



Adverse Effects to Northern Shovelers from Exposure to Treated Wastewater from Central Front Range, Colorado, Wastewater Treatment Plants



Administrative Report

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Contents

Abstract.....	1
Introduction	2
Material and Methods	4
Wastewater Exposure Trials	4
LC–MS Chemical Analysis	6
Statistical Analysis	7
Results	8
Wastewater Exposure Trials	8
LC–MS Chemical Analysis	16
Discussion.....	20
Acknowledgments.....	28
References Cited	28

Figures

1. Temporary tank at the secondary clarifier site, Metro Wastewater Reclamation District, Denver, Colorado	4
2. Air and water temperatures recorded from HOBO [®] temperature loggers	9
3. Individual body temperatures for ducks and ambient air and water temperatures at the control tank site	10
4. Individual body temperatures for ducks and ambient air and water temperatures at the secondary clarifier site.....	11
5. Individual body temperatures for ducks and ambient air and water temperatures at the chlorine contact basin site	12
6. Example of control tank duck	14
7. Example of secondary clarifier duck	15
8. Example of chlorine contact basin duck.....	15
9. Total nonionic polyethylene glycol (PEG) concentration comparison by treatment.....	21

Tables

1. Sample preparation and concentration weights of duck feather rinsates and water samples.	17
2. Mass-to-charge ratio of nonionic surfactant ions (as ammonium adducts) and their assignments for the alkyl polyethylene glycol (PEG) ethoxylate ion progressions observed in the duck feather extracts.....	19
3. Total nonionic polyethylene glycol (PEG) surfactant quantitation from duck feather rinsates.	20

Conversion Factors

Inch/Pound to SI

Multiply	By	To obtain
	Length	
mile (mi)	1.609	kilometer (km)
	Volume	
gallon (gal)	3.785	liter (L)
gallon (gal)	0.003785	cubic meter (m ³)
gallon (gal)	3.785	cubic decimeter (dm ³)

SI to Inch/Pound

Multiply	By	To obtain
Length		
centimeter (cm)	0.3937	inch (in.)
millimeter (mm)	0.03937	inch (in.)
meter (m)	3.281	foot (ft)
meter (m)	1.094	yard (yd)
Area		
square meter (m ²)	0.0002471	acre
square centimeter (cm ²)	0.001076	square foot (ft ²)
square meter (m ²)	10.76	square foot (ft ²)
square centimeter (cm ²)	0.1550	square inch (in ²)
Volume		
liter (L)	33.82	ounce, fluid (fl. oz)
liter (L)	2.113	pint (pt)
liter (L)	1.057	quart (qt)
liter (L)	0.2642	gallon (gal)
cubic meter (m ³)	264.2	gallon (gal)
cubic decimeter (dm ³)	0.2642	gallon (gal)
cubic centimeter (cm ³)	0.06102	cubic inch (in ³)
cubic decimeter (dm ³)	61.02	cubic inch (in ³)
liter (L)	61.02	cubic inch (in ³)
cubic decimeter (dm ³)	0.03531	cubic foot (ft ³)
cubic meter (m ³)	35.31	cubic foot (ft ³)
cubic meter (m ³)	1.308	cubic yard (yd ³)
Mass		
gram (g)	0.03527	ounce, avoirdupois (oz)

Temperature in degrees Celsius (°C) may be converted to degrees Fahrenheit (°F) as follows:

$$^{\circ}\text{F}=(1.8\times^{\circ}\text{C})+32$$

Temperature in degrees Fahrenheit (°F) may be converted to degrees Celsius (°C) as follows:

$$^{\circ}\text{C}=(^{\circ}\text{F}-32)/1.8$$

Concentrations of chemical constituents in water are given either in milligrams per liter (mg/L) or micrograms per liter (µg/L).

NOTE TO USGS USERS: Use of hectare (ha) as an alternative name for square hectometer (hm²) is restricted to the measurement of small land or water areas. Use of liter (L) as a special name for cubic decimeter (dm³) is restricted to the measurement of liquids and gases. No prefix other than milli should be used with liter. Metric ton (t) as a name for megagram (Mg) should be restricted to commercial usage, and no prefixes should be used with it.

Adverse Effects to Northern Shovelers from Exposure to Treated Wastewater from Central Front Range, Colorado, Wastewater Treatment Plants

By William M. Iko, Jenny Berven, Laurie A. Baeten, Colleen E. Rostad, David W. Rutherford, Carolyn J. Otten, and Paul Winter

Abstract

From January through February of 2007, more than 900 waterfowl, the majority of which were northern shovelers (*Anas clypeata*), died in apparent association with prolonged exposure to water in or near treatment tanks at a number of wastewater treatment plants located along the central Colorado Front Range. Preliminary postmortem assessments were negative for waterfowl diseases, ingested toxins, or heavy metal contaminants. The probable cause of death for most of these mortalities was the induction of a fatal hypothermia resulting from the wetting of the ducks' feathers. To test whether prolonged exposure of waterfowl to chemical compounds in secondary treated wastewater from municipal sources may play a role in the loss of waterproofing, a controlled experimental exposure scenario was developed in which captive, and previously unexposed, live ducks would be subjected to treated wastewater to determine if that exposure, the duration of their exposure, or other factors associated with the experiment adversely affected the waterproofing capabilities of the birds' feathers. Experimental ducks (mallards, *Anas platyrhynchos*) were exposed to treated wastewater from the secondary clarifier tanks (n = 5), chlorine contact basin (n = 5), and a control water source (n = 5), and physically assessed every three to six hours for hypothermic responses over the duration of their exposure trial. Ducks exposed to secondary clarifier and chlorine contact basin water began to display signs of hypothermic response (lowered body temperature, shivering, and a reduced ability to maintain buoyancy) within 17 and 12 hours of exposure, respectively, while ducks exposed to the control water showed no hypothermic response over the duration of their exposure trials. To assess whether chemical compounds from the treated wastewater may have affected duck waterproofing in the experiment, liquid chromatography mass-spectrometry (LC-MS) was performed on feather rinsates extracted from the exposed ducks and water samples from each exposure tank. The presence of nonionic polyethylene glycol (PEG) surfactant-group compounds were detected in all the feather rinsates (n = 8), including ducks from the control group, and no surfactant chemicals were identified in the water samples. Although the chemical results are inconclusive regarding the impact of nonionic surfactants on duck waterproofing, given the chemical complexity present in municipal wastewater treatment impoundments, more detailed chemical analysis quantifying surfactants in wastewater systems would be required to determine if surfactant groups, combination of groups, or combination of chemical groups and classes, may be causing the effects on waterproofing that were observed during this experiment.

Introduction

From January through February 2007, more than 900 waterfowl died in or near ten different wastewater treatment plants located in the Denver, Colorado, metropolitan area and other locations along the Front Range of central Colorado (ProMED-mail, 2007a,b,c; M. Kaknes, CDOW, written commun., 2007). Most of these mortalities were from one duck species, the northern shoveler (*Anas clypeata*), with the highest number of ducks being recovered at the Metro Wastewater Reclamation District (MWRD) facility located in north Denver, Colorado. Dead and moribund birds were found at the north secondary clarifier tanks and chlorine contact disinfecting basin, the last two stages of wastewater treatment before treated water flows into the nearby South Platte River (Metro Wastewater Reclamation District, 2008). MWRD staff reported that it was normal for large flocks of waterfowl to frequent the 2.2-million-gallon tanks located on the north end of this facility for feeding and roosting during the winter months because the temperature of the water undergoing treatment process prevents these tanks from freezing. Although annual waterfowl mortalities do occur (S. Rogowski, MWRD, oral commun., 2007), the number of ducks that died during the winter of 2007 in or near wastewater treatment plants was unprecedented for wintering waterfowl populations along the Colorado Front Range. Concerns were immediately raised as to the possible causes for this mortality event, such as avian disease, toxicological exposure, or chemical contamination (Nero, 1968; Moulton and others, 1976; Lewis, 1999; Fischel, 2001; Hamilton, 2007).

In January 2007, the U.S. Fish and Wildlife Service Region 6 (FWS, Denver, Colo.), Colorado Division of Wildlife (CDOW), U.S. Geological Survey, National Wildlife Health Center (NWHC, Madison, Wis.), MWRD, and the other wastewater treatment facilities began monitoring the waterfowl die-off to assess its severity and the potential causes for this event. MWRD monitored water and effluent chemistry for unusual water quality of chemical inputs or biological growths within the treatment tanks (V. Hahn, MWRD, written commun., 2007). Standard water chemistry analysis included measurements of volatile organics, semi-volatile organics, nonyl phenols, surfactants, petroleum organics, pesticides, and polychlorinated biphenyls (PCBs), and biological samples were monitored for zooplankton and phytoplankton levels in the water. However, their analyses did not find any unusual chemical inputs or biological growth during the die-off. MWRD, CDOW, and FWS began collecting dead ducks found in the tanks for diagnostic examination, retrieving moribund ducks for rehabilitation efforts, and discouraging tank use by other waterfowl through hazing efforts (M. Kaknes, CDOW, written commun., 2007). Ducks were observed having problems maintaining buoyancy, floating low in the water, and were visibly water-logged in both their body and flight feathers (M. Kaknes, CDOW, oral and written commun., 2007). Over 100 ducks in a moribund state were retrieved and transferred to rehabilitation centers, of which 60 were rehabilitated and released (M. Kaknes and L. Baeten, CDOW, oral and written commun., 2007). Diagnostic examinations on approximately 30 dead ducks were performed by the CDOW Wildlife Health Laboratory (Fort Collins, Colo.), U.S. Fish and Wildlife Service National Forensics Laboratory (Ashland, Oreg.), USGS NWHC, and the Veterinary Diagnostic Laboratory, Colorado State University (Fort Collins, Colo.) to determine potential causes of death. Preliminary postmortem assessments were negative for avian influenza, West Nile virus, avian cholera, avian botulism, or other significant infectious waterfowl diseases. Likewise, no ingested toxins or heavy metal contaminants were found in these ducks (T. Spraker, Colorado State University, and M. Jankowski, NWHC, oral and written commun., 2007). Physical examination of the duck carcasses from MWRD found that many were soaked to the skin, confirming earlier observations of waterlogged ducks in the treatment tanks. Histopathology performed on internal organs (liver, kidney, spleen, heart, and lung) did not find gross lesions of pathological significance. Based upon the cumulative clinical and necropsy findings from these laboratories, the probable cause of death for most of these mortalities was

the induction of a fatal hypothermia resulting from the wetting of the ducks' feathers (L. Baeten, CDOW, oral and written commun., 2007).

Why the waterproofing of some overwintering ducks failed at the wastewater facilities remains unknown. The possibility that ducks were exposed to various chemicals in the treated wastewater, which affected their waterproofing, was investigated further. Waterfowl transferred to local wildlife rehabilitation centers did not regain buoyancy or waterproofing until after their feathers had been thoroughly washed, suggesting the possibility that a surface contaminant may have been present on the feathers that was affecting their water repellency (L. Baeten and M. Kaknes, CDOW, oral and written commun., 2007). Researchers at NWHC and the University of Wisconsin (Madison, Wis.), using a scanning electron microscope, further examined duck-feather surfaces and found a crystallized substance imbedded within the microstructure of the feather, suggesting the possible presence of a surfactant polymer substance (M. Jankowski, NWHC, oral and written commun., 2007). Surfactants are an important ingredient in a broad spectrum of household cleaning products, pharmaceuticals, and industrial applications. These large-volume chemicals are extensively manufactured worldwide and are frequently found in raw wastewater (Greenberg and others, 1992a,b; Knepper and others, 2003) as well as in treated wastewater effluent (Chiron and others, 2000; Shon and others, 2006; Loos and others, 2007). Standard general tests for overall surfactants include the qualitative methylene blue active substances (MBAS) test for anionic surfactants (Greenburg and others, 1992a) and the cobalt thiocyanate active substances (CTAS) test for nonionic surfactants (Greenburg and others, 1992b). Both of these tests have interferences and are neither very sensitive nor specific (Greenburg and others, 1992a,b). An initial attempt to identify chemical surfactant compounds was performed at Mississippi State University (Starkville, Miss.) using the MBAS test on the feather samples, but the results were negative for the presence of anionic surfactants (D. Crawford, oral commun. to J. Wegrzyn, FWS, 2007). To identify the chemical substance observed by NWHC and University of Wisconsin researchers on the feather surface, a more detailed and wider spectrum chemical analysis, such as liquid chromatography–mass spectrometry (LC–MS), was recommended (Ardrey, 2003). LC–MS is a powerful technique used for many applications where very high sensitivity and specificity is desired. Chemir Analytical Services (Chemir) was contacted by FWS to complete preliminary LC–MS tests of duck feather rinsates from the 2007 die-off. The Chemir LC–MS analytical results indicated the presence of nonionic polyethylene glycol (PEG) surfactant-group compounds extracted from the feather rinsates, leading to further speculation that this surfactant group may be responsible for the loss of duck waterproofing (C. Otten, Chemir, oral and written commun., 2007).

