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Toxicity and Hazard of Vanadium to Mallard Ducks (*Anas platyrhynchos)* and Canada Geese (*Branta canadensis*)

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Running Head: Vanadium toxicity to mallards and geese

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ABSTRACT

A recent Canada goose (*Branta canadensis*) die-off at a petroleum refinery fly ash pond in Delaware, USA was attributed to vanadium (V) toxicity. Because of the paucity of V toxicity data for wild birds, a series of studies was undertaken using the forms of V believed to have resulted in this incident. In 7-day single oral dose trials with mallard drakes (*Anas platyrhynchos*), the estimated median lethal dose (LD50) for vanadium pentoxide was 113 mg/kg body weight, while the LD50 for sodium metavanadate was 75.5 mg/kg. Sodium metavanadate was found to be even more potent (LD50 = 37.2 mg/kg) in male Canada geese. The most distinctive histopathological lesion of both forms of V was lympho-granulocytic enteritis with hemorrhage into the intestinal lumen. Vanadium accumulation in liver and kidney was proportional to the administered dose, and predictive analyses based on these data suggest that V concentrations of 10 μg/g dw in liver and 25 μg/g dw in kidney are associated with mortality (>90% confidence that exposure >LD50) in mallards acutely exposed to sodium metavanadate. Chronic exposure to increasing dietary concentrations of sodium metavanadate (38.5 to 2651 ppm) over 67 days resulted in V accumulation in liver and kidney (25.2 and 13.6 μg/g dw, respectively), mild intestinal hemorrhage, blood chemistry changes and evidence of hepatic oxidative stress in mallards, although some of these responses may have been confounded by food avoidance and weight loss. Dietary exposure of mallards to 250 ppm sodium metavanadate for 4 weeks resulted in modest accumulation of V in liver and kidney (<5 μg/g dw) and mild intestinal hemorrhage. Based upon these data and other observations, it is unlikely that chronic low-level dietary exposure to V poses a direct lethal hazard to wildlife. However, point sources, such as the V laden fly ash pond encountered by geese at the petroleum refinery in Delaware, may pose a significant hazard to waterbirds.

INTRODUCTION

In January 2003, a report of dead and dying Canada geese (*Branta canadensis*) at a petroleum refinery fly ash pond was received by the Delaware Department of Natural Resources and Environmental Control. The incident involved at least 55 geese (45 carcasses and 10 apparently debilitated individuals), and occurred during a period of very cold weather (-10 to 0°C average daily temperature) when most fresh water sources were frozen. Geese were apparently attracted to the 4-hectare fly ash pond, parts of which were not frozen. The pond had been used to settle fly ash containing vanadium pentoxide from the refinery's power plant for about 20 years. Once vanadium pentoxide entered the pond, it was probably converted to vanadium oxides including metavanadate, tetravanadate and pyrovanadate sodium salts (Crans et al., 1998). The pond was slated for closure by dewatering and capping, but this was delayed by heavy rain in 2002 and 2003 that filled it to an estimated volume of 11 million liters. At the time of the die-off, high concentrations of vanadium (V; 467,000 μg/L), nickel (16,100 μg/L) and thallium (1,190 μg/L) were present in water samples from the pond (K.A. Knowles, Delaware Department of Natural Resources and Environmental Control, personal communication), while other metals (arsenic, cadmium, chromium, lead, mercury and silver) were below National Recommended Water Quality Criteria for aquatic life (U.S. Environmental Protection Agency, 1999). The pH of pond water samples was 4.5.

Staff of the U.S. Geological Survey National Wildlife Health Center (NWHC) (L. Sileo & G.S. McLaughlin, Case 18510, personal communication) and the U.S. Fish and Wildlife Service National Fish and Wildlife Forensics Laboratory (NFWFL) (R.K. Stroud, Lab Case 02-000236, personal communication) examined 6 of the dead geese. No pathogenic bacteria or viruses were isolated from tissue samples, and there was no evidence of organophosphorus or

carbamate pesticide poisoning based on brain cholinesterase activity. Necropsies revealed that the geese were in good flesh with abundant body fat reserves, and there were no lesions suggesting trauma. Lung, liver and kidney tissue were congested, and parts of the intestine were inflamed and necrotic, and contained mucoid material and blood. A composite liver sample from 2 geese contained 57.3 μg/g dw of V and composite kidney sample from 2 geese contained 226 μg/g dw of V, while values of arsenic, cadmium, chromium, lead, mercury, nickel, selenium and thallium were low, generally <0.5 μg/g dw (K.A. Knowles, Delaware Department of Natural Resources and Environmental Control, personal communication). Subsequently, liver samples of geese necropsied at the NWHC were analyzed for V and contained 87 to 200 μg/g dw. Intestinal lesions and accompanying dehydration associated with V toxicity was reported to be the suspected cause of death (NWHC Case 18510 and NFWFL Case 02-000236). Another die-off involving 60 Canada geese occurred at this same site in December of 2004 (D.E. Collins, U.S. Fish and Wildlife Service, Division of Law Enforcement, personal communication).

V toxicity has been observed in occupationally exposed workers that maintain oil-fired boilers or were involved in the manufacture of high-strength steel alloys. Such exposure occurs through inhalation and adverse effects are often confined to the respiratory system (ATSDR, 1992; Ghio et al., 2002). *In vitro* and *in vivo* studies in laboratory mammals indicate that V is a potent inhibitor of Na^+,K^+-ATPase, alters cardiovascular function and induces oxidative stress with lipid peroxidation (Harvey & Klaassen, 1983; Younes & Strubelt, 1991; Byczkowski & Kulkarni, 1998; Thompson et al., 1998). Results of acute oral and chronic dietary studies in rodents demonstrated that pentavalent forms of V are the most potent, falling in the moderate to highly toxic range (Llobet & Domingo, 1984; Toxicological Research Lab Dukou Sanitary Antiepidemic Station, 1987), and are the forms most readily bioaccumulated by

liver, kidney and bone (Parker & Sharma, 1978). Some forms of V appear to be genotoxic and produce adverse reproductive and developmental effects (Thompson et al., 1998), but V does not appear to be carcinogenic (ATSDR, 1992). Limited data are available on V toxicity in birds, making interpretation of the goose die-off difficult. In newly hatched chickens (*Gallus domesticus*), a 4-wk dietary exposure to 100 ppm ammonia metavanadate impaired growth, while 200 ppm resulted in 20% mortality (Hafez & Kratzer, 1976). As little as 40 ppm ammonia metavanadate reduces egg production in hens (Ousterhout & Berg, 1981). Vanadium pentoxide fell in the moderately toxic range in a standardized 5-day feeding trial in Japanese quail chicks (*Coturnix japonica*) (Hill & Camardese, 1986). In a 12-wk feeding trial in mallards (*Anas platyrhynchos*), 100 ppm of vanadyl sulfate yielded no signs of toxicity and only modest concentrations of V were present in liver, kidney and bone (<5 μg/g dw) (White & Dieter, 1978), which presumably reflects the lower potency and uptake of this tetravalent form.

