantly different than paired mean by t-test ($p < 0.05$).
** Mean is significantly different than paired mean by t-test ($p < 0.01$).

Table C.7. Occurrence of histopathological changes in gastrointestinal tract tissues following 1, 3, and 7 days post-treatment with SAMW treatment in mallards.

| Pathology | Mortalities | Day 1 | Day 3 | Day 7 |
|---|---|---|---|---|
| Esophagus | 2/2 | 0/4 | 0/4 | 0/5 |
| Ventriculus | 1/2 | 4/4 | 2/4 | 0/5 |
| Proventriculus | 2/2 | 2/4 | 1/4 | 0/5 |

## Study D: Dose-Response Acute Toxicity of Synthetic Acid Mine Water to Mallards

### Introduction

Study A demonstrated acute toxicity of a synthetic AMW representative of a high level of AMW contamination. Other tailings ponds and pregnant leach solutions associated with the Tyrone, Morenci, and Chino mines contain lower levels of AMW contamination (Stratus, 2003). The goals of study D were to 1) compare the acute toxicity of SAMW with SAMWs that are representative of lower levels of AMW contamination 2) provide a first step towards the determination of dose-response relationship and 3) determine if findings from this dose-response study are consistent with other reports from SAMW and AMW acute toxicity studies.

### Methods

*Treatment Water Source and Preparation*

SAMW treatment group mallards were dosed with same SAMW that was used in Study A. The pH of SAMW was 2.00. Twenty liters of 1/10 SAMW and 1/100 SAMW were formulated by sequentially diluting the stock SAMW solution 10-fold and 10-fold again with deionized water, producing $1/10^{th}$ and $1/100^{th}$ strength nominal SAMW solutions, respectively (Table D.1). Each step in the dilution was accompanied by 2 hr of mixing prior to removal of the stock SAMW for use in the dilution. Following dilution, the pH of the 1/10 SAMW solution was 2.70 and the 1/100 SAMW was 3.28. Eighteen mL of 18M nitric acid was added to the 1/10 SAMW to adjust the pH to 2.02. Thirteen mL of 18M nitric acid was added to the 1/100 SAMW solution to adjust the pH to 2.04. The pHs of tap water, 1/100 SAMW, 1/10 SAMW, and SAMW dosing solutions ranged from 8.18 to 8.31, 2.10 to 2.11, 2.10 to 2.11, and 2.06 to 2.08, respectively, during the study.

*Study Design*

Thirty-two 8-week old mallards (16 males, 16 females) were obtained from a commercial gamebird grower (see Study A), raised at The Institute of Environmental and Human Health avian facility, and later transported to the Texas Tech University Animal Care Resources Center when the birds had reached approximately 20 to 22 weeks of age. Mallards were

approximately 22 - 24 weeks of age at the time of dosing. Birds were banded and maintained individually in 2.5 $ft^3$ stainless steel rabbit cages at 20°C, 40-70% relative humidity, and 12h:12h light:dark photoperiod. All mallards were allowed at least two weeks to acclimate to indoor, caged conditions prior to initiation of testing, with their body weight near or exceeding that recorded upon receipt into the animal facility. Ducks had *ad libitum* access to feed (Mazuri waterfowl maintenance diet in pellet form; PMI Nutritional, LLC, Brentwood, MO, USA) and tap water during the acclimation period. All animal care was performed in accordance with the Texas Tech University ACRC and the Institutional Animal Care and Use Committee.

The study was performed over an 11-day period, with two groups of sixteen (equal sex per trial) tested at a time. All mallards were fasted and dehydrated for a 24-hr period prior to dosing. During each half of the study, four groups of four mallards were provided *ad libitum* access to SAMW, 1/10 SAMW, 1/100 SAMW, or tap water for a maximum of 48 hours. Mallards were not allowed access to food for the duration of the study. Body mass measurements were collected for each mallard before the initiation of the dehydration and fasting period, at the initiation of dosing, at 24 hrs post-dose for survivors, and at the time of euthanasia. Body mass was collected with an electronic balance to the nearest 0.1 g.