In 2007, the USGS Fort Collins Science Center (FORT), in collaboration with CDOW, FWS, MWRD, and Chemir, developed a study to test whether prolonged exposure of waterfowl to chemical compounds in secondary treated wastewater from municipal sources may play a role in the loss of waterproofing in ducks. This hypothesis was based upon (1) the location of dead and affected birds primarily occurring at or near a number of municipal wastewater treatment facilities, (2) the collection of adversely affected birds from areas at or near these sites, (3) the lack of detection of significant avian pathogens, (4) gross and microscopic pathology (histopathology) evaluations being consistent with hypothermia as the suspected cause of death for a large number of these birds, (5) the presence of a crystallized substance imbedded within the microstructure of duck feathers (examined by scanning electron microscope), and (6) preliminary chemical analysis pointing toward chemical exposure as the suspected cause for the loss of the waterproofing capability of the birds' feathers. This hypothesis was addressed by developing a controlled experimental exposure scenario in which captive, and previously unexposed, live ducks would be subjected to treated wastewater to determine if that exposure, the duration of their exposure, or other factors associated with the experiment adversely affected the waterproofing capabilities of the birds' feathers.

Material and Methods

Wastewater Exposure Trials

An experiment was designed to expose live ducks to treated wastewater derived from two different points within the treatment process at the MWRD facility, as well as a clean water source as a control, to determine whether exposure of waterfowl to treated wastewater from municipal sources caused the wetting of feathers to the point of hypothermic response and mortality. The experimental design of this study was similar to a concentration response study assessing mortality impacts (Hartung, 1967; Rand and Petrocelli, 1985; Shane, 1994; Vyas and others, 2006). The experiment was conducted during February 2008 to expose ducks to similar biological and meteorological conditions to which waterfowl were exposed during the 2007 die-off. Ducks were housed in temporary treatment tanks made of stainless steel (cattle watering tanks; fig. 1) that were noncorrosive and nonreactive to the water treatments (Jon Powers, MWRD, oral commun., 2007). These tanks were approximately 3.7 m in diameter, 1.2 m in depth (10.8 m² surface area), insulated to prevent the treatment water from freezing, and fitted with continuous-flow pumps so that water moving through the tanks matched the flow rates found in the MWRD facility's original water sources. Water for the temporary tanks was drawn from one of three different sources found at MWRD: (1) Burlington Ditch water—water diverted from the South Platte River upstream of the MRWD outfall, (2) secondary clarifier tanks, and (3) the chlorine contact basin. As a consequence of normal wastewater treatment processing, water temperatures in the secondary clarifier tanks and chlorine contact basin are maintained at approximately 14–16°C throughout the year. To maintain ambient water temperature in the control tank comparable to the temperatures that normally occur in the Burlington Ditch during this time of year, a tank water heater was used in to keep the water temperature at approximately 5°C. Each of the temporary tanks was enclosed within chain-link fencing and chicken wire to prevent escape by the experimental ducks or access by wild, free-ranging waterfowl and predators. An interior mesh-cloth tent was used to keep the experimental ducks exposed to the water surface and to reduce visual disturbance from operational activities occurring at the facility.



Figure 1. Temporary tank at the secondary clarifier site, Metro Wastewater Reclamation District, Denver, Colorado (February 2008).

To use waterfowl in similar physiological and molt cycle conditions to those ducks involved in the 2007 die-off, wild ducks were captured in January 2008 from a wintering aggregation found along the Colorado Front Range (J. Gammonley, CDOW, oral and written commun., 2007). Although the majority of duck mortalities in 2007 were northern shovelers, for this experiment wild mallards (*Anas platyrhynchos*) were used, due to the difficulty in capturing and maintaining wild shovelers and because some mallards were also observed to have been affected during the 2007 die-off (J. Gammonley, CDOW, oral and written commun., 2007). Ducks were captured at Tamarack State Wildlife Area located approximately 30 miles northeast of Sterling, Colo., using corn bait and swim-in metal mesh traps. Once the ducks were captured, they were transported to a quarantine pen and held for seven days at the CDOW Foothills Wildlife Research Facility in Fort Collins, Colo. Captive ducks were randomly assigned to a treatment or control tank and individually marked both with colored leg bands and a color mark on their bill. Ducks were transported to the MWRD facility in Denver and placed in their pre-assigned tank. Food was provided freely from feeding buckets suspended within the treatment tanks throughout the study.

A total of 15 wild mallard ducks were used for the experiment, five ducks per treatment water and five ducks in the control group. As the exposure trials progressed, ducks were evaluated every three to six hours to assess their physical condition. Due to personnel constraints, only one exposure trial with control tank comparison could be performed at a time. Also, due to personnel constraints and handling time, comparable assessment periods (treatment versus control group) were usually completed within one to two hours of each other (and before the next assessment period began). To limit the number of birds used in this experiment, the same ducks were used in a single control group (n = 5) for comparison with both wastewater exposure trials (control tank ducks were in their treatment water for the entire 93 hours of the experiment). The exposure trial at the secondary clarifier tank (n = 5 ducks) lasted for 21 hours, with a time gap between exposure trials for the control duck group of 53 hours (during this time interaction was limited to reduce handling stress). The exposure trial at the chlorine contact basin (n = 5 ducks) lasted for the final 19 hours of the experiment.

During each assessment period, the following physiological measurements were collected from each duck: (1) body temperature, (2) body mass, (3) estimates of displacement and buoyancy, and (4) an estimate of feather wetting. Body temperature was measured using a standard digital thermometer inserted into the cloaca (Banta and others, 2004; Bakken and others, 2006). Body mass was measured by placing the duck in an enclosed dishwashing tub positioned on a digital balance. Estimates of water displacement and buoyancy were recorded by using a graduated fish aquarium filled with tap water and measuring the difference in water level before, during, and after placement of the duck within the aquarium. Also, photographs were taken of the ducks while they were in the aquarium to record any visual changes in flotation posture and buoyancy between sequential assessment periods (Banta and others, 2004; Bakken and others, 2006). Estimates of feather wetting were subjectively scored using the following numerical scale: 0—Entire duck is dry, water beading noted over entire body; 1—Dampness noted on 1–2 parts of the duck, down is dry, and some beading noted; 2—Dampness noted on only three parts of the duck, down is dry, and beading noted only on head and back; 3—Downy feathers are wet on chest and/or abdomen but are not saturated and beading noted only on head/neck; 4—Downy feathers are wet on chest and abdomen, back and flight feathers are wet, and beading only noted on head/neck; and 5—Downy feathers are saturated on chest and abdomen, back and flight feathers are wet, and beading not noted. After each duck was examined and assessed, it was returned to its treatment tank for further exposure to water until the next assessment period.

Over the duration of the experiment, ambient temperatures were recorded using automatic Hobo[®] temperature loggers for a continuous record of air and water temperatures. During each assessment period, ambient air and tank water temperature were also recorded using digital aquarium thermometers. To develop time activity budgets of ducks in the exposure tanks (for example, how

much time spent feeding, preening, or resting) (Webb and Brotherson, 1988; Thompson and Baldassarre, 1991), behaviors were recorded using motion sensing cameras (Moultrie[®] I-40 digital trail cameras) set on tripods within each of the tanks. Anecdotal observations were also made of ducks in exposure tanks between and during assessment periods throughout the experiment.

Observations on wild, free-ranging, wintering ducks using the MWRD facility were recorded opportunistically between assessment periods over the duration of the experiment. Six of the 12 north secondary clarifier tanks, the chlorine contact basin, and the MWRD outflow basin to the South Platte River were monitored sporadically between assessment periods as an estimate of the number of ducks present during the recorded time period and their general behavioral activity. During these observations, the number of ducks of each species (if species could be identified), date, time, behavioral activity, and general weather conditions were recorded.

The main goal of this experiment was to elucidate a hypothermic response in ducks exposed to treated wastewater. To ensure exposure to treated wastewater during these trials, the ducks were forced to maintain continuous contact with the water surface in each treatment tank. However, early in the secondary clarifier exposure trial (the first of the two exposure trials), ducks were observed out of the water along the edge of the tanks in both the treatment and control tanks (assessment period 2, 3 hours into the exposure trial). This was rectified early in the experiment by weighting down the interior mesh clothing, preventing ducks from leaving the water surface. The ducks were subjected to their treatment water until the end of their exposure trial, or until mortality or acute behavioral signs associated with hypothermia were observed (for example, listlessness, depressed wing and body position, inability to keep head above water and drowning, feather wetting to point of drowning, or declines of 10°C below the average 42.1°C mallard body temperature) (Choules and others, 1978; Gill, 1995; Dawson and Whittow, 2000; Banta and others, 2004). Ducks displaying acute signs associated with hypothermia were removed from their exposure trial and humanely euthanized by lethal injection with pentobarbital IV. When two or more of the exposed ducks in a treatment tank began to show acute signs associated with hypothermia, the exposure trial was terminated (American Veterinary Medical Association, 2007; Animal Welfare Act, 2008). At the completion of the experiment, all remaining ducks were humanely euthanized by lethal injection with pentobarbital IV, necropsied to assess their physiological condition, and tissue samples were collected from the birds for further analyses.

LC–MS Chemical Analysis

Water samples (250–1,000 mL) from each treatment site were collected prior to, during each assessment period, and after the completion of the exposure trials. Water samples were kept at 0°C in a standard refrigerator until chemical analysis. Feather rinsate samples were collected from all the ducks following laboratory methods developed by Chemir (C. Otten, Chemir, written commun., 2008). Feathers (outer and downy feathers were not separated nor dried prior to the solvent extraction procedure) were removed from the abdomen, where ducks made the most contact with the water, and the back, in response to the wetting patterns observed on the 2007 duck carcasses. Approximately 2.8 to 3.0 g of feathers were placed in a 120 mL glass jar to which approximately 100 mL of dichloromethane (CH₂Cl₂) was added to completely submerge the feathers. The sealed jars were shaken at approximately 180 rpm for 24 h at 25–30°C. The dichloromethane extract was filtered through PS 90mm Whatman[®] phase filter paper to remove solid material. The extract was then concentrated to approximately 10 mL of volume under a gentle stream of dry nitrogen at laboratory facilities located at the USGS Water Resources Division (Denver, Colo.). Briefly, the dichloromethane extracts had a consistent flow of dry nitrogen blowing over the extract's surface causing evaporation of the solvent without loss of the analytes. Once the extracts were concentrated, the glass vials were capped with

Teflon[®]-lined caps and stored at room temperature. These concentrated feather rinsate extracts and water samples were sent to Chemir for LC–MS analysis.

LC–MS analysis combines the techniques of high performance liquid chromatography (HPLC) and mass spectrometry (MS) to structurally characterize organic components of a complex matrix. HPLC is used for the separation of the compounds in the sample. A mixture of solvents, called the mobile phase, is forced at high pressure through a column packed with coated silica particles, called the stationary phase. Components in the mixture are separated based on the difference in their affinities for the stationary phase and the mobile phase, and can be detected and measured as they elute from the column. The time a chemical component spends in the column from injection until detection is known as retention time, and is an indicator of component identity when compared with the retention time of known standards under the same conditions. The measured peak area or height is concentration dependent and may be used to quantify the component. For LC–MS analysis, the effluent from HPLC is transferred to the mass spectrometer for mass analysis of the resolved components. Using an ion trap mass spectrometer, the components can be characterized by fragmentation, providing structural information for characterization of unknowns, or complete identification of known species by matching both the retention time and fragmentation pattern between the sample and a reference standard.

LC–MS samples were analyzed on a Finnigan Surveyor[®] HPLC coupled to a Finnigan[®] LCQ Advantage mass spectrometer (ThermoScientific, Waltham, Mass.). The solutions were analyzed by a general method that screened for nonionic surfactants, specifically those based on the alkyl ethoxylate or polyethylene glycol (PEG) backbone. Samples were analyzed by atmospheric pressure chemical ionization (APCI), liquid chromatography (LC), and mass spectrometry (MS) appropriate for the analysis of nonionic surfactants, such as ethoxylate or PEG and related compounds (Schroder, 2001). A total of 13 samples (10 abdominal feather rinsate samples and three water samples) were sent to Chemir for LC–MS analysis. Feather rinsate samples from eight of the exposed mallard ducks were selected on the basis of their wetting estimates. From the secondary clarifier and the chlorine contact basin tanks, rinsates from the two ducks rated the wettest from each group, and one of the three remaining samples from each group, respectively, were sent for analysis ($n = 3$ for each wastewater treatment). Two feather rinsate samples were randomly selected from the control group (all five ducks in this group ranked at the same wetness level). To compare differences in chemical levels between duck species, two northern shoveler abdominal feather rinsate samples (both of which had been exposed to wastewater at MWRD) were analyzed. In addition, three of the 56 water samples collected were also analyzed: one water sample from the secondary clarifier and chlorine contact basin tanks, respectively (collected within the last six hours of the exposure trial), and one water sample from the control water tank (collected at the midway point of the exposure trial).