In an effort to more reliably interpret the observations and tissue V concentrations from the goose die-off in Delaware, a series of studies was undertaken to determine the median lethal dose, chronic toxicity, signs of intoxication and pathology produced by the forms of V presumed to be present in the fly ash pond. In addition, predictive analyses were conducted to estimate the diagnostic concentrations of V in tissues associated with mortality. These studies were conducted in mallards, a model species used extensively in wildlife toxicology, and in Canada geese, the species affected in the Delaware event.

MATERIALS AND METHODS

Animals

Adult game farm mallard drakes were commercially obtained (Oak Ridge Game Farm, Gravette, AK, and later from Y-Farms, Zachary, LA that purchased the entire Oak Ridge Game Farm flock), weighed and banded upon receipt. Drakes were housed individually in outdoor covered 1 x 1 m vinyl coated wire pens, raised above a concrete slab. Adult male Canada geese (Stromberg's Chick & Game Birds Unlimited, Pine River, MN) were weighed, banded and housed individually in unheated 1.5 x 3 m pens in a partially enclosed building on a concrete slab. Each pen had a feed container and fresh water. Both ducks and geese had *ad libitum* access to feed (Mazuri Waterfowl Maintenance Diet in pellet or mash form with 14% protein; PMI Nutritional, LLC, Brentwood, MO) and were maintained in a natural photoperiod. Birds were housed for at least 2-wk prior to the initiation of each study, with their body weight near or exceeding that recorded upon receipt. During the course of each study, birds that succumbed were necropsied within hours. At the termination of each study, surviving birds were individually euthanized using carbon dioxide and necropsied within 15 minutes.

Acute Oral Toxicity Trials

In January and February of 2004, mallards were weighed and fasted overnight, and then dosed with gelatin capsules containing vanadium pentoxide (V_2O_5; CAS # 1314-62-1, 99.99% purity; Sigma-Aldrich Corp., St. Louis, MO) or sodium metavanadate ($NaVO_3$; CAS # 13718-26-8, >98% purity, Sigma-Aldrich Corp.) to the level of the proventriculus using a pilling device (JorVet J-384, Jorgensen Laboratories, Inc.,

Loveland, CO). The number of test animals/dose and arrangement of dosage levels were selected based on available data for rodents (Llobet & Domingo, 1984; Toxicological Research Lab Dukou Sanitary Antiepidemic Station, 1987), small range finding trials and humane efforts to minimize the number of test subjects. Drakes (n = 4/dose) received 10, 18, 34, 62, 113, 208, 382 or 700 mg/kg body weight (BW) of vanadium pentoxide or sodium metavanadate, or empty capsules (sham-dosed controls), and were observed for 7-days.

In March of 2003, a smaller toxicity trial was undertaken in male Canada geese. Geese were weighed and fasted overnight, and then administered gelatin capsules containing sodium metavanadate (18 mg/kg BW, n = 1; 38 mg/kg, n = 2; 76 mg/kg , n = 6; 151 mg/kg, n = 2) or empty capsules (n = 3 controls). Ganders were observed for 7-days.

Chronic Dietary Feeding Trials

In order to examine the potential hazard of chronic V exposure to waterfowl, two feeding studies were undertaken using mallard drakes. Various quantities of sodium metavanadate were weighed and mixed into feed in mash form and provided *ad libitum*. In the first trial (March to May 2004), control drakes (n = 6) received waterfowl maintenance diet and treated drakes (n = 12) received mash containing sodium metavanadate. The initial concentration of sodium metavanadate was 38.5 ppm and its concentration was increased by a factor of 1.6 each wk for 10 weeks (61.7, 98.8, 158, 252, 405, 647, 1036, 1657 and 2651 ppm). A second trial was undertaken in the summer of 2004. Mallards were at various stages of completing their eclipse molt, so they were

assigned to groups in a stratified manner in an attempt to account for molt stage. Six drakes were provided control mash and 14 drakes received 250 ppm sodium metavanadate in mash for 4 weeks. Body weight and food consumption/kg BW (actually mash removal from the feeder) were monitored during these trials.

Blood Chemistry and Measures of Oxidative Stress

At the end of chronic exposure studies, a 5-ml jugular or brachial venipuncture sample was collected from each drake for comprehensive avian blood chemistry analysis (Antech Diagnostics, Lake Success, NY). Hematocrit and differential cell counts were conducted using whole blood, and concentrations of total protein, albumin, globulin, glucose, cholesterol, uric acid and electrolytes and activities of aspartate aminotransferase (AST; EC 2.6.1.1) and creatine phosphokinase (EC 2.7.3.2) were determined in serum.

A 1-g portion of each liver or kidney was homogenized (1:10 w/v) in ice-cold 1.15% KCl-0.01 M sodium/potassium phosphate buffer (pH 7.4) containing 0.02 M EDTA. The 10,000 x g supernatant was used to assay enzymes related to antioxidant activity and glutathione metabolism including glutathione peroxidase (C-GSH peroxidase, coupled reaction at 30^{o}C with glutathione reductase using cumene hydroperoxide as substrate and H-GSH peroxidase, using hydrogen peroxide as substrate; EC 1.11.1.9), glutathione reductase (GSSG reductase, EC 1.6.4.2), glutathione-S-transferase (GSH S-transferase; EC 2.5.1.18) and glucose-6-phosphate dehydrogenase (G-6-PDH; EC 1.1.1.49) using a centrifugal analyzer as previously described (Hoffman & Heinz, 1998).

Glutathione and other thiol status measurements included reduced glutathione

(GSH), oxidized glutathione (GSSG), total sulfhydryls (total SH) and protein-bound sulfhydryls (PBSH; calculated as the difference between total SH and GSH). In addition, thiobarbituric acid-reactive substances (TBARS) were measured as an estimate of hepatic lipid peroxidation (Hoffman & Heinz, 1998).

Histopathology

Formalin-fixed tissues (heart, lung, liver, kidney, intestine, spleen and adrenal) were processed commercially (American Histolabs, Inc., Gaithersburg, MD) using standard methods (Luna, 1968). Hematoxylin and eosin stained sections were examined for structural alterations and lesions by light microscopy.

Analytical Methods

Vanadium analyses were conducted at the Environmental Laboratory of the State of Delaware. Frozen tissue samples were lyophilized for 48-hr, stored desiccated at room temperature and subsequently pulverized in an acid-washed mortar and pestle. Aliquots (0.5 g) were removed for digestion and up to 1 g aliquots were removed for determination of moisture content. Sample aliquots were digested utilizing a DigiPREP (SPC Science, Champlain, NY) hot block and refluxed at 95°C in an acid peroxide solution according to U.S. Environmental Protection Agency Method 3050B (1996). Sample size and reagent volumes were reduced 50% from the method specifications, however, reagent concentrations, temperature conditions and digestion times were used as specified in the method. Following digestion, each sample was filtered under vacuum through a Whatman 42 filter (Florham Park, NJ) and brought to 50 ml final volume.