Dosing of each mallard was staggered at 2-min intervals to allow for changing of water bottle reservoirs and waste-collection bottles. Consumption of different treatment water sources was not adjusted for the density of the solutions and is reported in g of water/kg body mass. Animals were observed continuously throughout the exposure period while behavioral observations and water consumption data were collected. Water consumption measurement methods were the same as those used in Study A.

*Animal Euthanasia and Sample Collection*

Birds were determined to be in moribund condition by visual signs of wing droop, immobility, lack of response to touch/visual/auditory stimuli, and/or inability to hold head erectly. All treatment ducks were observed until they were *in extremis*, at which time were weighed and then euthanized via carbon dioxide asphyxiation. Control birds were euthanized as close as possible to the time treatment animals died. Due to the moribund

condition and reduced blood flow of treatment ducks, we were unable to collect blood samples via jugular or brachial venipuncture and instead collected blood using cardiac puncture. Whole blood was collected with 1 mL syringes and placed in 5 mL serum separator tubes for serum clinical chemistry, 2 ml microcentrifuge tubes for metal residues, and microhematocrit tubes for determination of erythrocyte PCV. An additional 1 mL heparinized blood sample was collected from mallards from the second half of the study for the purpose of complete blood counts. Blood smears were performed during sample collections. Whole blood in serum separator tubes was allowed to clot at room temperature for 30 minutes and then centrifuged at 6,000 rpm for 10 minutes. Serum was decanted into 2-ml microcentrifuge tubes and frozen at -80°C until being shipped for analysis. Serum volumes from 6 of 7 SAMW samples, 2 of 8 1/10 SAMW samples, and 1 of 8 1/100 SAMW samples were not sufficient for clinical chemistry analysis and were diluted two-fold with 18 mega-ohm deionized water. All other samples were submitted undiluted. Serum samples were analyzed with a Hitachi 911 Analyzer at the TVMDL for total serum protein, albumin, globulins, calcium, phosphorus, glucose, CK, AST, uric acid, cholesterol, ALP, hemoglobin, and electrolytes. Serum clinical chemistry data were corrected for dilutions. Heparinized whole blood samples with blood smears were shipped on ice for determination of complete blood counts.

Gross pathological lesions were documented, and tissues were collected for histopathological analyses for all birds. Sections of right lung, testes or ovaries, right kidney, liver, spleen, pancreas, brain, tongue, esophagus, trachea, heart, proventriculus, ventriculus, duodenum, jejunum, ileum, ceca, and large intestine were fixed in 10% buffered formalin and stored in jars until processed. Histopathology samples have been stored for possible analysis at a future date.

*Statistical Methods*

Measures of central tendency were expressed as the mean ± standard deviation. All data analyzed using parametric methods were tested for normality and homogeneity of variances. When the assumptions of parametric tests were not met, data were reanalyzed after log transformation or analyzed using non-parametric methods. Differences in total and first hour dose among groups were analyzed using analysis of variance or Kruskal-Wallis Tests. Body

mass and hematology parameters were analyzed using analysis of variance or Kruskal-Wallis tests. Not all clinical chemistry parameters could be analyzed using either analysis of variance or Kruskal-Wallis tests; therefore, these parameters were analyzed using both tests. Because analysis of variance is generally a robust test for sample testing among means and tests for homogeneity of variance are generally poor (Zar, 1998) only the assumption of normality was applied to the clinical chemistry dataset. If the assumption of normality was met, results from the analysis of variance test were used in place of Kruskal-Wallis test results. All statistical analyses were performed with R (version 2.2.0; R Foundation, Vienna, Austria). Results of statistical tests were considered to be significant at $p < 0.05$.