Feather rinsate extracts were analyzed initially as-received, and re-analyzed after further concentration at Chemir. Briefly, 3.0 to 4.0 g of the solution were dried under a gentle stream of dry nitrogen to residue. Approximately 1.0 g of methanol was added to the residue. The residue was resuspended when the mixture was shaken, and then the insoluble material was allowed to settle. A portion of the supernatant liquid was analyzed for surfactants by LC–MS. Water samples were also analyzed as-received (poured directly into auto sampler vials) and re-analyzed following further concentration (by a factor of 2). A portion of the water samples were lyophilized to residue and reconstituted in water for analysis.

Statistical Analysis

Data collected on ambient physical conditions (air and water temperature) and the physiological responses in the exposed ducks (body temperature, body mass, water displacement, buoyancy, and feather wetting) were used to determine if subsequent changes from initial baseline values over the

duration of the exposure trial were significantly related. Data were compared using n-way and repeated measures analysis of variance (ANOVA) for within-factor interactions. All statistical analyses were performed using Systat[®] statistical program (Chicago, Ill.) at the $\alpha = 0.05$ level of significance.

Results

Wastewater Exposure Trials

The exposure trials were performed between February 2–5, 2008, at the MRWD facility to expose ducks to winter meteorological conditions and photoperiod similar to those that occurred during the 2007 die-off. Monthly average temperatures (National Climatic Data Center, 2008) for December 2007 were -2.9°C (-2.0°C from average), for January 2008, -2.3°C (-0.7°C from average), and for February 2008, 1.1°C (-0.4°C from average). Monthly precipitation rates ranged from 53.1 cm of snowfall in December 2007, 7.9 cm in January 2008, and 13.0 cm in February 2008. In comparison with the previous winter 2007, monthly average temperatures for December 2006 was -0.2°C (-0.8°C from average), for January 2007, -6.2°C (-4.7°C from average) and for February 2007, -1.6°C (-2.3°C from average). Monthly precipitation rates ranged from 74.7 cm of snowfall in December 2006, 40.4 cm in January 2007, and 14.0 cm in February 2007. Overall ambient air and tank water temperatures during the exposure trials are presented in figure 2. Over the duration of the exposure trials, ambient air temperature averaged -0.3°C , while treatment tank water temperatures depended on water source—control water temperatures averaged 4.6°C , secondary clarifier and chlorine contact basin water temperatures averaged between 13.6 to 14.1°C , similar to ambient water temperatures found in each of the original water sources.

During each assessment period, body temperature was measured to monitor hypothermic response of ducks in each treatment tank (figs. 3–5). A significant difference in body temperature existed between treatment groups ($F_{(2,112)} = 8.305$, $P < 0.05$). Neither ambient air temperature ($F_{(1,111)} = 0.621$, $P = 0.432$) nor tank water temperature ($F_{(1,111)} = 1.200$, $P = 0.276$) demonstrated a significant influence on observed body temperature. The control group had the highest average body temperature (41.9°C), followed by those in the chlorine contact basin (41.1°C), and lastly those in the secondary clarifier treatment group (40.8°C). At the control tank, all five ducks were able to maintain a relatively steady body temperature throughout the entire 93 hours of exposure (fig. 3B), showing no external signs associated with hypothermia. In comparison, two of five ducks in the secondary clarifier tank could not maintain body temperature for the duration of their exposure trial (fig. 4B). The most drastic change occurred in duck 5 at the secondary clarifier tank, whose body temperature dropped from 39.1°C to below 34.4°C in a four-hour period (between assessment periods 6 and 7, 17 hours into its exposure trial). The second most drastic body temperature change occurred in duck 3 at the secondary clarifier tank, whose body temperature dropped from 39.8°C to 36.4°C in a four-hour period (between assessment periods 6 and 7, 17 hours into its exposure trial). Both ducks 3 and 5 exhibited external signs associated with hypothermia (shivering, depressed head and wing position) and an increase in feather wetting as the exposure trial progressed. In the chlorine contact basin tank, one of five ducks could not maintain body temperature (fig. 5B). The body temperature of duck 1 at the chlorine contact tank dropped from 40.2°C to 36.0°C in a three-hour period (between assessment periods 4 and 5, 12 hours into its exposure trial). Also, two of the other five ducks at the chlorine contact basin (ducks 1 and 3) began exhibiting external signs associated with hypothermia (shivering, depressed head and wing position) and an increase in feather wetting between assessment periods 4 and 5 (12 hours into the exposure trial).

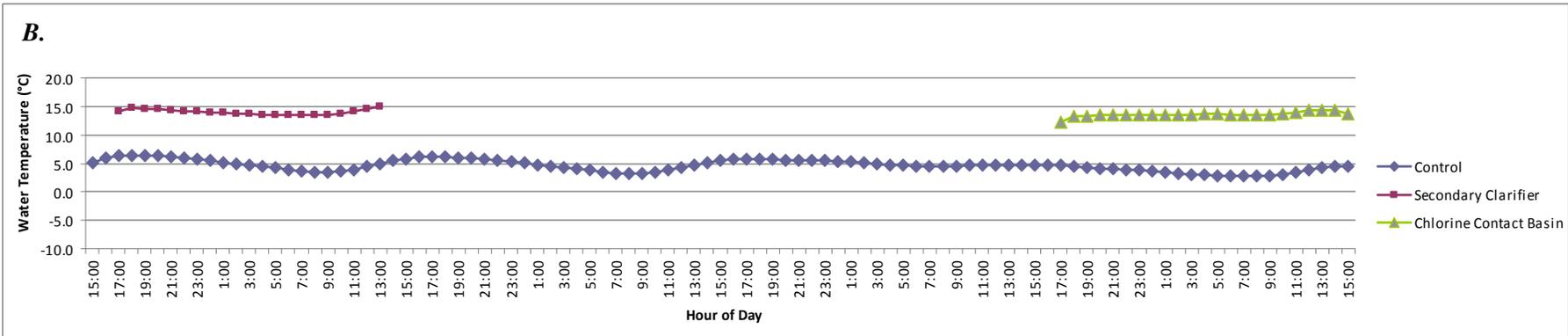
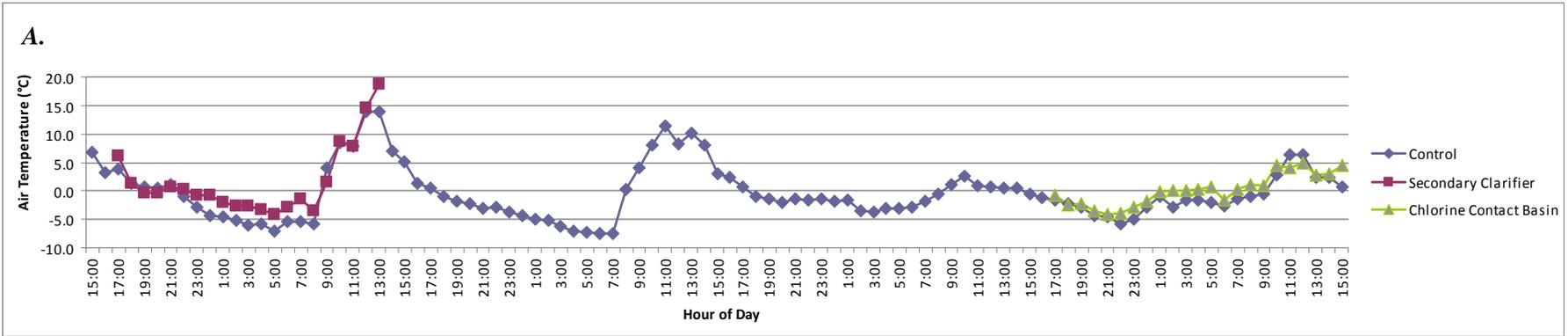


Figure 2. A. Air temperatures recorded from HOBO[®] temperature loggers placed approximately 30.5 cm above the water line in each temporary tank for the duration of each trial. B. Water temperatures recorded from HOBO[®] temperature loggers placed approximately 30.5 cm below the water line in each temporary tank for the duration of each trial.

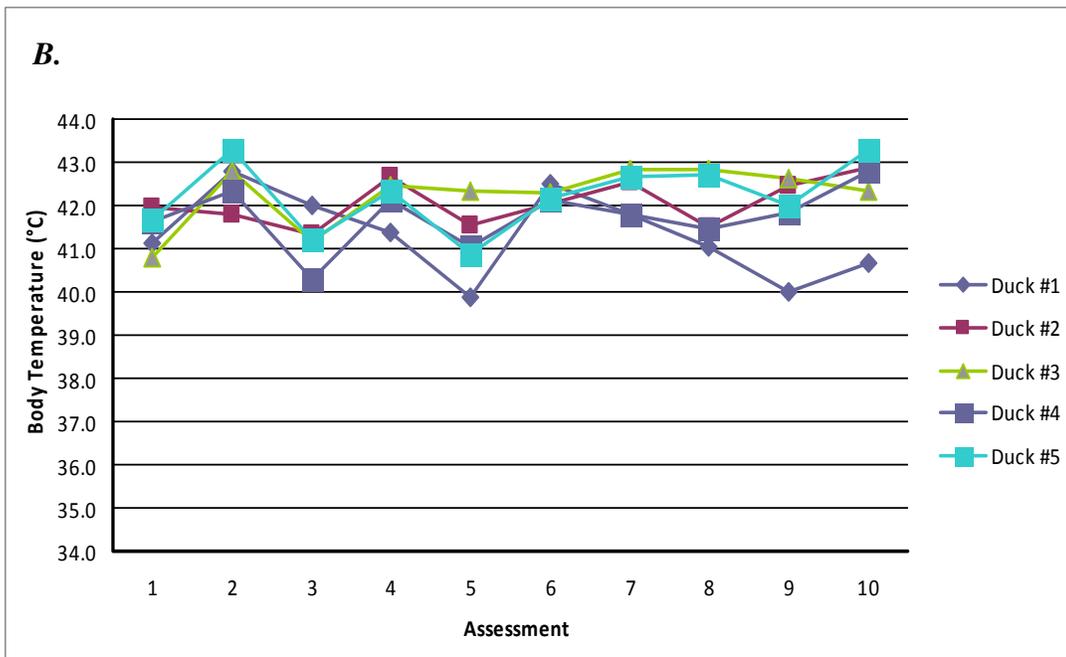
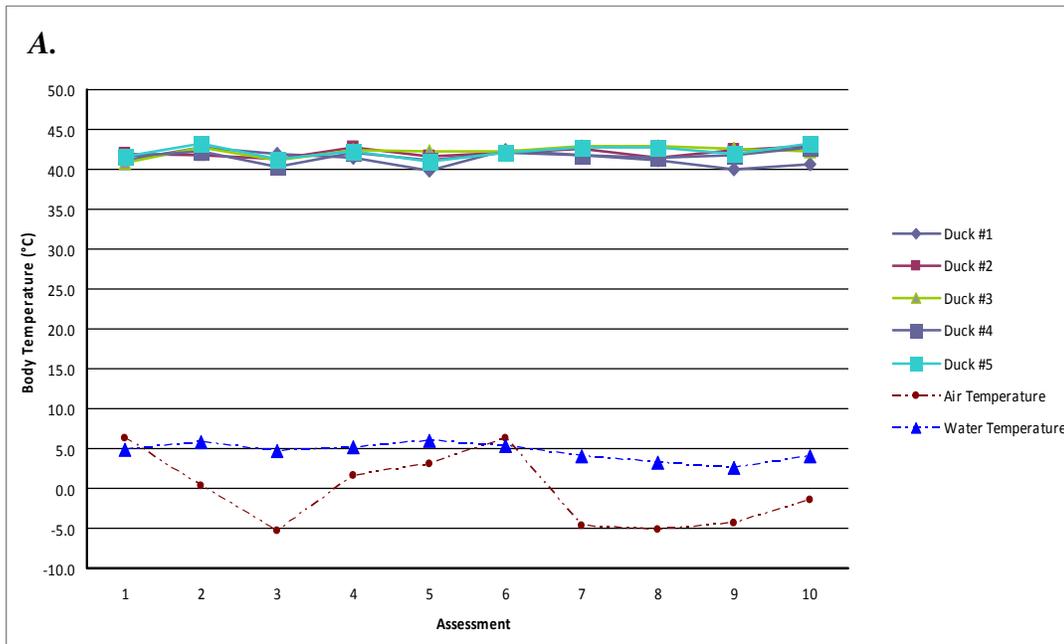


Figure 3. *A.* Individual body temperatures for ducks and ambient air and water temperatures at the control tank site. *B.* Enlarged (note change in temperature scale) comparison among individual duck body temperatures at the control tank site. Assessments were from hour 0 (Assessment 1) to hour 93 (Assessment 10) of the trial.