Digested duck and goose tissue samples were analyzed for V by inductively coupled plasma atomic emission spectrometry according to modified U.S. Environmental Protection Agency Method 200.7 (1983) using a Perkin Elmer Optima 3300DV ICP-AES instrument (Avondale, PA). Instrument calibration was performed using a two point curve; a blank (zero point) plus a standard containing V, copper and zinc each at 1000 ppb. Linearity was verified daily by analyzing 6 additional standards. The daily linear regression correlation coefficient averaged 0.9999. Initial and continuing calibration verification standards were analyzed to verify calibration accuracy. Continuing calibration % recoveries ranged between 93 and 105%. Precision and accuracy of the laboratory analyses were evaluated using quality control samples including method blank, blank spike, laboratory control sample (soil), matrix spike (in duplicate) and certified reference material (National Institute of Standards and Technology Standard Reference Material 1577b bovine liver, Gaithersburg, MD). Quality control samples were analyzed at a rate of 5%, with at least 1 set per analytical run. Vanadium was not detected above the detection limit (0.2 μg/g) in any method blank. Recovery of vanadium in matrix spike samples ranged from 88 to 106%, with a mean of 99%. Relative % difference of matrix spike duplicate samples ranged from 0 to 7%, with a mean of 2.5%. The standard reference material (SRM) was analyzed for copper and zinc due to limited availability of V tissue SRM above the limit of detection. Copper and zinc recoveries in the SRM ranged from 90 to 110% and from 86 to 106%, respectively, with mean recoveries of 100% and 97%, respectively. All concentrations were reported in μg/g on a dw basis. The lower limit of detection for V varied from 0.17 to 0.33 μg/g dw, depending on sample size and moisture content. Sample concentrations were not

adjusted for recovery. Percent moisture values in the dried tissue ranged from 0 to 6%, with a mean of 2%.

Statistical Analyses

Dose-response probability *p* was modeled as a species-specific linear function of the log of the dose level, namely

$$\text{logit}(p) = \log(p/(1-p)) = \alpha_S + \beta_S \log(Dose),$$

for S = mallard or goose. This model corresponds to the assumption that individual subjects have tolerance sampled from a logistic distribution[1], with

$$\Pr(Tolerance \le Dose) = \frac{A_S \; Dose^{\beta_S}}{1 + A_S \; Dose^{\beta_S}} \quad ,$$

where $A_S = \exp(\alpha_S)$; it follows that the median lethal dose (LD50) is $\exp(-\alpha_S/\beta_S)$.

The logarithms of the concentrations of V in liver and kidney of mallards were treated as having linear regressions on the logarithm of the dose, i.e.,

$$\log(V \; in \; liver) = a_L + b_L \log(dose) + \varepsilon_L$$

and

$$\log(V \; in \; kidney) = a_K + b_K \log(dose) + \varepsilon_K \quad ;$$

the error terms ε_L and ε_K were modeled as following a mean zero bivariate normal distribution with correlation ρ. Our analysis allowed for the possibility of correlation between these error terms, corresponding to individual bird effects not explained by the regression on dose.

[1]The logistic distribution (logit transformation) is virtually indistinguishable from the normal distribution (probit transformation) except with very large sample sizes. Given the complexity of the models we subsequently considered and the insensitivity of the inference to the choice, we chose the logit transformation for its superior operating characteristics.

Objective Bayesian analyses were implemented using program *WinBUGS* (Spiegelhalter et al., 1999), reporting posterior medians as point estimates and credible intervals[2] for interval estimates. All Bayesian inference is based on posterior distributions of unknown quantities (Link et al., 2002). One of the benefits of this approach is the ease with which predictions of unobserved quantities can be made, while appropriately accounting for the uncertainties associated with estimation of unknown parameters. In particular, one may regard each bird in the study as having four associated quantities: a dose level, kidney V concentration, liver V concentration and a mortality response. All of these are observed for birds in the present controlled exposure study; for birds encountered in the field, one can only observe the liver V concentration, kidney V concentration and mortality response: the dose level is an unobserved quantity. Under the Bayesian paradigm, the posterior distribution of the dose level was calculated for birds encountered in the field, as a function of the liver and kidney V concentrations and the mortality response, based on the posterior distributions of model parameters from the present controlled exposure study. Thus, it was possible to tabulate predictions of the dose level to which a bird was exposed, given measurements of liver and kidney V concentrations and taking into account whether the bird was recovered alive or dead.

Body and organ weights, V concentrations in tissues and blood chemistry and other biochemical endpoints were tested for homogeneity of variance and transformed when necessary. Differences among treatment groups were compared by analysis of variance (ANOVA). The relation among some endpoints was examined using Pearson product-moment correlation.

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[2] Credible intervals are the Bayesian analog of confidence intervals.

RESULTS

Acute Oral Toxicity Studies

Ambient temperature during mallard trials ranged from –10 to 7°C. Reliable dose-responsiveness was obtained, and the estimated LD50 of both forms of V in mallards fell in the range often classified as moderately toxic (Table 1). Overt signs of intoxication in mallards included head tremor, sustained swallowing behavior, staggering gait and diarrhea. The time course of intoxication ranged from 2- to 48-hr, with nearly all mortality occurring by day 3 of the trials. All mallards, including those that succumbed to V intoxication, were in good to moderate flesh at necropsy. Gross lesions of drakes receiving high doses of vanadium pentoxide included intestinal hemorrhage, sloughing of the intestinal mucosa and areas of ventricular myocardial hemorrhage, while drakes receiving high doses of sodium metavanadate exhibited petechial to ecchymotic hemorrhage on the ventricles, congestion and swelling of the liver and intestinal hemorrhage.

Microscopic examination of tissues from control drakes were for the most part unremarkable, with the exception of foamy hepatocellular cytoplasm and some perivascular cuffing in the liver. All drakes receiving low doses (10-34 mg/kg) of vanadium pentoxide and sodium metavanadate survived and were euthanized at the end of the trial. Most exhibited slight disruption and lack of staining of tips of some villi, focal granulocytic lesions of the heart, foamy hepatocellular cytoplasm and some perivascular cuffing. At moderate doses (62 and 113 mg/kg) encompassing the estimated LD50s of these compounds, several individuals exhibited thickening of the base of villi or loss of villi with associated hemorrhage and severe, acute, lympho-granulocytic enteritis, cardiac petechial hemorrhage, and occasional multifocal necrosis and mild renal congestion. At highest doses (208-700 mg/kg), there was often extensive loss of intestinal

villi including crypts, sloughing of cellular debris into the lumen, accompanied by hemorrhage and a diffuse infiltration of lymphocytes and granulocytes. Heart tissue exhibited petechial hemorrhage and mild congestion was apparent in the kidney and liver. In general, the severity of these histological lesions was dose-dependent (Figure 1), with the appearance of lesions in drakes receiving sodium metavanadate occurring at lower dosages than in drakes receiving vanadium pentoxide.