## Results

### *First Hour and Total Water Consumption*

There were no significant differences in first hour water consumption among treatment groups ($p = 0.17$; Table D.2). Two mallards in the SAMW treatment group consumed greater than 50 mL/kg in the first hour. Total water consumption increased significantly as metal content decreased (Table D.2) and was significantly different ($p < 0.001$) between all pairs of treatment groups except for 1/10 SAMW and 1/100 SAMW treatment groups.

### *Signs of Toxicity*

Signs of toxicity in SAMW and 1/10 SAMW treatment group mallards were similar to those reported in study A. SAMW-treated mallards were euthanized *in extremis* 241 ± 137 minutes following initiation of dosing. Two 1/10 SAMW-treated mallards were euthanized *in extremis* 226 and 328 minutes following initiation of dosing. Survivor mallards from the 1/10 SAMW treatment group were lethargic, consumed very little water, and were occasionally heard wheezing while breathing on the second day of dosing. Mallards from the 1/100 SAMW treatment group initially responded to the treated water with vigorous lateral head shaking and regurgitation, however similar signs of toxicity were observed less on the second day of dosing. Despite these signs of toxicity , no 1/100 SAMW-treated mallards died. Signs of toxicity were absent in control mallards.

There were no significant among group differences in body mass for the pre-dose dehydration period (Table D.3). Multiple between group differences in body mass change existed for the other two time periods (Table D.3).

*Clinical Chemistry*

Multiple parameters were significantly affected by SAMW treatments. SAMW and 1/10 SAMW treatment mallards had significantly elevated creatine kinase, aspartate transaminase, and uric acid levels when compared to both control and 1/100 SAMW mallard treatment groups (Table D.4). Alkaline phosphatase levels were significantly elevated in SAMW-treated mallards when compared only to 1/100 SAMW and control group mallards. Phosphorus levels of 1/10 SAMW and SAMW-treated mallards were significantly elevated when compared to 1/100 SAMW-treated mallards only, and potassium levels of SAMW-treated mallards were significantly elevated when compared to 1/100 SAMW-treated mallards. SAMW-treated mallard serum glucose levels were significantly lower compared to 1/10 SAMW-treated mallard levels.

*Hematology*

Percent heterophil counts in SAMW and 1/10 SAMW treatment mallards were significantly elevated when compared to both control and 1/100 SAMW treatment groups ($p < 0.001$; Table D.5). Conversely, percent lymphocyte counts in SAMW and 1/10 SAMW treatment mallards were significantly reduced when compared to control and 1/100 SAMW treatment groups ($p < 0.001$; Table D.5). Absolute lymphocyte counts were also reduced in SAMW and 1/10 SAMW treatment groups when compared to the 1/100 SAMW group ($p < 0.001$; Table D.5). Mean cell volumes of 1/10 SAMW mallards were significantly elevated when compared to 1/100 SAMW mallards ($p < 0.05$; Table D.5). Blood samples from SAMW mallards were too hemolyzed to determine mean cell volumes. Mean hematocrit values were highest in SAMW treatment mallards and decreased as dilution of metal content increased.

*Gross Pathology*

Common grossly observable abnormalities in mallards that died in the SAMW and 1/10 SAMW treatment groups were consistent with those identified in Study A. Gross lesions in survivors from the 1/10 SAMW treatment group were similar and sometimes appeared more severe than those that belonged to their lethally-treated counterparts. Additionally, surviving mallards from the 1/10 SAMW group sustained lesions on the ventral surfaces of the tongue and upper and lower mandibles. There were no treatment-related gross lesions observed in the 1/100 AMW or control groups.

Examples of macroscopic changes in internal tissues are provided in the appendix.