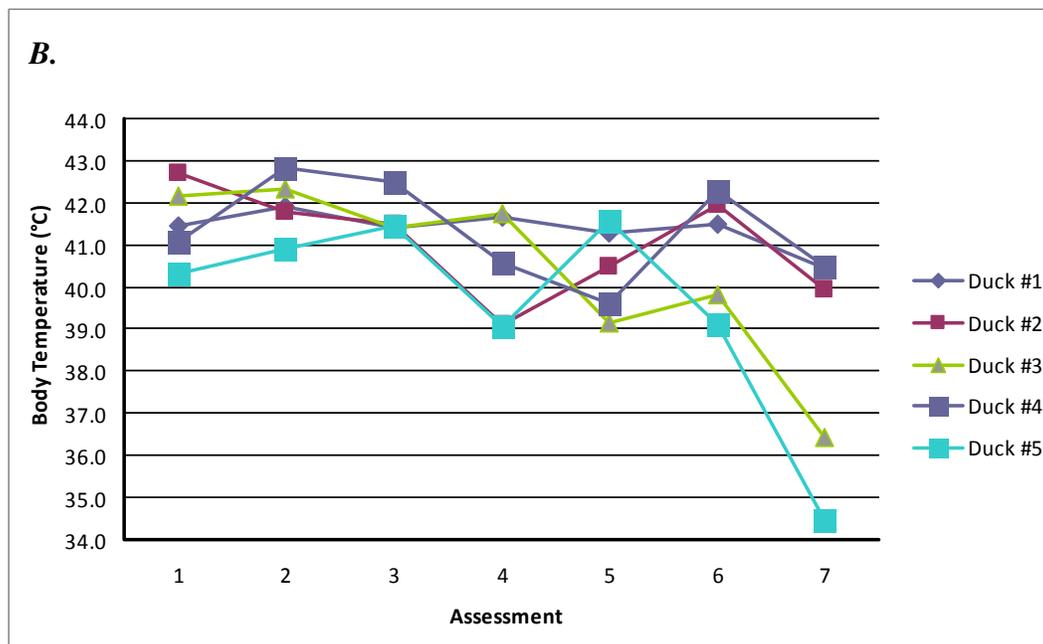
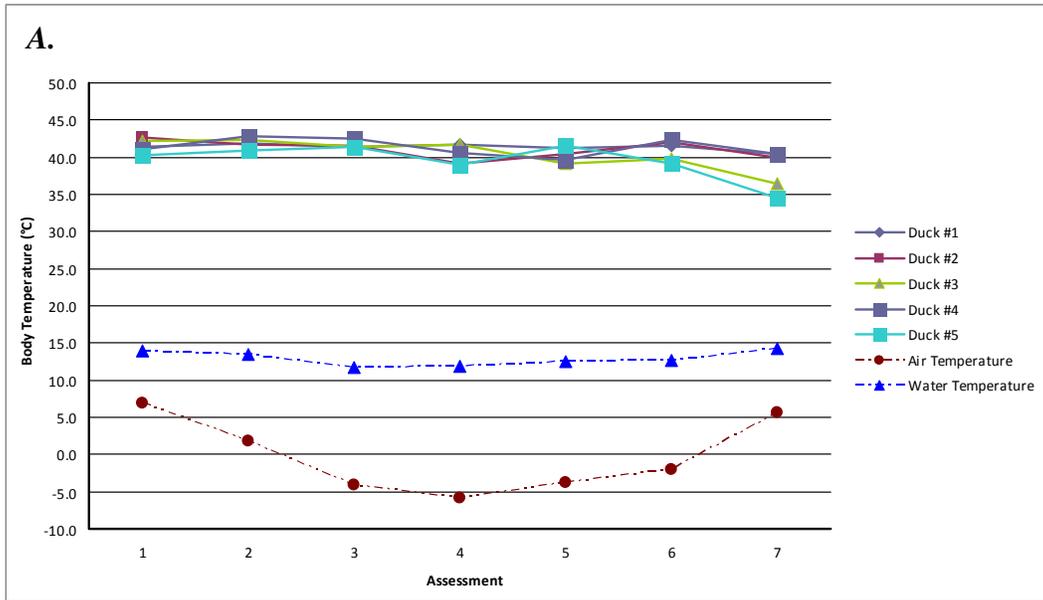


Figure 4. A. Individual body temperatures for ducks and ambient air and water temperatures at the secondary clarifier site. B. Enlarged (note change in temperature scale) comparison among individual duck body temperatures at the secondary clarifier site. Assessments were from hour 0 (Assessment 1) to hour 21 (Assessment 7) of the trial. Note that ducks #3 and #5 were showing clinical signs of hypothermia during Assessment 7.

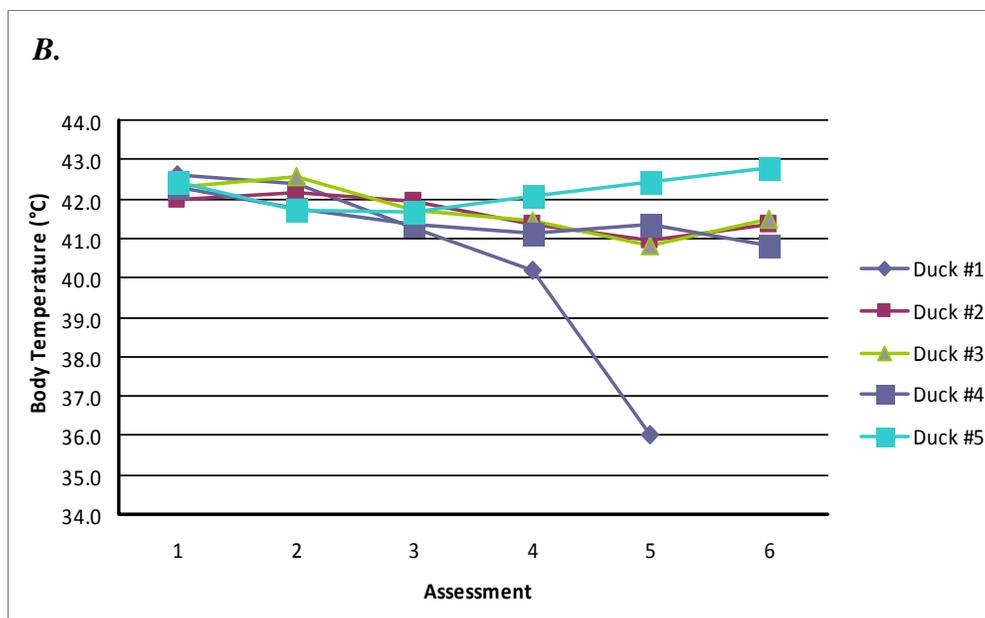
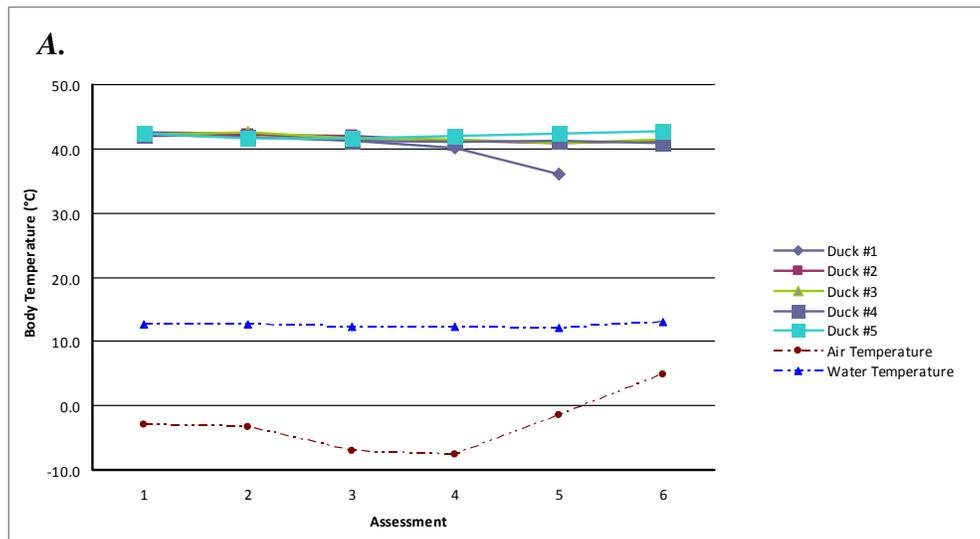


Figure 5. A. Individual body temperatures for ducks and ambient air and water temperatures at the chlorine contact basin site. B. Enlarged (note change in temperature scale) comparison among individual duck body temperatures for chlorine contact basin site. Assessments were from hour 0 (Assessment 1) to hour 19 (Assessment 6) of the trial. Note that duck #1 was showing clinical signs of hypothermia during Assessment 5 and was euthanized.

Body mass was recorded to assess whether increases in mass related to wetting of the feathers could be measured. However, a high level of variability in individual mass measurements between assessment periods (as great as ± 400 g over a three-hour period) indicated that these measures were not reliable enough for further statistical analysis.

Water displacement measurements ranged between 0.6 to 1.7 L among control tank ducks, 0.025 to 2.0 L among secondary clarifier ducks, and 0.6 to 1.7 L among chlorine contact basin ducks. However, a high degree of variation in displacement values among control group ducks ($F_{(4,45)} = 4.35$, $P = 0.005$) indicated that comparison of these values between treatment groups was not reliable enough for further statistical analysis.

Photographs taken of ducks in the aquarium visually recorded changes in buoyancy among the different individuals and treatment groups (figs. 6–8). “Normal” duck buoyancy was characterized by birds floating in a fairly horizontal plane with tail and wings generally maintained above the waterline and exhibiting good water beading on its plumage. Control tank ducks appeared to maintain their buoyancy and waterproofing despite the extended period that they were kept in their tank (fig. 6). However, duck 5 in the secondary clarifier tank showed an appreciable change in its buoyancy from assessment period 3 (7 hours into its exposure trial) to its final assessment period 7 (19 hours into its exposure trial; fig. 7). This duck struggled to maintain its head above water, demonstrated signs of increased feather wetting over this time period, flotation lower in horizontal plane (that is, it appeared to be lower in the waterline), and wings drooped below the waterline. The bird was removed from the exposure trial and immediately euthanized after assessment period 7. Duck 3 in the secondary clarifier tank showed signs of increased feather wetting and reduced buoyancy by assessment period 5 (13 hours into its exposure trial), but was not struggling to keep its head above water. Duck 1 in the chlorine contact basin tank began showing signs of increased wetting and swimming lower in the water beginning at assessment period 4 (12 hours into its exposure trial) until its final assessment period 5 (15 hours into its exposure trial). By this final assessment period, this duck was swimming much lower in the water, shivering, and showing signs of wetting through its down feathers (fig. 8); it was removed from the exposure trial at this time and euthanized. Duck 3 in the chlorine contact basin showed similar signs of shivering, increased feather wetting, and lower wing and body posture in the water by assessment period 5 (15 hours into its exposure trial).

Scores for feather wetting were subjectively assessed on a numerical scale of 0 to 5. Ducks in the control group were consistently rated as dry (0) or mildly damp (1) throughout the exposure trial, despite the extended duration (93 hours) that these ducks spent in this tank. In comparison, two of five ducks in the secondary clarifier group received wetting scores of 3 or greater at some point during the study trial—duck 5 had a maximum wetting value of 4 (at hours 11, 13, and 16 of the exposure trial), and duck 3 had a maximum wetting value of 3 (at hours 13, 16, and 19 of the exposure trial). All five ducks in the secondary clarifier group received wetting scores of 2 or greater at some point in the study trial. At the chlorine contact basin tank, two of five ducks received wetting scores of 3 or greater at some point during the study trial—duck 1 had a maximum wetting score of 4 (at hour 15 of the exposure trial), and duck 3 had a maximum wetting score of 3 (at hours 15 and 19 of the exposure trial). One of the remaining five ducks in this group received a wetting score of 2, while the other two ducks received wetting scores of 0 or 1. To assess if wetting values were related to changes in body temperature, a repeated-measures ANOVA was performed. All birds were pooled for analysis to assess the relationship between wetness scores and body temperature, which was significant ($F_{(5,9)} = 7.05$, $P = 0.006$), indicating that increased level of wetness score was related to declines in body temperature.

To record time activity budgets of the exposure trial ducks, infrared motion-sensing trail cameras were used to remotely monitor behavioral activity in the tanks. However, the majority of the



Figure 6. Example of control tank duck. Control duck 2, at end of exposure trial (93 hours exposure to control tank water). Note tail and wing above the water line and upright position of the head.

images was of poor quality (due to rapid movement of birds in the experimental tanks and flash glare) and did not adequately capture specific behavioral activities of individuals or groups as intended. Some behavioral information gleaned from the photographs showed that despite being forced to maintain contact with the water surface, ducks were preening, feeding, head dipping, and raising their bodies off the surface of the water as their exposure trials progressed. Although these behaviors may also indicate that ducks were possibly responding to some irritant in the water (P. Henry, written commun., 2008), in general, the constant movement, paddling, water shaking, and wing flapping seemed more consistent with behaviors described as increasing discomfort due to thermal imbalance (Fabricius, 1956; Choules and others, 1978; de Vries and van Eerden, 1995) and behaviors associated with increasing metabolic rate to raise body temperature (Welty, 1982; Gill, 1995; Dawson and Whittow, 2000).



Figure 7. Example of secondary clarifier duck. Secondary clarifier duck 5 was wettest in this group, showing signs of wetting at 19 hours into its exposure trial. Note tail and wings well below water line and depressed position of head.