Based on findings in mallards, the acute oral toxicity of sodium metavanadate was determined in geese. Temperature during this trial ranged from 5 to 17°C. Sodium metavanadate was found to be highly toxic to geese (Table 1), significantly more so ($P = 0.044$) than observed in mallards. The time course of toxicity was similar to that of mallards, but overt signs of intoxication were more pronounced and included sloughing of portions of the intestinal mucosa (found on floor of pen) in 4 of 11 treated geese; 3 of these 4 died with 24 hr. At necropsy, all geese were in good flesh, and those receiving the highest dosages of sodium metavanadate exhibited intestinal hemorrhage.

Histopathological evaluations of geese receiving 38-151 mg/kg of sodium metavanadate exhibited dose-dependent effects ranging from loss of some villi to complete loss of villi, with increasing lymphocyte and granulocyte infiltration. Relative to controls, multifocal necrosis in the liver was noted at the low dose of sodium metavanadate (18 mg/kg), while higher doses (38-151 mg/kg) resulted in liver congestion in all individuals. No lesions were seen in the heart or kidney.

Vanadium Concentrations in Liver and Kidney

Concentrations of V were determined in liver and kidney tissue for a subset of mallards and geese from the acute oral toxicity studies. Samples were selected from sham-dosed control and treated mallards and geese across a range of doses encompassing the estimated LD1 to LD99. Liver and kidney V concentrations of 4 sham-dosed mallards were below the detection limit (<0.4 μg/g dw), while values in 2 sham-dosed geese were quite low (liver: 0.62 and 1.40 μg/g dw; kidney: 0.60 and 0.74 μg/g dw). Log transformed V concentrations in liver and kidney of dosed mallards were linearly related to both the log of the concentration of administered vanadium pentoxide ($r = 0.88$ and 0.90, $P < 0.001$, $n = 7$) and sodium metavanadate ($r = 0.88$ and 0.89, $P < 0.001$, $n = 15$; Figure 2). For sodium metavanadate-treated geese, the log transformed V concentration in liver and in kidney was only marginally related to the log of the dose ($r = 0.68$ and 0.71, $P = 0.098$ and 0.073, $n = 7$), presumably because of the small sample size. Vanadium uptake by kidney exceeded that of liver in both species (e.g., mallard: 113 mg/kg dose, geometric means of V in kidney and liver were 14.7 μg/g dw and 7.14 μg/g for vanadium pentoxide, and 35.7μg/g and 9.68 μg/g for sodium metavanadate; geese: 76 mg/kg dose, geometric mean V in kidney and liver were 19.7 μg/g dw and 9.49 μg/g for sodium metavanadate).

Prediction of Exposure Based on Tissue V Concentrations and Toxic Response

Recall that composite samples from 2 dead geese collected at the fly ash pond contained 57.3 μg/g dw of V in liver and 226 μg/g dw of V in kidney. Here results are reported of analyses

examining what levels of acute exposure to sodium metavanadate are indicated by these measurements.

Suppose that a mallard drake was administered sodium metavanadate under the conditions of our controlled acute exposure study, but that for some reason, the dosage was not recorded. Given measurements of V concentration in liver and kidney, and given the bird's response, inference regarding the administered dose is a prediction problem aptly handled under the Bayesian paradigm. The posterior distribution of administered dose is computed based on the modeled relations among dose, liver and kidney V concentrations, and toxic response (lethality). This posterior distribution is used to make inference about the unknown dose. Dose levels are converted into response probabilities (lethality), simultaneously accounting for uncertainty about the dose and parametric uncertainty in the modeled relations.

If the bird died, and had V concentrations of 10 μg/g dw in liver and 25 μg/g dw in kidney, the median value for the posterior distribution of response probability would be 90.1%. This value was used as our prediction: the dose to which this bird was exposed was such that 90.1% of mallard drakes exposed to this level under similar circumstances would die.

There is, of course, statistical uncertainty in this prediction. It could certainly be that this drake was exposed to a lower dose, but happened to have a lower tolerance. Consulting the same posterior distribution, one finds that the 10^{th} percentile of response probability is 58.5%. It was concluded with 90% confidence that the drake was exposed to at least the LD58.5. Data refer to 90.1% as the estimated lethality of the exposure, and to 58.5% as a 90% confidence lower bound of the exposure.

Estimated lethality of sodium metavanadate exposure and lower bounds are reported in Table 2, for a set of liver and kidney V concentrations similar to those observed in our controlled

exposure studies. These values were reported for birds encountered dead and for birds encountered alive; not surprisingly, for a given liver and kidney V concentration the indicated response is lower for birds encountered alive. The final row of Table 2 reports summaries based on the values observed in geese at the fly ash pond that prompted this study. A mallard with such liver and kidney V concentrations would have been subjected to a dose well above the LD50**,** and by species extrapolation one would have 90% confidence that it was exposed to at least the LD96.4.

Chronic Dietary Toxicity Studies

In the first feeding trail (sodium metavanadate concentration increasing weekly from 38.5 to 2651 ppm), food consumption appeared to decline by wk 7 (Figure 3). During wk 8, 2 drakes receiving sodium metavanadate exhibited mild signs of intoxication (unusual swallowing behavior). This study was terminated on day 67 when half of the treated drakes had lost >25% of their initial body weight. Repeated measures ANOVA of average daily food consumption/kg BW weight indicated a treatment x week interaction ($P < 0.001$), with food consumption by treated mallards during wk 9 and 10 being less ($P < 0.05$) than during wk 1-5 (Figure 3). Average daily food consumption/kg BW between the control and treatment groups differed ($P < 0.05$) only during the final partial wk of the study. Body weight loss became apparent in treated mallards by wk 8 (Figure 3). Repeated measures ANOVA for BW indicated a treatment x week interaction ($P < 0.001$), with weight of treated individuals declining during wk 9 and 10 ($P < 0.05$) compared to their weight during wk 1-8, presumably due to food avoidance, intoxication, or a combination thereof.

Gross liver lesions were observed in 5 of 12 treated mallards (4 livers were mottled in appearance and 1 had a 0.6 cm cyst), and mild intestinal hemorrhage was apparent in 7 of 12 treated drakes. Histological findings for controls were generally unremarkable except for the presence of foamy hepatocellular cytoplasm and perivascular cuffing. Of the treated drakes that had gross liver lesions, 2 exhibited zonal hepatocytic necrosis, 2 had vacuoles of varying size and the liver tissue of the individual with the cyst appeared normal (cyst not microscopically examined as it was lost in processing). Renal congestion was apparent in 6 of the treated ducks, and nearly every drake receiving sodium metavanadate had some disruption of the tips of villi and thickening, along with diffuse granulocytic infiltration of the remaining villi. None of the controls exhibited comparable renal or intestinal lesions.

Concentrations of V in the liver of mallards fed sodium metavanadate approached values observed in the goose die-off (Table 3). However, V values in kidneys of treated drakes were an order of magnitude lower than observed at the die-off. Compared to controls, hematocrit, serum AST activity and phosphorus concentrations were elevated in drakes ingesting sodium metavanadate, while serum globulin, sodium and chloride values were reduced (Table 3).