Discussion

Behavioral, clinical, and pathological responses of SAMW and 1/10 SAMW-treated mallards were similar to those observed from mallards acutely poisoned with SAMW in study A. Three of eight mallards in the SAMW treatment group consumed a dose in the first hour that is above the lowest lethal dose (31.0 mL/kg). Such a finding suggests that birds have the potential to obtain a lethal dose in a short period of time. The surviving six of eight survivors from the 1/10 SAMW treatment group sustained severe gastrointestinal injuries and alterations of some clinical chemistry endpoints that are indicative of liver, kidney, and/or muscle damage or dysfunction. Gastrointestinal lesions can induce anorexia in birds, as seen in Study C, and can have potentially lethal implications for birds with high bioenergetic demands or that are experiencing physiologically stressful circumstances.

One confounding factor in Studies A, C, and D, is the contribution of the acidic nature of the solution to the overall toxicity of SAMW and AMW. Acidic water containing low levels of hazardous metals, such as the 1/100 SAMW tested in this study, appear to pose less of a hazard to birds. However, ducklings exposed to drinking water at pH 3.0 without any added metals experience 67% mortality over a 5-day exposure period (Foster, 1999). Acid-only solutions may produce age-dependent toxicity in avian species, however data are limited.

The observed pathologies suggest that AMWs with character similar to the 1/10 SAMW pose a significant health risk to wild birds that ingest them. These findings are consistent with the literature pertaining to the acute toxicity of lower levels of AMW contamination or copper supplementation (ENSR, 1996; Stubblefield *et al*., 1997; Jensen *et al*., 1991).

Table D.1. Concentrations of metals in the SAMW solution analyzed by flame/furnace atomic absorption spectroscopy and inductively-coupled plasma atomic emission spectroscopy and estimated concentrations of metals in 10-fold and 100-fold SAMW dilutions.

| Element | Stock SAMW (mg/L at pH = 2) | 1/10 SAMW (mg/L at pH = 2) (nominal concentrations) | 1/100 SAMW (mg/L at pH = 2) (nominal concentrations) |
|---|---|---|---|
| Cu | 5943 | 594 | 59.4 |
| Al | 3718 | 372 | 37.2 |
| Zn | 2071 | 207 | 20.7 |
| Mg | 1596 | 160 | 16.0 |
| Fe | 1351 | 135 | 13.5 |
| Mn | 746 | 74.6 | 7.46 |
| Ca | 493 | 49.3 | 4.93 |
| Cd | 22.2 | 2.22 | 0.22 |
| Co | 21.8 | 2.18 | 0.22 |
| Na | 17.3 | 1.73 | 0.17 |
| Ni | 10.8 | 1.08 | 0.11 |
| Cr | 4.8 | 0.48 | 0.05 |
| Se | 0.639 | 0.06 | 0.01 |
| V | 0.352 | 0.04 | 0.004 |
| As | 0.344 | 0.03 | 0.003 |

Table D.2. Mean ± SD and ranges of first hour and total water consumption (g/kg) and associated percent mortality and time to death for tap water, 1/100 SAMW, 1/10 SAMW, and SAMW treatment groups. N = 8 for all treatment groups. Treatment groups with different letters differed significantly. NA is not applicable.

| Treatment | Water Consumption | | % Mortality | Time To Death (mean ± SD) |
|---|---|---|---|---|
| | First Hr (g/kg) (mean ± SD) [range] | Total (g/kg) (mean ± SD) [range] | | |
| Tap Water | 53.9 ± 32.8 [25.9 – 123.6] | 689 ± 225 [a] [344.7 – 1028.6] | 0 | NA |
| 1/100 SAMW | 31.7 ± 17.6 [2.3 – 53.2] | 269 ± 112 [b] [105.6 – 425.4] | 0 | NA |
| 1/10 SAMW | 42.2 ± 22.0 [10.5 – 71.0] | 190 ± 59.9 [b] [77.0 – 279.5] | 25 | 277 (n = 2) |
| SAMW | 28.9 ± 21.5 [2.6 – 59.6] | 69.1 ± 21.4 [c] [39.3 – 100.7] | 100 | 241 ± 137 (n = 8) |