Figure 8. Example of chlorine contact basin duck. Chlorine basin duck 1 was wettest from this group (at hour 15) and shows the tail, a majority of the wing, and a large proportion of the anterior aspect of the body below the water line.

The observations of free-ranging ducks provided additional information on the activity and behavior of ducks at this facility. Data were collected over 22 time periods between 1800 hours on February 2, 2008, through 1100 hours on February 5, 2008. Observations were made primarily in the early evening (approximately 1700 hours) through the night and early morning (approximately 1100 hours). The minimum observed number of waterfowl using the treatment facility water was 13 birds on February 2nd, 1700 hours. The maximum number of waterfowl was 357 birds on February 4th, 2300 hours. The greatest number of waterfowl observed in and around MWRD (including the South Platte River) was approximately 635 birds (195 on the secondary clarifier tanks, 40 on the chlorine contact basin, and 400 on the South Platte River adjacent to the MWRD facility). The average number of waterfowl using MWRD impoundments containing the treatment facility water throughout the day (not including between 1200 hours and 1600 hours) was approximately 115 birds. During nocturnal observations, ducks (predominantly shovelers, but also mallards and American wigeon, *Anas americana*) in the secondary clarifier tanks were exhibiting feeding behaviors throughout these tanks, both near the water inlet to the clarifier tank (where activated sludge containing microorganisms enter the tank), and in the scum layer (grease and oil materials that rise to the surface). Ducks were actively feeding in both areas and did not appear to be avoiding the scum layer or skimming boom areas. The number of ducks using the secondary clarifier tanks seemed to increase during inclement weather conditions, characterized by lower overnight temperatures and higher wind chills (W. Iko, personal observation). Nocturnal behavior in the chlorine contact basin was similar, such that ducks were exhibiting feeding behaviors, but also were observed preening. Behaviors of ducks during daylight hours included feeding and preening, as well as loafing/preening and sleeping, both in and out of the water in the MWRD tanks and along the rocky shorelines of the South Platte River.

LC-MS Chemical Analysis

Feather rinsate extracts were initially analyzed as-received, without concentration, using liquid chromatography with three forms of detection: (1) positive ionization APCI, (2) negative ionization APCI, and (3) ultraviolet (UV) response at 214 nanometers (Knepper and others, 2003). The solutions were analyzed by a general LC-MS method that screened for surfactants, specifically those based on nonionic surfactants. No major components were observed in these solutions. A UV peak at about 32 minutes in the control mallard no. 1 sample was associated with a MS ion at m/z of 391, likely due to bis(2-ethylhexyl)phthalate, a common plasticizer often observed as background response from the HPLC tubing and other plastic components in the analytical system.

The three water samples, initially analyzed as-received, all contained a LC peak at about 2.7 minutes, eluting approximately with the solvent front for the injection. The positive and negative MS analysis reflected only an increase in background noise, consistent with a salt front passing through the instrument. This type of peak usually is due to inorganic salt or buffer in the solution. No other peaks were identified that differed significantly from those observed in the water blank samples used as controls.

Because of lack of detection of any chemicals during the initial as-received analyses, both feather rinsate and water samples were further concentrated by gently evaporating the solvent and reconstituting the residue in methanol for reanalysis by LC-MS. Table 1 summarizes the sample preparation for the feather rinsate and water samples. The amount of residue remaining after concentration from the recorded weight of the sample that was concentrated was used to calculate the residue concentration in the reconstituted solution. This was used to calculate the amount of nonionic surfactant present in each extract solution, as presented below. Concentrated rinsates from feather sample residues in methanol were reanalyzed. Unlike the as-received solutions, these concentrated feather solutions showed significant response in both positive and negative ionization modes. For the

Table 1. Sample preparation and concentration weights of duck feather rinsates and water samples.

Sample description	Treatment tank	Total extract weight g	Total weight of residue g	Weight of residue	Methanol to reconstitute g	Residue concentration mg/L
Control mallard #1	Control	3.5459	0.0065	0.18 %	1.0020	6,487
Control mallard #2	Control	3.3968	0.0047	0.14 %	1.0000	4,700
Secondary clarifier mallard #3	Secondary clarifier	3.2937	0.0072	0.22 %	1.0470	6,877
Secondary clarifier mallard #4	Secondary clarifier	3.6842	0.0041	0.11 %	1.0038	4,084
Secondary clarifier mallard #5	Secondary clarifier	3.4287	0.0097	0.28 %	1.0235	9,477
Chlorine contact basin mallard #1	Chlorine contact basin	3.4967	0.0052	0.15 %	1.0281	5,058
Chlorine contact basin mallard #3	Chlorine contact basin	3.3285	0.0043	0.13 %	1.0025	4,289
Chlorine contact basin mallard #5	Chlorine contact basin	4.0737	0.0397*	0.97 %	1.0057	3,9475
Northern shoveler #1	MWRD facility, rehab. center	3.8063	0.009	0.24 %	1.0045	8,960
Northern shoveler #2	Chlorine contact basin	3.8315	0.0063	0.16 %	1.0379	6,070
Control water	Control	10.1031	0.0093	0.092 %	5.0262	1,850
Secondary clarifier water	Secondary clarifier	10.1807	0.0063	0.062 %	5.0037	1,259
Chlorine contact basin water	Chlorine contact basin	10.3207	0.0049	0.047 %	5.2138	940

*Sample may not have been completely dry at time of weighing.

control feather extracts, significant differences were present between the two samples (in fact, the control mallard no. 1 exhibited a significant difference from all of the other feather rinsate samples). As a consequence of the sample concentration step, the peak in the UV chromatogram at about 32 minutes, observed in the initial screen of the as-received sample, was much greater in the concentrated sample. This component, with an ion at m/z of 391, likely bis(2-ethylhexyl)phthalate, was not observed in any of the other feather samples after sample concentration. As noted above, this is a common plasticizer that is often observed in water blank samples used as controls. Second, this sample had a significantly larger LC-MS response in the elution range from 15 to 30 minutes. Much of this was due to nonionic ethoxylate PEG-related surfactants. Among the secondary clarifier feather extracts, some differences were observed in peak intensities, but no significant difference in composition was observed. The same was true among feather extracts from birds in the chlorine contact basin. Among the northern shoveler feather extracts, there again appeared to be a difference in the peak intensities between the two samples; however, these differences likely were due to concentration level rather than the presence of different components.

Various nonionic PEG surfactants were identified from both the treatment and control tank ducks, including progressions that matched those for the octyl (C_8), decyl (C_{10}), and dodecyl (C_{12}), alkyl PEG surfactants as well as a progression that matched octyl and nonyl phenol ethoxylates, listed as homolog groups of ions (table 2). The distribution and range of the homolog groups varied considerably in each sample extract. Any progression of nonionic PEG surfactants that were observed in the laboratory controls (water blanks) was ignored in the PEG quantitation of the samples as compounds found in the controls were considered normal background concentrations. Only the control mallard no. 1 feather rinsate sample contained all eight of these progressions. Five of these progressions could be assigned based on the ions observed (table 2). All of the ethoxylate PEG species were detected as clusters associated with the ammonium cation, common under these conditions in positive APCI. The five assigned PEG species are assigned as follows (Lara-Martin and others, 2006):

octyl alcohol ethoxylates	C_8AEO	$C_8H_{17}-(OCH_2CH_2)_n-OH$
decyl alcohol ethoxylates	$C_{10}AEO$	$C_{10}H_{21}-(OCH_2CH_2)_n-OH$
dodecyl alcohol ethoxylates	$C_{12}AEO$	$C_{12}H_{25}-(OCH_2CH_2)_n-OH$
octyl phenol ethoxylates	OPEO	$C_8H_{17}-C_6H_4-(OCH_2CH_2)_n-OH$
nonyl phenol ethoxylates	NPEO	$C_9H_{19}-C_6H_4-(OCH_2CH_2)_n-OH$

These PEGs are common surfactants that are present in many household and industrial products.

Due to high complexity of the sample mass spectra, the assignments listed in table 2 were not confirmed by comparison with standards, but the ions observed and the elution order presented is consistent with these assignments. The ions listed in table 2 were used to determine the peak areas for each of these progressions in the samples in which they were observed. The peak areas for all of the surfactant components in each sample were summed and used to estimate the observed relative concentration of PEG in each sample. Relative concentration amounts could be measured, and an approximate concentration amount was determined using a calibration curve run under similar conditions within a week of the formal analyses. These calibration values provide an estimate for the concentration amounts detected among the examined samples, which can assist with comparing the effects across the examined samples, as well as in understanding the possible effects upon the birds from the presence of these ethoxylate PEG species on the feathers.

Estimates for the amount of nonionic ethoxylate PEG surfactant in each feather rinsate extract are shown in table 3, both as a relative concentration amount (with the high control extract [control

Table 2. Mass-to-charge ratio of nonionic surfactant ions (as ammonium adducts) and their assignments for the alkyl polyethylene glycol (PEG) ethoxylate ion progressions observed in the duck feather extracts.

[C₈AEO attributed to octyl alcohol ethoxylates; OPEO attributed to octyl phenol ethoxylates; C₁₀AEO attributed to decyl alcohol ethoxylates; NPEO attributed to nonyl phenol ethoxylates; C₁₂AEO attributed to dodecyl alcohol ethoxylates]

C ₈ AEO	unknown	OPEO	C ₁₀ AEO	NPEO	unknown	C ₁₂ AEO	unknown
280		312	370	350	336	350	
324	332	356	352	414	394	380	394
368	376	400	396	458	438	424	438
412	420	444	440	502	482	468	482
456	464	488	484	546	526	512	526
500	508	532	528	590	570	556	570
544	552	576	572	634	614	600	614
588	596	620	616	678	658	644	658
632	640	664	660	722	702	688	702
676	684	708	704	766	746	732	746
720	728	752	748	810	790	776	790
764	772	796	792	854	834	820	834
808	816	840	836	898	878	864	878
					922		922

mallard 1] as the reference) and as a calculated concentration amount (an overall estimated concentration). Nonionic PEG surfactant concentrations ranged from 0.98 mg/L in the feather rinsate sample from mallard 5 from the secondary clarifier tank, to 10.4 mg/L in the feather rinsate sample from mallard 1 from the control tank. Looking across the range of samples, the concentration found in the feather rinsate sample from mallard 2 from the control tank (1.44 mg/L) is within the range of the concentrations found in feather rinsate samples from ducks exposed to secondary clarifier and chlorine contact basin water (fig. 9). However, the concentration of 10.4 mg/L from the feather rinsate sample from control mallard 1 was well above that of the range from ducks exposed to secondary clarifier and chlorine contact basin water. Furthermore, samples from mallards 4 and 5 exposed to the secondary clarifier water and mallard 1 exposed to chlorine contact basin water were lower in concentration amounts than both samples taken from ducks from the control water. In comparing differences between duck species, the concentrations in the two northern shoveler extracts (average = 2.46 mg/L) fell approximately in the middle range of all the feather rinsate samples. Given the levels estimated in the extract for these samples, the lack of detection in the screen of the as-received samples is not unreasonable. The typical limit of detection (LOD) for ethoxylate PEG species with the method used is on the order of 1–10 µg/L in solution; all these solutions were near that threshold as determined by using this estimation procedure.

After the lack of detection of nonionic surfactants or other compounds in the three water samples during the initial as-received screening, these water samples were further concentrated by a factor of

Table 3. Total nonionic polyethylene glycol (PEG) surfactant quantitation from duck feather rinsates. [mg/L, milligrams per liter]

Sample description	Treatment tank	Relative amount	Calculated amount in extract (mg/L)
Control tank mallard #1	control	1.00	10.40
Control tank mallard #2	control	0.14	1.44
Secondary clarifier mallard #3	secondary clarifier	0.12	1.25
Secondary clarifier mallard #4	secondary clarifier	0.49	5.06
Secondary clarifier mallard #5	secondary clarifier	0.28	2.90
Chlorine contact basin mallard #1	chlorine contact basin	0.15	1.57
Chlorine contact basin mallard #3	chlorine contact basin	0.10	1.07
Chlorine contact basin mallard #5	chlorine contact basin	0.09	0.98
Northern shoveler #1	MWRD facility, rehab. center	0.26	2.72
Northern shoveler #2	chlorine contact basin	0.21	2.20

two by gently evaporating the solvent and reconstituting the residue in methanol for reanalysis by LC–MS (table 1). Other than minimal peaks that eluted near the solvent peak, which is typical of buffers or salts, the water samples were generally free of detectable surfactants. No nonionic ethoxylate PEG surfactants were detected in the concentrated water samples, and therefore no quantitation of PEG surfactants or comparison to the surfactants detected on the duck feathers could be performed.