Chronic sodium metavanadate exposure resulted in lower activity of hepatic C-GSH-peroxidase, H-GSH-peroxidase, GSH S-transferase and G-6-PDH compared to controls (Table 4), while GSSG reductase activity was unaffected. In liver, log transformed V concentration was inversely related to activities of GSH S-transferase and G-6-PDH ($r = -0.93$ and -0.68, $P < 0.05$, $n = 9$). Three of the four measurements of hepatic thiol status were affected by dietary V, and included a decrease in the concentration of GSSG by 47%. This resulted in a proportionally smaller GSSG to GSH ratio. Total SH concentration increased by 21% due to an apparent corresponding increase in PBSH since there was not any significant change in GSH

concentration. Significant positive correlations occurred between the log of hepatic V and both total SH and PBSH ($r = 0.70$ and 0.72, $P < 0.05$, $n = 9$). A 52% increase in hepatic TBARS provided evidence of lipid peroxidation, although the relation between log hepatic V concentration and TBARS was only marginally significant ($r = 0.639$; $P = 0.06$, $n = 9$).

Vanadium concentrations in kidney were lower than in liver, and only one effect was apparent: a 20% decrease in renal G-6-PDH activity ($P < 0.05$). There was no significant relation between V concentration and oxidative stress endpoints in kidney.

Because of concerns that signs of vanadium toxicity might be confounded by reduced food intake, a second dietary feeding study was undertaken with treated drakes receiving 250 ppm sodium metavanadate (a concentration not avoided in the previous trial) for 4 weeks. Food consumption and BW were comparable between control and treated mallards during this trial, and no signs of intoxication were noted. At necropsy, some treated individuals exhibited very mild intestinal hemorrhage, and only 1 drake had a white discolored region along the edge of the liver. Histological examination revealed thickening of the intestinal villi with diffuse granulocytic and lymphocytic infiltration in 11 of 14 mallards receiving sodium metavanadate. Localized hepatocellular necrosis was apparent in the drake with the discolored liver. Half of the treated drakes exhibited disruption or destruction of the tips of the villi; this was not observed in any of the controls. Liver and kidney V concentrations in a subset of controls ($n = 3$) was <0.2 μg/g dw. Concentrations of V in a subset of treated mallards ($n = 6$) were considerably less than observed in the previous feeding trial and goose die-off (geometric mean, range; liver: 4.37 μg/g dw, 2.0-5.4; kidney: 3.13 μg/g dw, 1.8-4.9). Blood chemistry, and hepatic and renal measures of oxidative stress were comparable between drakes receiving 250 ppm sodium metavanadate and controls.

DISCUSSION

Acute Toxicity of Vanadium Compounds

Both vanadium pentoxide and sodium metavanadate were moderately toxic to mallard drakes, with estimated LD50s (113 and 75.5 mg/kg) being remarkably similar to that reported in laboratory mice (64 and 74.6 mg/kg) and rats (86 and 98 mg/kg) (Llobet & Domingo, 1984; Toxicological Research Lab Dukou Sanitary Antiepidemic Station, 1987). On a formula weight basis, sodium metavanadate contains substantially less V than vanadium pentoxide, yet the estimated LD50s of these two compounds did not differ in mallards. Based on allometric toxicity extrapolations for wildlife (Sample & Arenal, 1999), one might predict that the Canada goose, whose average mass is 5-fold greater than that of a mallard, might be more tolerant to V than mallards. However, in the present acute toxicity trials geese were significantly more sensitive to sodium metavanadate than mallards. Notably, Canada geese are also apparently more sensitive to lead than mallards (Hoffman et al., 2000a).

Some overt signs of intoxication in mallards and Canada geese (viz., abnormal gait and diarrhea) were similar to those observed in laboratory rodents orally dosed with vanadium pentoxide (Toxicological Research Lab Dukou Sanitary Antiepidemic Station, 1987) and sodium metavanadate (Llobet & Domingo, 1984). At necropsy, the most distinctive finding associated with acute oral exposure to these two V compounds was the presence of intestinal hemorrhage.

V accumulation in liver and kidney tissue was directly proportional to administered dose in acute toxicity studies. The extreme concentration of V in liver and in kidney of mallards dosed with vanadium pentoxide (27.9 and 118 μg/g dw) and those dosed with sodium metavanadate (42.1 and 295 μg/g dw) were of the same magnitude observed in dead geese collected at the fly ash pond (57.3 and 226 μg/g dw). However, the extreme values of V in liver

and kidney of geese dosed with sodium metavanadate were somewhat lower (16.9 and 37.2 μg/g dw), possibly because the greatest administered dose in geese (151 mg/kg BW) was much less than that for mallards.

Sublethal Responses to Chronic Sodium Metavanadate Exposure in Mallards

Chronic exposure of mallard drakes to sodium metavanadate at dietary concentrations exceeding 1000 ppm resulted in decreased food intake, weight loss, some overt signs of intoxication, pathology and V accumulation in liver and kidney. At a dietary concentration of 250 ppm, there was little or no evidence of toxicity and only modest accumulation of V in liver and kidney (<5 μg/g), not unlike observations in mallards fed 100 ppm vanadyl sulfate for 12-wk (White & Dieter, 1978). Domestic chickens may be more sensitive to dietary metavanadate than mallards based on threshold concentrations for impaired growth and mortality (Hafez & Kratzer, 1976; Ousterhout & Berg, 1981). Food intake, weight change and toxicity of laboratory rodents exposed to pentavalent V varies considerably depending on diet, method of administration and test species; V uptake by liver and kidney of laboratory rodents seems to be quantitatively less (Thompson et al., 1998) than observed in mallards and geese.

Chronic exposure of mallards to increasing dietary concentrations of sodium metavanadate was accompanied by elevated hematocrit, which has been observed in some rodent V toxicity studies and attributed to dehydration (Russanov et al., 1994; Thompson et al., 1998). Elevated serum AST activity, a biomarker of cellular damage in liver and other tissues, and reduced concentrations of serum total protein were suggestive of V-induced hepatotoxicity, which was further substantiated by the presence of liver lesions in 5 of 12 individuals ingesting sodium metavanadate. Decreased total protein and globulin concentrations might also reflect

protein loss associated with intestinal pathology and, in the case of globulin, possible effects of V on immune cell function. Serum sodium and chloride concentrations were reduced in V-treated mallards, with the probable cause being inhibition of renal Na^+,K^+-ATPase (Phillips et al., 1982) accompanied by impaired renal tubular reabsorption of sodium (Braunlich et al., 1989) and electrolyte imbalance associated with enteritis. It is also possible that anorexia, and dehydration associated with food avoidance, could have contributed to some of these blood chemistry alterations.