Table D.3. Mean ± SD body mass changes of control, SAMW, 1/10 SAMW, and 1/100 SAMW treatment group mallards in study D. N = 8 for each group. All SAMW treatment mallards and two 1/10 SAMW treatment mallards died on the day of dosing. All other mallards survived the entire study duration of 48 hrs. Treatment groups with different letters differed significantly.

| | Percent Body Mass Change [Mean ± SD] | | | |
|---|---|---|---|---|
| Time Period | Control | 1/100 SAMW | 1/10 SAMW | SAMW |
| Hydrated Condition To Initiation of Dosing (After 24-Hr Dehydration - -24 hr to 0 hr) | -6.2 ± 0.8 | -6.4 ± 1.5 | -5.7 ± 0.6 | -6.6 ± 1.3 |
| Initiation Of Dosing To Death | -1.9 ± 0.9 [a] | -3.3 ± 1.1 [ab] | -9.7 ± 3.9 [c] | -5.6 ± 1.8 [bc] |
| Hydrated Condition To Death | -7.9 ± 1.4 [a] | -9.5 ± 1.5 [ab] | -14.9 ± 3.9 [c] | -11.9 ± 2.3 [bc] |

Table D.4. Serum clinical biochemistry (mean ± SD) results from SAMW, 1/10 SAMW, 1/100 SAMW, and tap water treatment group mallards. N = 8 for all treatment groups except the SAMW group, where N = 7. Treatment groups with different letters differed significantly.

| | Parameter Mean ± SD | | | |
|---|---|---|---|---|
| Serum Parameter | Tap Water | 1/100 SAMW | 1/10 SAMW | SAMW |
| Total Serum Protein (g/dl) | 4.31 ± 0.524 | 3.94 ± 0.38 | 4.14 ± 0.81 | 4.12 ± 0.89 |
| Albumin (g/dl) | 2.17 ± 0.25 | 2.03 ± 0.25 | 1.96 ± 0.37 | 1.94 ± 0.50 |
| Globulin (g/dl) | 2.2 ± 0.4 | 1.9 ± 0.2 | 2.2 ± 0.5 | 2.2 ± 0.5 |
| A/G Ratio | 1.04 ± 0.22 [a] | 1.20 ± 0.41 [ab] | 1.12 ± 0.35 [a] | 1.64 ± 0.37 [b] |
| Calcium (mg/dl) | 13.1 ± 0.99 | 12.3 ± 0.37 | 14.3 ± 2.44 | 18.3 ± 10.9 |
| Phosphorus (mg/dl) | 5.88 ± 1.65[a] | 4.28 ± 0.66[ab] | 8.32 ± 2.96[ac] | 8.17 ± 4.24[ac] |
| Glucose (mg/dl) | 236 ± 48.9[a] | 195 ± 10.7[a] | 338 ± 141[ab] | 193 ± 100[ac] |
| Alkaline Phosphatase (U/l) | 107 ± 55.0 [a] | 132 ± 80.7 [a] | 155 ± 95.5 [ab] | 252 ± 107 [b] |
| Creatine Kinase (U/l) | 405 ± 262 [a] | 468 ± 236 [a] | 1280 ± 1009 [b] | 1058 ± 362 [b] |
| Aspartate Aminotransferase (U/l) | 45 ± 25 [a] | 34 ± 17 [a] | 146 ± 98 [b] | 220 ± 87 [b] |
| Uric Acid (mg/dl) | 4.76 ± 2.17 [a] | 3.67 ± 1.68 [a] | 27.3 ± 17.8 [b] | 54.4 ± 27.2 [c] |
| Cholesterol (mg/dl) | 409 ± 161 | 309 ± 50.1 | 334 ± 52.9 | 353 ± 113 |
| Sodium (meq/l) | 157 ± 4.4 | 160 ± 6.0 | 154 ± 13.7 | 166 ± 3.9 |
| Potassium (meq/l) | 5.17 ± 2.24[a] | 3.73 ± 0.42[ab] | 6.30 ± 2.60[a] | 6.64 ± 1.73[ac] |
| Na/K ratio | 35.0 ± 12.4 | 48.9 ± 17.7 | 33.2 ± 11.3 | 49.5 ± 18.6 |
| Chloride (meq/l) | 108 ± 2.0 | 110 ± 5.0 | 105 ± 12.9 | 112 ± 7.0 |
| Hemoglobin | 15 ± 5 | 13 ± 9 | 165 ± 262 | 18 ± 16 |