Discussion

The results of these waterfowl exposure trials demonstrated that the feathers of some of the ducks exposed to treated wastewater originating from the secondary clarifier tanks and the chlorine contact basin at MWRD had their waterproofing compromised. However, no clear conclusions can be drawn regarding what specifically caused this effect. Exposed birds displayed signs associated with hypothermia within 17 hours of exposure in the secondary clarifier tank, and within 12 hours of exposure in the chlorine contact basin tank. As their exposure trials progressed, body temperature of two of the five ducks in the secondary clarifier and two of the five ducks from the chlorine contact basin tanks declined, visual changes in buoyancy occurred, and increased wetting of their feathers was observed. These ducks were also observed shivering, constantly paddling, and flapping their wings to raise their body off the water surface (behaviors associated with increasing metabolic rate to raise body temperature; Welty, 1982; Gill, 1995; Dawson and Whittow, 2000). In comparison, ducks exposed to water in the control tank maintained a relatively constant body temperature, buoyancy, and displayed no signs of hypothermia, despite the much longer duration of their exposure and the colder water temperatures they were exposed to in the control tank. These results also suggest that the type of water

Total Nonionic Surfactant from Duck Feather Rinsates

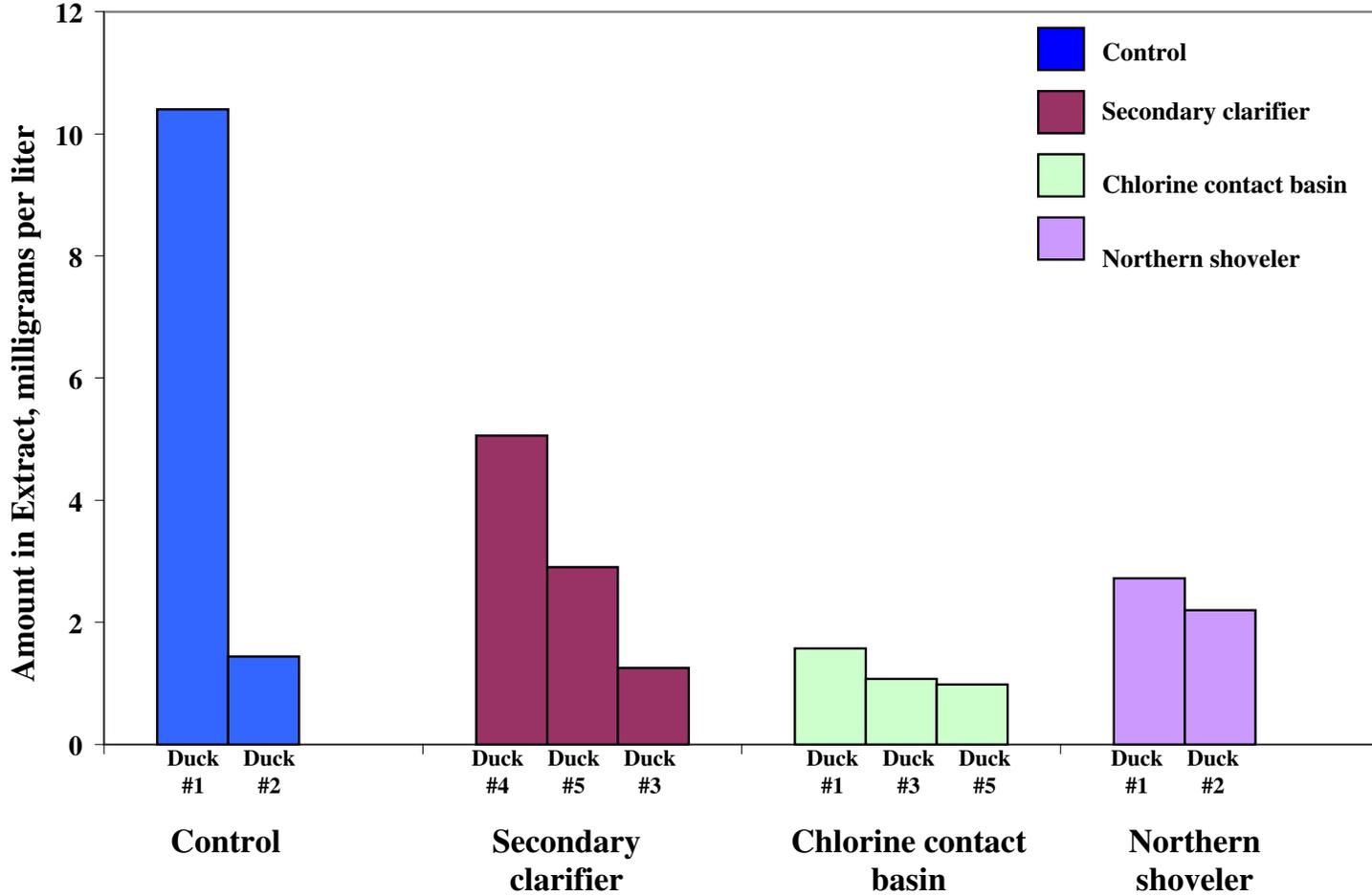


Figure 9. Total nonionic polyethylene glycol (PEG) concentration comparison by treatment. Control ducks were exposed to ditch water, secondary clarifier ducks were exposed to water from secondary clarifier tank, chlorine contact basin ducks were exposed to water from the chlorine contact basin, and the northern shoveler ducks had been exposed to wastewater prior to mortality.

(treated wastewater versus ambient water) ducks had been exposed to had a greater impact on body temperature than ambient conditions alone.

As mentioned, to elucidate a hypothermic response in the ducks exposed to treated wastewater, the experimental birds were forced to maintain contact with the water surface in each treatment tank for the duration of their exposure trial. Although some of the ducks were able to remove themselves from the water early in the experiment (during the secondary exposure trial), birds exposed to treated wastewater still showed a loss of waterproofing whereas ducks exposed to the control water did not. In fact, it is possible that the effects of secondary clarifier treatment water exposure on duck body temperature and estimates of buoyancy and feather wetting may have occurred sooner in the exposure trial (rather than after 17 hours of exposure), if continuous water surface contact had been maintained throughout the trial.

The feather rinsate extracts were analyzed by LC-MS to identify components present in rinsates from the experimental ducks, under the hypothesis that such components would exist in the secondary clarifier and chlorine contact basin but not in the control site. Based on indications from the previous preliminary chemical analysis performed in 2007, these analyses were specifically looking for nonionic ethoxylate PEG surfactants that may have been present in the feather rinsate extracts and the treatment water samples. However, the LC-MS analysis of feather rinsates detected the presence of ethoxylate PEG surfactants in all of the feather rinsates, including the control group samples, indicating an inconsistency between the physiological effects that occurred to ducks during the exposure trial and the subsequent LC-MS analysis results. Nonionic PEG polymers are commonly used as surfactants and thickeners in a wide range of commercial and pharmaceutical products, and have been studied extensively (Gonzalez and others, 2007). The base for these polymers is the repeating unit $-\text{CH}_2\text{CH}_2\text{O}-$. Most also contain some type of functional alkyl group at one end of the chain, and hydroxyl at the other end $[\text{R}-(\text{OCH}_2\text{CH}_2)_n-\text{OH}]$. The chemical nature of this functional group will change depending on the desired properties of the polymer. Nonionic PEG based polymers were easily identified in the mass spectral data because of their close sequential elution and characteristic spacing of 44 units between successive chain lengths.

Because of the variation and range of concentrations of the ethoxylate PEG surfactants in both treatment and control duck feather rinsates, no clear conclusions can be established that PEG surfactants found on affected ducks exhibiting increased feather wetting, loss of body temperature, and subsequently, symptoms of hypothermia, were responsible for the impact on feather waterproofing that was observed. That is, LC-MS results indicated no statistical difference in PEG surfactant levels between birds from the secondary clarifier and chlorine contact basin compared to ducks in the control group. These results indicate that analysis of residual nonionic PEG surfactants alone is not sufficient to differentiate between the effects observed between treatment and control ducks in this study.

Why there is an apparent discrepancy between the exposure trial results and the LC-MS analysis may be due to a number of factors. One explanation may have been the small sample size of ducks used in this experiment, making comparisons between the physical responses of individual birds and their feather chemical loads difficult. Also, in general, quantification of surfactants is difficult due to the wide range of compounds that are manufactured and used, and the lack of reference standards due to the fact that commercial PEG surfactants are a mixture consisting of a series of PEG with different molecular weights. The APCI analytical method is specific for nonionic surfactants such as PEGs and may therefore have been limited in addressing other surfactant groups present. Other explanations for these results could be possible cross-contamination with ethoxylate PEG surfactants during sample preparation in the laboratory, due to the presence of chemicals in many commercial products (Im and others, 2008). However, with only two control samples, it was not possible to determine if this is an outlier result. It was also assumed that chemical residues detected in feather rinsates would be a probable causal agent affecting waterproofing. These samples were derived from rinsing body feathers

with a solvent and therefore represent residue material left on the feathers, not necessarily all the chemicals that ducks were exposed to but rinsed off of the plumage prior to sampling. If a causal chemical agent was present in wastewater but not accumulated in the duck feathering, it is less likely that this substance would have remained present on the feathers and subsequently detected using this rinsate method (although the previous data collected in 2007 from scanning electron microscopy, which detected a substance on the feather microstructure, and the lack of waterproofing observed in rehabilitated ducks may make this explanation less likely).

The presence of ethoxylate PEG compounds found in all the feather rinsates indicate that the ducks had been exposed to this surfactant, either prior to, or during, the exposure trial. PEG surfactants are ubiquitous in wastewater effluent, receiving waters, and other downstream water bodies in North America (Knepper and others, 2003). The Burlington Ditch used as a control for this experiment diverts water from the South Platte River upstream of the Metro Wastewater outfall. The water in that portion of the South Platte River largely comes from releases from Chatfield Dam and effluent from the Littleton/Englewood wastewater treatment plant located upstream. Other minor tributaries such as Cherry Creek and Bear Creek also contribute flow during times of the year. The relative quantity of the waters varies throughout the year in response to factors that include water-right calls on the river. During the winter months when the experiment was conducted, the water is typically effluent dominated.

In the facility itself, degradation of surfactants takes place with high effectiveness in wastewater treatment plants (Matthijs and others, 1999), removing greater than 96 percent of anionic and 99 percent of nonionic surfactants through aerobic processes that generate carboxylated acids, sulfophenyl carboxylic acids, and alkyl phenol ethoxycarboxylates (Barco and others, 2003). The degradation of already very complex mixtures of surfactants subsequently generates additional very complex mixtures in wastewater treatment plant effluents (Lara-Martin and others, 2006). Given the prevalence of surfactants in the environment, it is possible that the high variability of ethoxylate PEGs detected in all the feather samples is due to a natural range of concentrations on the ducks in the wild (either through lengthy accumulative exposure at modest concentrations or short-term exposure to high concentrations). It is also possible that ethoxylate PEG chemicals were present in all of the water treatments in the experiment and therefore its detection on duck plumage may not be unusual among free-ranging waterfowl. However, its presence on all the ducks (including the control group that showed no hypothermic response) also suggests that ethoxylate PEG-related compounds may not even be a causal agent affecting the loss of duck waterproofing observed during the exposure trials. Because of the high variability in ethoxylate PEG concentration between feather rinsates from the control and treatment ducks, a larger number of samples would be required to detect statistically significant differences, which may be prohibitively expensive in determining the causal chemical agent. However, given the results from this study, despite its small sample size, further consideration of additional experiments should not be precluded, including further chemical assessments of anionic and cationic surfactant compound effects on the waterproofing of ducks.

The lack of detection of any nonionic surfactants in the three water samples analyzed is unusual given the number of surfactants known to be found and intensively studied in other wastewater research (Gonzalez and others, 2007; Lara-Martin and others, 2006; Loos and others, 2007; Schroder, 2001). Although it is possible that surfactant compounds sorbed onto the sides of the holding container or that the chemicals degraded during sample storage, it is also possible that the water samples needed to be more concentrated for detection to occur. When samples were concentrated by a factor of two, some surfactants were detected, but these concentrations were near the detection limit for the instrumentation and analytical method. Most studies involving surfactant detection in wastewater usually involved concentration by a factor of at least 500 to 1,000 (Knepper and others, 2003); therefore, it is possible that the water samples were not sufficiently concentrated by the methods employed to produce useful

information. The lack of PEG detection in the water samples further confounds a reasonable explanation for the presence of ethoxylate PEG compounds on all the ducks.