Chronic dietary exposure to sodium metavanadate resulted in a number of manifestations of hepatic oxidative stress, including a 52% increase in hepatic lipid peroxidation. Vanadium-induced hepatotoxicity and lipid peroxidation was previously reported in laboratory rats (Harvey & Klaassen, 1983; Younes & Strubelt, 1991), including an association between TBARS production and V accumulation in tissue (Oster et al., 1993). In addition, chronic dietary exposure to sodium metavanadate resulted in decreased activity of several hepatic antioxidant enzymes (C-GSH-peroxidase, H-GSH-peroxidase, GSH S-transferase and G-6-PDH) in mallards, not unlike antioxidant enzyme changes observed in liver and kidney of V-exposed rats (Russanov et al., 1994; Byczkowski & Kulkarni, 1998). Mercury, lead and selenium have also been reported to produce oxidative stress, lipid peroxidation and neurologic impairment in mallards and other avian species (Henny et al., 2002; Hoffman, 2002; Hoffman and Heinz, 1998; Hoffman et al., 2000a, 2000b; Mateo et al., 2003).

Although hepatic GSH concentration was unaffected in the present study, total SH increased, apparently due to elevated PBSH concentration. A decrease in GSSG and the ratio of GSSG to GSH was apparent, indicating a physiological attempt to combat oxidative stress. Chronic low level exposure to V in drinking water of rats (0.5 ppm) was found to increase

hepatic GSH concentration and GSH S-transferase activity without evoking signs of toxicity (Bishayee & Chatterjee, 1995). Once pentavalent V enters cells, it may be reduced to the tetravalent form by GSH forming GSSG. Activity of GSSG reductase was not affected by V in the present study, which may explain the absence of increased in hepatic GSSG concentration and the corresponding ratio of GSSG to GSH. Other metals and metalloids, including methylmercury, lead and selenomethionine, have all been reported to alter GSH status, by mobilizing GSH at lower concentrations which tends to deplete it, and by increasing the ratio of GSSG to GSH at higher concentrations (Hoffman & Heinz, 1998; Henny et al., 2002; Hoffman, 2002). According to a recent report, GSSG is actually a more efficient oxovanadium(IV) binder than GSH in the pH range of 6-7 (Baran, 2003). Therefore, both GSH and GSSG may participate in the stabilization and transport of VO^{+2} immediately after GSH-mediated reduction of pentavalent V in cellular systems. This would also probably tend to reduce GSSG content as seen in the present study.

Vanadium Exposure and Prediction of Toxicity

This study generated data on tissue V concentrations and thresholds of toxicity that will assist in the interpretation of V exposure in free-ranging wildlife. Concentrations of V in tissues of healthy vertebrates are generally quite low, rarely >3 μg/g dw in mammals (Costigan et al., 2001) and wild terrestrial vertebrates (Rattner et al., 2005). In the present study, V concentrations in liver and kidney of unexposed mallards and geese were $\leq$1.4 μg/g dw.

With the exception of the goose die-off in Delaware reported herein, the highest mean values for V in waterbirds reported in the literature include 7.36 μg/g dw in the liver of apparently healthy American coots (*Fulica americana*) collected in San Francisco Bay (Hui,

1998) and 8.11 μg/g dw (maximum value: 31.8 μg/g dw) in the kidney of apparently healthy spotbill ducks (*Anas poecilorhyncha*) from an industrial site in Japan (Mochizuki et al., 1999). In addition, the liver of an apparently healthy gadwall (*Anas strepera*) collected at an oil refinery waste pond in Wyoming was observed to contain V at a concentration of 34.7 μg/g dw (P. Ramirez Jr., U.S. Fish and Wildlife Service, unpublished observation). Although these values fall in the toxic effects range derived from the present study, it has been suggested that some waterbirds (e.g., coots) feed on plants that contain moderate concentrations of V, and thus may be able to tolerate moderate levels in liver (Hui, 1998).

Results of acute toxicity trials in mallards and geese suggest that histopathological changes in the intestine, liver, kidney and heart become discernable when liver and kidney V concentrations were approximately 5 μg/g dw. Vanadium concentrations of 10 μg/g dw in liver and 25 μg/g dw in kidney were associated with mortality (>50%) in mallards receiving an acute oral dose of sodium metavanadate. In chronic feeding trials, blood chemistry changes, indications of oxidative stress and liver lesions and kidney congestion were apparent when V concentrations equaled or exceeded values observed in acute toxicity trials that were associated with mortality. It is noteworthy that in our acute exposure studies, V was preferentially accumulated in kidney, while in our chronic studies V was preferentially accumulated in liver. This difference in organ accumulation could be useful in assessing environmental exposure scenarios (e.g., single oral or sub-chronic exposure at a point source versus chronic low level dietary exposure), and certainly warrants further investigation.

Hazard of Vanadium to Wildlife

Concern for the toxicity of V to wild birds originally stemmed from its high concentration in petroleum crude oil and the potential for adverse effects that might accompany oil spills or discharges (White & Dieter, 1978; Hoffman, 1979). Vanadium mobilization in the environment occurs naturally, and also from mining activities, steel production, petroleum processing and fuel combustion (Hilliard, 1994; Nriagu, 1998). As with most other metals, the principal exposure route in wildlife is presumed to be through ingestion of food, sediment and water. Based upon the present study and others (White & Dieter, 1978), it seems unlikely that chronic low-level dietary exposure to V often poses a direct lethal hazard to wildlife. However, point sources, such as the V laden fly ash impoundment encountered by geese that died in Delaware, certainly do pose a hazard to wildlife. Whether or not the V-linked goose die-off is an isolated event is currently under investigation by the Interagency Testing Committee of the Toxic Substances Control Act (Federal Register, 2005). Notably, data from the U.S. Environmental Protection Agency Toxics Release Inventory indicates that 18 million pounds of V was released into surface impoundments at 172 facilities in 2002, and several of these sites are located in key migratory waterbird flyways (Federal Register, 2005).

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FIGURE 1. (A) Photomicrograph of hematoxylin and eosin stained section of intestine with distinct villi and crypts of a control mallard that was euthanized (100x; scale bar is 100 μm); (B) disrupted intestinal villi (missing tips, lack of staining) of mallard dosed with 62 mg/kg BW sodium metavanadate that was euthanized (100x); (c) loss of villi, hemorrhage, lymphocytic and granulocytic infiltration and luminal cellular debris of mallard dosed with 113 mg/kg sodium metavanadate necropsied within hours of death (100x); and (D) pronounced loss of villi and crypts and luminal cellular debris of mallard dosed with 113 mg/kg sodium metavanadate necropsied within hours of death (40x; scale bar is 300 μm).

FIGURE 2. Relation between log transformed V concentration and administered acute oral dose of sodium metavanadate for liver [$R^2 = 0.77$, $P < 0.001$, $n = 15$; $\log_{10}$ Liver V = -0.946 + 0.907($\log_{10}$ Dose)] and kidney [$R^2 = 0.80$, $P < 0.0001$, $n = 15$; $\log_{10}$ Kidney V = -2.220 + 1.7259($\log_{10}$ Dose)] of mallards.

FIGURE 3. Average daily food consumption and body weight (mean $\pm$ SE) at weekly intervals of mallards fed increasing dietary concentrations of sodium metavanadate (38.5 to 2651 ppm).