Table D.5. Mean ± SD complete blood count values from control, 1/100 SAMW, 1/10 SAMW, and SAMW treatment groups. N = 4 for all groups except hematocrit where N = 8. Eosinophils and basophils were not detected for SAMW treatment group complete blood counts. Treatment groups with different letters differed significantly.

| Treatment Group | Total WBCs (per uL) | Differential White Blood Cell Count (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Heterophils | Lymphocytes | Monocytes | Eosinophils | Basophils | Polychro-masia (RBCs) |
| Control | 27225 ± 5463 | 42 ± 11 [a] | 55 ± 13 [a] | 1.0 ± 0.8 | 0.8 ± 1.5 | 1.0 ± 2.0 | 0/4 |
| 1/100 SAMW | 15700 ± 4188 | 42 ± 3 [a] | 52 ± 4 [a] | 0.5 ± 0.6 | 2.3 ± 2.1 | 3.3 ± 2.1 | 0/4 |
| 1/10 SAMW | 33238 ± 18834 | 86 ± 3 [b] | 12 ± 5 [b] | 1.8 ± 1.5 | 0.3 ± 0.5 | 0.3 ± 0.5 | 2/4 |
| SAMW | 20825 ± 15934 | 90 ± 7 [b] | 10 ± 7 [b] | 1.0 ± 2.0 | 0 | 0 | 0/4 |

Table D.5. Continued.

| Treatment Group | Differential White Blood Cell Count (Absolute No. / uL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Heterophils | Lymphocytes | Monocytes | Eosinophils | Basophils | RBCs (x $10^6$/uL) | Hematocrit (%) | Mean Cell Volume |
| Control | 11026 ± 1394 | 15544 ± 6535 [a] | 280 ± 206 | 161 ± 321 | 214 ± 428 | 3.01 ± 0.53 | 45 ± 1 [a] | 151 ± 22.7 [ab] |
| 1/100 SAMW | 6626 ± 2257 | 8123 ± 1710 [ab] | 74 ± 85 | 392 ± 404 | 485 ± 251 | 3.90 ± 0.60 | 47 ± 4 [a] | 119 ± 19.3 [a] |
| 1/10 SAMW | 28009 ± 14943 | 4598 ± 4040 [b] | 468 ± 412 | 82 ± 163 | 82 ± 164 | 2.30 ± 1.09 | 58 ± 10 [b] | 184± 28.6 *[b] |
| SAMW | 19355 ± 16114 | 1242 ± 908 [b] | 228 ± 456 | 0 | 0 | 2.79 ± 1.33 | 67 ± 4 [c] | na |

* N = 3

## Conclusion

Based on the findings from our studies and findings from other reports, we conclude that acid metalliferous water bodies pose a significant hazard to wildlife that come in contact with them. Birds that are not averse to AMW have a potential increased risk of injury following oral exposure, and possibly dermal/feather or ocular exposure as well. Thousands of migrating birds are likely to be injured every year in the western U.S. due to exposure to AMW; however limited monitoring effort prevents the ability to accurately assess the severity of this issue. We have presented data concerning the acute toxicity of multiple SAMWs that likely reflect worst-case, yet realistic exposure scenarios that correspond to actual conditions measured at the Phelps Dodge mine sites. While it appears that more typical concentrations of hazardous metals may be on the order of 2-10 times lower than those investigated in study A (Stratus, 2003), ponds of relatively lower levels of metal contamination, such as those examined in study D, may still pose a significant hazard to wildlife (ENSR, 1996; Stratus, 2003).

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