The LC-MS results, however, do not preclude that other surfactant chemicals could have been involved in the loss of duck waterproofing observed in this study. Although nonionic PEG surfactants may not have caused the problems, there are numerous other surfactant compounds found in the environment. In general, surfactants are an important ingredient in a broad spectrum of household cleaning products, pharmaceuticals, and industrial applications. Surfactants are used to reduce the surface tension of water and the interfacial tension between oil and water, allowing dirt or grease adhered to various articles to be washed off. Surfactants are classified into three main types: anionic, nonionic (such as ethoxylate PEG compounds), and cationic, based on the type of charged hydrophilic group present. They are manufactured and used in the United States in proportions of approximately 45 percent anionic, 35 percent nonionic, and 20 percent cationic types, and are often used in complex mixtures or combination with other types (Im and others, 2008). Commercial mixtures of surfactants also consist of several tens to hundreds of homologues, oligomers and isomers of anionic, nonionic, and cationic compounds, and therefore, identification and quantification in the environment is complicated and cumbersome (Schroder, 2001). These large-volume chemicals are extensively manufactured worldwide and are frequently found in wastewater treatment plant effluent and other natural water systems (Chiron and others, 2000; Shon and others, 2006; Loos and others, 2007). Also, although the term “surfactant” lumps together a wide variety of compounds in one word, the characteristics of each class are widely diverse and complex. Cleaning, emulsifying, foaming, reducing eye and skin irritation, viscosity, softness, and anti-static are some aspects that these compounds can impart upon use. For example, hair shampoos and conditioners both contain surfactants, but they contain very different types (Im and others, 2008). The surfactant effects that would be most damaging to the waterproofing of the duck feathers would more likely be those from the conditioner surfactants (enabling water to penetrate), rather than those from shampoo surfactants (cleaning and foaming). A specific surfactant class responsible for the effects observed on the ducks may not be the most widely used or at the highest concentration.

Therefore, although the ethoxylate PEG surfactants identified in the feather rinsates may not have been primarily responsible for affecting the loss of waterproofing in ducks, one of the numerous other surfactants (or combination of surfactants) could still have played a role. However, without better quantification of the different surfactant groups and chemicals found in the MWRD wastewater, more accurate identification of a chemical causal agent affecting duck waterproofing is currently not possible.

Although a causal chemical agent affecting the loss of duck waterproofing in this study was not definitively identified, the potential for the adverse impact of surfactants on birds is well documented. In fact, surfactants have been used as a control agent on avian agricultural pest species (Lustick, 1976). Choules and others (1978) found that despite a mixture of industrial chemicals present at a waste basin pond they were investigating, the presence of surfactants in the wastewater had the greatest impact on duck feather wetting and mortality. In their experiment, they found that ducks placed in laboratory tanks of wastewater, or water with a similar concentration of detergents (19 ppm of sodium dodecyl sulfate, an anionic surfactant compound), became soaked through their feathers within 30 minutes. These birds rapidly lost body temperature and showed hypothermic responses within 2.5 hours.

Surfactants may be responsible in physically removing preen oils on the feathers (used by birds to enhance the integrity of plumage structure and layering; Fabricius, 1956; Shawkey and others, 2003; Sandilands and others, 2004; Soini and others, 2007), but also may increase water penetration of plumage by lowering surface tension along the water-feather interface (Stephenson, 1997; Stephenson and Andrews, 1997; Gremillet and others, 2005). Regardless of the mechanism(s) involved, once plumage integrity has been compromised, wetting of feathers can cause thermal imbalance and hypothermia (Fabricius, 1956; Lustick, 1976; Simon and others, 1981; Stephenson and others, 1992;

Wolf and Walsberg, 2000; Banta and others, 2004). The indirect effect of surfactants on impairing thermoregulation also may be exacerbated by severe weather conditions, the duration of contact with a water source, and the lack of more suitable thermal microclimate (Lustick, 1976; Jorde and others, 1984; Goudie and Ankney, 1986; de Vries and van Eerden, 1995; Wolf and Walsberg, 2000; Bakken and others, 2002; McKinney and McWilliams, 2005).

Birds can potentially offset the adverse effects of surfactants on their feather waterproofing by altering behavioral patterns and habitat selection to restore thermoregulation. Key to thermoregulation in birds is the maintenance of waterproofing by cleaning feathers and maintaining overall plumage structural integrity (Fabricius, 1956; Nero, 1968; Stephenson and others, 1992; Wolf and Walsberg, 2000; Bakken and others, 2006). Fabricius (1956) found that waterproofing was dependent on maintaining the structural integrity of the feathers and layering, regardless of the presence of uropygial glands and the preen oils they produce. If birds were not allowed to preen, however, they eventually lost their waterproofing. de Vries and van Eerden, (1995) found that the longer the contact a bird makes with a water surface, the greater thermal loss it will experience unless mitigated by changes in bird activity patterns and behaviors. Under natural conditions, increased foraging activity and solar heating can help reduce these thermal losses, but may be limited by body condition, body size, species foraging behavior and competition, or availability of preferred habitat (Lustick and others; 1979; Jorde and others, 1984; Goudie and Ankney, 1986; Bakken and others, 2002; McKinney and McWilliams, 2005; Arzel and others, 2006).

In this study, anecdotal behavioral observations made of ducks during assessment periods, and from some of the photographs recording activity within the tanks, showed that despite being forced to maintain contact with the water surface, ducks were able to continue preening and cleaning activities as their exposure trials progressed (W. Iko, J. Berven, and L. Baeten, personal observations). Despite the extended duration of exposure of the ducks in the control group, these birds seemed able to maintain their waterproofing and thermoregulation, possibly by modifying the preening and cleaning activities observed. Conversely, as the wastewater treatment exposure trials progressed, it was observed that ducks in these tanks seemed to display increased cleaning and preening activity, shaking water off their plumage, as well as constant movement and wing-flapping to raise their body off the water (behaviors associated with increasing metabolic rate to raise body temperature; Welty, 1982; Gill, 1995; Dawson and Whittow, 2000). Although these observations are anecdotal, and the forced contact with the water treatments in this experiment would be unusual among wild free-ranging ducks, it does suggest that waterfowl using these wastewater treatment tanks may need to thermally balance the duration of exposure time to wastewater with increased cleaning and preening efforts, and if necessary, by removing themselves from the water altogether.

A combination of factors may help explain the large waterfowl mortalities observed in 2007 at MWRD and other wastewater facilities. Weather conditions in the Denver Metro area from December 2006 through February 2007 were unusually cold, with higher than normal snowfall (66.5 cm above normal) and lower than normal ambient temperatures (-6.22°C in January 2007, the 8th coldest January in Denver weather history; National Weather Service, 2007). Given the severe weather conditions that extended over a six-week period, normally open water sources along the central Colorado Front Range were frozen, forcing waterfowl to concentrate on the few remaining available open water locations (J. Gammonley and M. Kaknes, CDOW, oral commun., 2007). The open water and warmer temperatures of the wastewater treatment tanks compared to winter conditions along the central Colorado Front Range likely concentrated waterfowl in greater than normal numbers at MWRD and the other treatment facilities. In past winters, waterfowl have been observed using these treatment tanks overnight, but then dispersing from the facilities during the day (M. Kaknes, CDOW, written commun., 2007). Under less-crowded aggregations and milder winter conditions, waterfowl use of these tanks may afford ducks greater opportunity to partition their time budgets appropriately between feeding and preening locations

to maintain their waterproofing and thermal balance. However in 2007, higher than normal use of the treatment tanks by waterfowl were noted throughout the winter months (S. Rogowski, MWRD, oral commun., 2007). The crowded conditions faced by waterfowl within the confined space of the treatment tanks would not only potentially increase their exposure time to treated wastewater, but possibly could impede their ability to effectively feed and preen, further increasing the potential for feather wetting and hypothermia (Fabricius, 1956; Lustick and others, 1979; Jorde and others, 1984; Goudie and Ankney, 1986; de Vries and van Eerden, 1995; Bakken and others, 2002; McKinney and McWilliams, 2005). In such crowded situations, reduced spacing between individuals, increased food competition, and increased agonistic behavior (which could further impact feather integrity), could also have affected overwinter survival among these ducks (Jorde and others, 1984; Nudds and Bowlby, 1984; Goude and Ankney, 1986; DuBowy, 1985, 1988; Stephenson and others, 1992; DuBowy, 1996, 1997; Guillemain and others, 2000; McKinney and McWilliams, 2005; Arzel and others, 2006).

Open water habitat is particularly important for northern shovelers, whose unique bill morphology is specialized for filter feeding in surface waters, unlike other dabbling duck species that can feed in both terrestrial and aquatic environments (Johnsgard, 1961; Swanson, 1977; Johnsgard, 1978; Bellrose, 1980; Dubowy, 1985, 1988; Euliss and others, 1991; Dubowy, 1996, 1997; Guillemain and others, 2000). Shovelers are known to frequent wastewater treatment sites throughout the year, and may use these man-made impoundments during winter months because of their reliable open water and food availability (Swanson, 1977; Maxson, 1981; DuBowy, 1985, 1988; Webb and Brotherson, 1988; Euliss and others, 1991; DuBowy, 1996, 1997; Guillemain and others, 2000; Hamilton and others, 2005; Hamilton, 2007). Given that the MWRD facility is the largest wastewater treatment facility in the Intermountain West region (Metro Wastewater Reclamation District, 2008), it is not surprising that wintering shovelers and other waterfowl regularly access this facility. During this study, northern shovelers were observed frequently feeding in secondary clarifier and chlorine contact basin tanks, particularly overnight, and seemed to be in greater numbers when overnight temperatures were lower or wind chill conditions greater than expected ambient environmental conditions (W. Iko, personal observation). During daylight hours, shovelers still used these tanks for feeding, but increased their preening, loafing, sunning, and sleeping activities, both in and out of the treatment impoundments and along the shorelines of the South Platte River.

Under less-crowded or milder winter conditions when alternate roost habitat is more readily available, shovelers may be able to spend more time out of, and away from, the wastewater tanks to effectively preen and wash their plumage. However, in overcrowded or severe winter conditions when alternate habitat is frozen over, shovelers may have had to choose between staying in the relative safety of the treatment tanks or leave and risk not only exposure to the severe weather conditions, but also possibly the loss of preferred locations within the confined space of the treatment tanks (Jorde and others, 1984; Nudds and Bowlby, 1984; DuBowy, 1985; Goude and Ankney, 1986; DuBowy, 1988, 1996, 1997; Guillemain and others, 2000; McKinney and McWilliams, 2005; Arzel and others, 2006). Given the use of wastewater treatment impoundments by northern shovelers over winter months, during the severe and prolonged winter conditions observed from December 2006 through February 2007, the majority of the wintering shoveler population in this region may have become trapped at these treatment facilities (Newton, 2007). As these severe weather conditions continued, shovelers and other ducks in poor winter condition may have become more susceptible to stress and mortality (Jorde and others, 1984; Goude and Ankney, 1986; DuBowy, 1988, 1996, 1997; Guillemain and others, 2000; McKinney and McWilliams, 2005). The number of shoveler mortalities that occurred in 2007 may have also been a consequence of a larger proportion of the wintering population of this species being present in these wastewater treatment plant locations due to the severe weather conditions. What is not known from the 2007 die-off is whether these severe winter conditions impacted other duck species/populations along the central Colorado Front Range in a similar fashion (Jorde and others, 1984; Goude and Ankney,

1986; Euliss and others, 1991; Arzel and others, 2006). More thorough assessments of winter duck populations and habitat use of natural and man-made water bodies along the central Colorado Front Range would help better address this issue.

Selection of overwintering habitat is critical not only in individual survivorship, but also in lifetime reproductive strategy (Clutton-Brock, 1988). In general, winter is the most stressful period in the annual life cycle of migratory birds, balancing their physiological and thermoregulatory needs for maintenance with greater potential opportunity for reproductive success (Pienkowski and Evans, 1985). For migratory birds, reduction in movement between breeding and wintering grounds increases long-term reproductive success and lifetime survival; therefore, wintering closer to summer breeding habitats will reduce the physiological stress of migration, but at the potential risk of falling victim to unpredictable winter weather conditions (Welty, 1982; Pienkowski and Evans, 1985; Newton, 2007). Once a wintering location is established, migratory birds also have a strong tendency to remain at that location rather than risk further exploration in unfamiliar winter environment (Welty, 1982; Jorde and others, 1984; Goudie and Ankney 1986; Pienkowski and Evans, 1985; Newton, 2007).

Given the use of wastewater treatment impoundments by northern shovelers, this species may be using these man-made environments during severe weather conditions in areas farther north of its previous historical wintering range, but at the risk of becoming trapped at these locations by unpredictable inclement weather, as witnessed in 2007 (Welty, 1982; Jorde and others, 1984; Goudie and Ankney 1986; Pienkowski and Evans, 1985; Arzel and others, 2006; Newton, 2007). The normally mild winter conditions found along the central Colorado Front Range and the exponential growth in human infrastructure support in this area (for example, increased number of reservoirs, wastewater treatment facilities, city parks, and natural areas) may allow northern shoveler populations to winter farther north and in larger numbers than previously recorded. A review of northern shoveler Christmas bird count data in Colorado and New Mexico does show an upward trend in wintering populations; however, the reasons for these trends are currently unknown (J. Dubovsky, FWS, written commun., 2008). However, if the northward winter population trends of shovelers continue, with an increased reliance on wastewater treatment impoundments, the potential risk of waterfowl mortality events as witnessed in 2007 may also continue.