TABLE 1. Acute Oral Toxicity of V Compounds to Mallards (*Anas platyrhynchos)* and and Geese (*Branta canadensis*)

Species	Compound	Median Lethal Dose (mg/kg)	95% Credible Interval (mg/kg)	Slope $\pm$ SD (logit/$\log_{10}$)
Mallard	V_2O_5	113	84.2-157	21.5 $\pm$ 38.4
Mallard	$NaVO_3$	75.5	44.8-125	2.43 $\pm$ 0.85
Canada goose	$NaVO_3$	37.2*	12.1-72.2	2.34 $\pm$ 0.77

* Estimated median lethal dose significantly different (P = 0.044, maximum likelihood analysis) from that of mallards receiving sodium metavanadate.

TABLE 2. Estimated Lethality of Sodium Metavanadate Exposure and 90% Confidence Lower Bounds for Birds Encountered in the Field Based on Liver and Kidney V Concentrations from Experimentally Treated Mallards

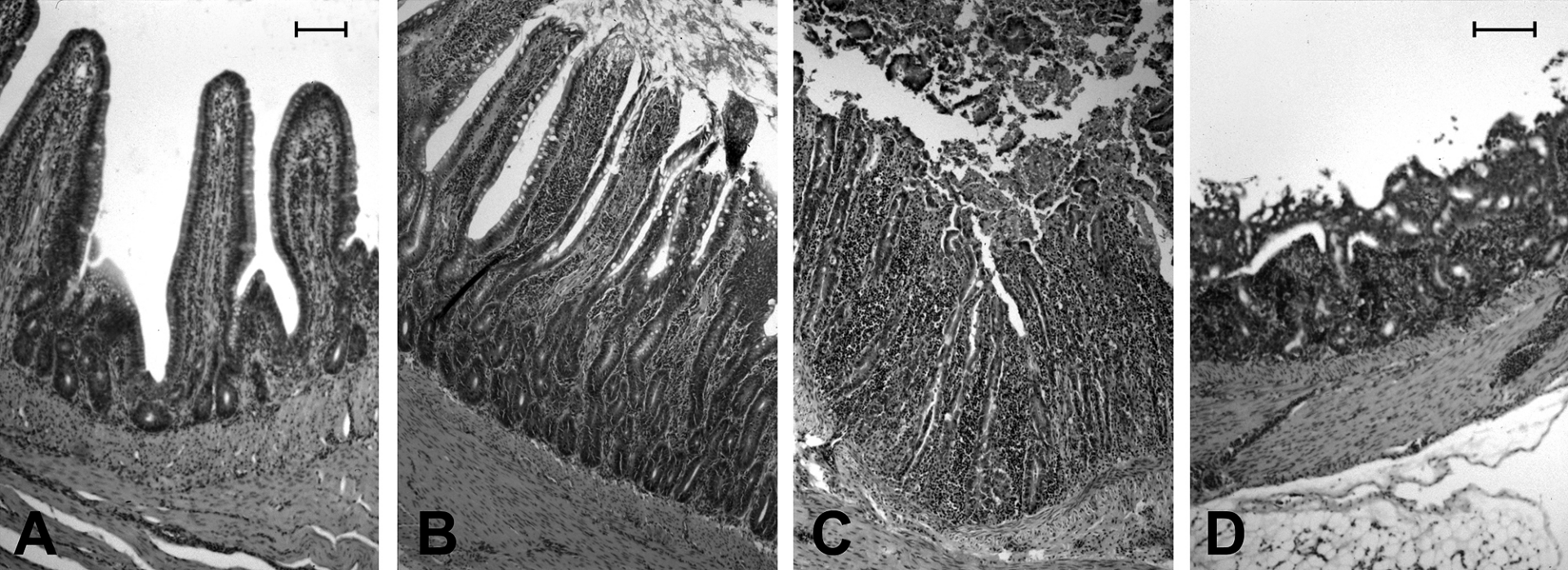
V Concentration (μg/g dw)		Birds Encountered Dead		Birds Encountered Alive	
Liver	Kidney	Estimated Lethality of Exposure (%)	90% Confidence Lower Bound	Estimated Lethality of Exposure (%)	90% Confidence Lower Bound
1	0.5	6.3	0.3	0.9	0.0
2	2	26.9	3.2	6.9	0.4
5	10	70.7	28.1	38.7	7.4
10	25	90.1	58.5	68.0	24.2
15	50	96.3	77.0	84.3	43.2
20	100	98.7	88.0	93.0	61.5
30	200	99.6	94.5	97.6	78.7
40	300	99.8	96.7	98.8	86.0
57.3[a]	226[a]	99.8	96.4	98.9	85.3

[a] Liver and kidney V concentrations observed at goose die-off.

TABLE 3. Tissue V Concentrations, Hematocrit and Serum Chemistry in Mallards following Experimental Chronic Dietary Exposure to Increasing Concentrations of Sodium Metavanadate

	Control	Sodium metavanadate (38.5-2651 ppm)[a]
Liver V (μg/g dw)	nd	25.2 (16.1 - 35.4)
Kidney V (μg/g dw)	nd	13.6 (7.0-24.0)
Hematocrit (%)	48.7 ± 0.9	55.2 ± 1.3*
Aspartate aminotransferase activity (IU/L)	15.7 ± 2.0	24.9 ± 2.6*
Creatine phosphokinase (IU/L)	236.5 ± 51.7	239.2 ± 30.3
Total Protein (g/dL)	4.1 ± 0.1	3.7 ± 0.1
Albumin (g/dL)	2.2 ± 0.1	2.1 ± 0.1
Globulin (g/dL)	1.9 ± 0.1	1.6 ± 0.1*
Glucose (mg/dL)	188.0 ± 7.3	181.6 ± 4.0
Cholesterol (mg/dL)	256.2 ± 20.9	236.8 ± 12.8
Uric Acid (mg/dL)	4.0 ± 0.6	12.8 ± 4.2
Sodium (meq/L)	147.8 ± 1.1	140.6 ± 2.3*
Potassium (meq/L)	3.4 ± 0.2	3.9 ± 0.3
Chloride (meq/L)	108.7 ± 1.4	88.2 ± 2.8*
Calcium (mg/dL)	9.9 ± 0.2	9.9 ± 0.1
Phosphorus (mg/dL)	2.4 ± 0.32	3.9 ± 0.43*

[a]Dietary concentrations were increased each week, starting at 38.5 ppm and ending at 2651 ppm.
Note - Tissue V concentrations (control group, n = 6; sodium metavanadate group, n = 6) are geometric mean (range); nd = not detected.
Hematocrit and serum measures are arithmetic mean ± SE (control group, n=6; sodium metavanadate, n=12), and were compared by ANOVA; * $P < 0.05$.


A
B
C
D

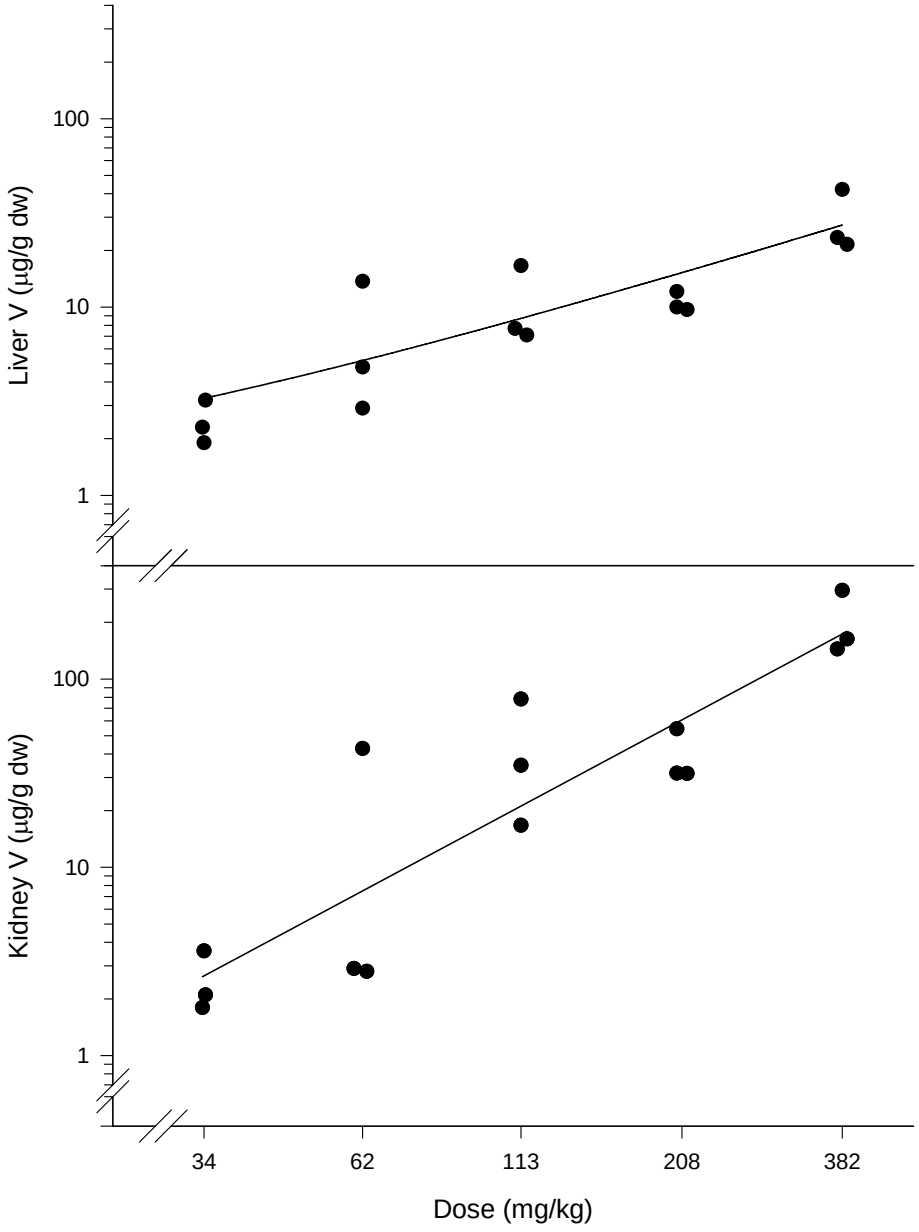

Liver V (µg/g dw)
100
10
1
Kidney V (µg/g dw)
100
10
1
34
62
113
208
382
Dose (mg/kg)

FIGURE 2

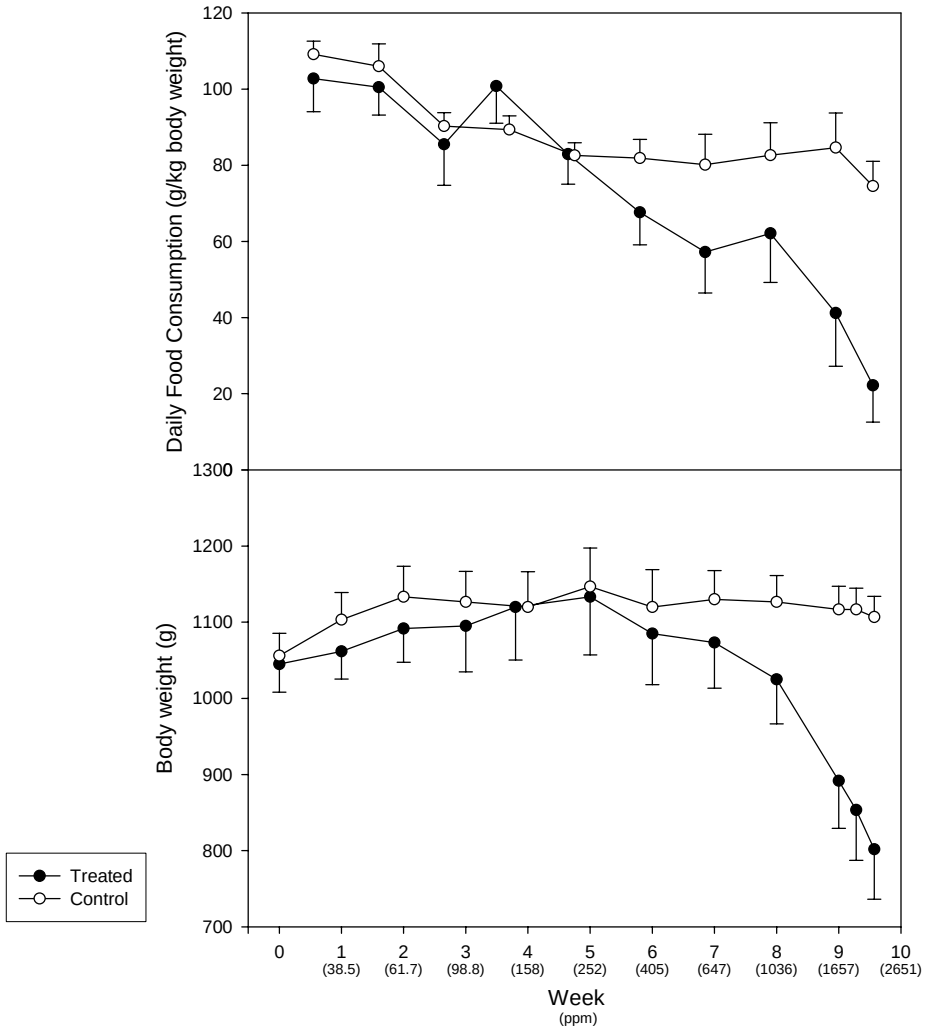

Daily Food Consumption (g/kg body weight)
120
100
80
60
40
20
0
Body weight (g)
1300
1200
1100
1000
900
800
700
Treated
Control
0
1
(38.5)
2
(61.7)
3
(98.8)
4
(158)
5
(252)
6
(405)
7
(647)
8
(1036)
9
(1657)
10
(2651)
Week
(ppm)

FIGURE 3