Numerous studies have shown the importance of wastewater treatment facilities and other industrial water impoundments to waterfowl populations (Swanson, 1977; Maxson, 1981; Webb and Brotherson, 1988; Euliss and other, 1991; Guillemain and others, 2000; Hamilton and others, 2005) as well as the potential health risks these habitats pose to these birds, especially related to petroleum oils, pesticides, heavy metals, and other contaminants (Hartung, 1967; Will and others, 1977; Holmes and others, 1979; Euliss and others, 1989; Gebauer and Weseloh, 1993; Custer and others, 1994; Jenssen, 1994; King and Andrews, 1997; Matsunaga and others, 1999; Gordus and others, 2002). However, the effect of surfactants on wild waterfowl populations under natural conditions, both in man-made and natural water systems, is less well understood (Nero, 1968; Parker and Barsom, 1970; Lustick, 1976; Choules and others, 1978; Stephenson, 1997; Stephenson and Andrews, 1997; Hamilton, 2007).

Given the chemical complexity present in municipal wastewater treatment impoundments and other man-made water systems, more detailed chemical analysis quantifying surfactants in wastewater systems would be required to determine if surfactant groups, combination of groups, or combination of chemical groups and classes, may be causing the effects on waterproofing that were observed during this experiment (Chiron and others, 2000; Shon and others, 2006; Gonzalez and others, 2007; Loos and others, 2007). Changes in the physical nature of these water bodies could also be further investigated to assess whether the potential for increased water penetration is more likely in the presence of surfactant compounds (Parker and Barsom, 1970; Lustick, 1976; Choules and others, 1978; Stephenson, 1997; Stephenson and Andrews, 1997).

The use of wastewater treatment facilities by waterfowl is well documented and unlikely to diminish in the near future as urban expansion, particularly in the Western United States, continues to provide suitable overwintering habitat along urban corridors (Swanson, 1977; Maxson, 1981; Webb and Brotherson, 1988; Euliss and other, 1991; Guillemain and others, 2000; Hamilton and others, 2005). Further assessments on winter waterfowl population numbers and natural and man-made habitat use, in combination with more detailed analytical chemical assessments of treated municipal wastewater, can help identify the causes associated with waterfowl die-offs and help address future considerations on management actions or mitigation to improve overwintering conditions for waterfowl populations.

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References Cited

- American Veterinary Medical Association, 2007, AVMA guidelines on euthanasia: Schaumburg, Ill., American Veterinary Medical Association, 36 p.
- Animal Welfare Act, 2008, Animal Welfare Act and Regulations, <http://www.nal.usda.gov/awic/legislat/usdaleg1.htm> (accessed Nov. 1, 2007).
- Ardrey, R.E., 2003, Liquid chromatography–mass spectrometry: An Introduction: Hoboken, N.J., John Wiley and Sons, 276 p.
- Arzel, C., Elmberg, J., and Guillemain, M., 2006, Ecology of spring-migrating Anatidae: a review: Journal of Ornithology, v. 147, p. 167–184.
- Bakken, G.S., Banta, M.R., Higginbotham, C.M., and Lynott, A.J., 2006, It's just ducky to be clean: the water repellency and water penetration resistance of swimming mallard *Anas platyrhynchos* ducklings: Journal of Avian Biology, v. 37, p. 561–571.
- Bakken, G.S., Williams, J.B., and Ricklefs, R.E., 2002, Metabolic response to wind of downy chicks of Arctic-breeding shorebirds (*Scolopacidae*): Journal of Experimental Biology, v. 205, p. 3,435–3,443.
- Banta, M.R., Lynott, A.J., VanSant, M.J., and Bakken, G.S., 2004, Partitioning heat loss from mallard ducklings swimming on the air-water interface: Journal Experimental Biology, v. 207, p. 4,551–4,557.
- Barco, M., Planas, C., Oscar Palacios, O., Ventura, F., Rivera, J., and Caixach, J., 2003, Simultaneous quantitative analysis of anionic, cationic, and nonionic surfactants in water by electrospray ionization mass spectrometry with flow injection analysis: Analytical Chemistry, v. 75, p. 5,129–5,136.
- Bellrose, F.C., 1980, Ducks, geese, and swans of North America: Harrisburg, Pa., Stackpole Books, 540 p.
- Chiron, S., Sauvard, E., and Jeannot, R., 2000, Determination of nonionic polyethoxylate surfactants in wastewater and sludge samples of sewage treatment plants by liquid chromatography–mass spectrometry: Analisis, v. 28, p. 535–542.
- Choules, G.L., Russell, W.C., and Gauthier, D.A., 1978, Duck mortality from detergent-polluted water: Journal of Wildlife Management v. 42, p. 410–414.
- Clutton-Brock, T.H., 1988, Reproductive success: Chicago, Ill., University of Chicago Press, 537 p.
- Custer, T.W., Bickham, J.W., Lyne, T.B., Lewis, T., Ruedas, L.A., Custer, C.M., and Melancon, M.J., 1994, Flow cytometry for monitoring contaminant exposure in black-crowned night-herons: Archives of Environmental Contaminants and Toxicology, v. 27, p. 176–179.

- Dawson, W.R., and Whittow, G.C., 2000, Regulation of body temperature, *in* Whittow, G.C., ed., Sturkie's avian physiology, 5th ed.: San Diego, Calif., Academic Press, p. 343–390.
- de Vries, J., and van Eerden, M.R., 1995, Thermal conductance in aquatic birds in relation to the degree of water contact, body mass, and body fat: implications of living in a strong cooling environment: *Physiological Zoology*, v. 68, p. 1,143–1,163.
- DuBowy, P.J., 1985, Feeding ecology and behavior of postbreeding male blue-winged teal and northern shovelers: *Canadian Journal of Zoology*, v. 63, p. 1,292–1,297.
- DuBowy, P.J., 1988, Waterfowl communities and seasonal environments: temporal variability in interspecific competition: *Ecology*, v. 69, p. 1,439–1,453.
- DuBowy, P.J., 1996, Northern Shoveler (*Anas clypeatea*), *in* Poole, A., and Gill, F., eds., *The birds of North America*, no.217: Philadelphia, Pa., The Academy of Natural Sciences, and Washington, D.C., The American Ornithologists' Union, 24 p.
- DuBowy, P.J., 1997, Long-term foraging optimization in northern shovelers: *Ecological Modelling*, v. 95, p. 119–132.
- Euliss, N.H., Jr., Jarvis, R.L., and Gilmer, D.S., 1989, Carbonate deposition on tail feathers of ruddy ducks using evaporation ponds: *Condor*, v. 91, p. 803–806.
- Euliss, N.H., Jr., Jarvis, R.L., and Gilmer, D.S., 1991, Feeding ecology of waterfowl wintering on evaporation ponds in California: *Condor*, v. 93, p. 582–590.
- Fabricius, E., 1956, What makes plumage waterproof?: *Zoologisk Revy*, v. 18, p. 71–83.
- Fischel, M., 2001, Evaluation of selected deicers based on a review of the literature: Report no. CDOT-DTD-R-2001-15, Colorado Department of Transportation, Research Branch, Denver, Colo., 117 p.
- Gebauer, M.B., and Weseloh, D.V., 1993, Accumulation of organic contaminants in sentinel mallards utilizing confined disposal facilities at Hamilton Harbour, Lake Ontario, Canada: *Archives of Environmental Contaminants and Toxicology*, v. 25, p. 234–243.
- Gill, F.B., 1995, *Ornithology*, 2nd ed.: New York, N.Y., W.H. Freeman and Co., p. 131–138.
- Gonzalez, S., Petrovic, M., and Barcelo, D., 2007, Advanced liquid chromatography–mass spectrometry (LC–MS) methods applied to wastewater removal and the fate of surfactants in the environment: *Trends in Analytical Chemistry*, v. 26, p. 116–124.
- Gordus, A.G., Shivaprasad, H.L., and Swift, P.K., 2002, Salt toxicosis in ruddy ducks that winter on agricultural evaporation basin in California: *Journal of Wildlife Diseases*, v. 18, p. 124–131.
- Goudie, R.I., and Ankney, C.D., 1986, Body size, activity budgets, and diets of sea ducks wintering in Newfoundland: *Ecology*, v. 67, p. 1,475–1,482.
- Greenburg, A.E., Clesceri, L.S., and Eaton, A.D., eds, 1992a, 5440C, Anionic surfactants as MBAS, *in* Standard methods for the examination of water and wastewater, 18th ed.: Washington, D.C., American Public Health Association, p. 5–36 – 5–38.
- Greenburg, A.E., Clesceri, L.S., and Eaton, A.D., eds, 1992b, 5440D, Nonionic surfactants as CTAS, *in* Standard methods for the examination of water and wastewater, 18th ed.: Washington, D.C., American Public Health Association, p. 5–36 – 5–38.
- Gremillet, D., Chauvin, C., Wilson, R.P., Le Maho, Y., and Wanless, S., 2005, Unusual feather structure allows partial plumage wettability in diving great cormorants *Phalacrocorax carbo*: *Journal of Avian Ecology*, v. 36, p. 57–63.
- Guillemain, M, Frtiz, H., and Guillon, N., 2000, Foraging behavior and habitat choice of wintering northern shoveler in a major wintering quarter in France: *Waterbirds*, v. 23, p. 355–363.
- Hamilton, A.J., 2007, Potential microbial and chemical hazards to waterbirds at the Western Treatment Plant: *Ecological Management and Restoration*, v. 8, p. 38–41.
- Hamilton, A.J., Robinson, W., Taylor, I.R., and Wilson, B.P., 2005, The ecology of sewage treatment gradients in relation to their use by waterbirds: *Hydrobiologia*, v. 534, p. 91–108.

- Hartung, R., 1967, Energy metabolism in oil-covered ducks: *Journal of Wildlife Management*, v. 31, p. 798–804.
- Holmes, W.N., Gorsline, J., and Cronshaw, J., 1979, Effects of mild cold stress on the survival of seawater-adapted mallard ducks (*Anas platyrhynchos*) maintained on food contaminated with petroleum: *Environmental Research*, v. 20, p. 425–444.
- Im, S.H., Jeong, Y.H., Ryoo, J.J., 2008, Simultaneous analysis of anionic, amphoteric, nonionic and cationic surfactant mixtures in shampoo and hair conditioner by RP-HPLC/ELSD and LC/MS: *Analytica Chimica Acta*, v. 619, p. 129–136.
- Jenssen, B.M., 1994, Effects of oil pollution, chemically treated oil, and cleaning on the thermal balance of birds: *Environmental Pollution*, v. 86, p. 207–215.
- Johnsgard, P.A., 1961, The taxonomy of the *Anatidae*—a behavioural analysis: *Ibis*, v. 103a, p. 71–85.
- Johnsgard, P.A., 1978, Ducks, geese, and swans of the world: Lincoln, Nebr., University of Nebraska Press, 404 p.
- Jorde, D.G., Krapu, G.L., and Crawford, R.D., 1984, Effects of weather on habitat selection and behavior of mallards wintering in Nebraska: *Condor*, v. 86, p. 258–265.
- King, K.A., and Andrews, B.J., 1997, Environmental contaminants in ducks collected from wastewater treatment plant ponds, Nogales, Arizona 1996: U.S. Fish and Wildlife Service Region 2 Contaminants Program, Project No. 22410-1 130-2F35. 13 p.
- Knepper, T.P., Barcelo, D., and de Voogt, P., eds., 2003, Analysis and fate of surfactants in the aquatic environment, *in* *Comprehensive analytical chemistry*, vol, 40: Amsterdam, The Netherlands, Elsevier Science, 994 p.
- Lara-Martín, P.A., Gomez-Parra, A., Gonzalez-Mazo, E., 2006, Development of a method for the simultaneous analysis of anionic and non-ionic surfactants and their carboxylated metabolites in environmental samples by mixed-mode liquid chromatography–mass spectrometry: *Journal of Chromatography A*, v. 1137, p. 188–197.
- Lewis, W.M., 1999, Studies of environmental effects of magnesium chloride deicer in Colorado: report no. CDOT-DTD-R-99-10, Final Report, Colorado Department of Transportation Research Branch, Denver, Colo., 78 p.
- Lustick, S., 1976, Wetting as a means of bird control, *in* *Bird control seminars proceedings*: Lincoln, University of Nebraska, p. 41–53.
- Lustick, S., Battersby, B., and Kelty, M., 1979, Effects of insolation on juvenile herring gull energetics and behavior: *Ecology*, v. 60, p. 673–678.
- Loos, R., Hanke, G., Umlauf, G., Eisenreich, S.J., 2007, LC–MS–MS analysis and occurrence of octyl- and nonylphenol, their ethoxylates and their carboxylates in Belgian and Italian textile industry, waste water treatment plant effluents and surface waters: *Chemosphere*, v. 66, p. 690–699.
- Matsunaga, H., Harada, K-I., Senma, M., Ito, Y., Yasuda, N., Ushida, S., and Kimura, Y., 1999, Possible cause of unnatural mass death of wild birds in a pond in Nishinomiya, Japan: sudden appearance of toxic cyanobacteria: *Natural Toxins*, v. 7, p. 81–84.
- Matthijs, E., Holt, M.S., Kiewiet, A., Rijs, G.B.J., 1999, Environmental monitoring for linear alkylbenzene sulfonate, alcohol ethoxylate, alcohol ethoxy sulfate, alcohol sulfate, and soap: *Environmental Toxicology and Chemistry*, v. 18, p. 2,634–2,644.
- Maxson, G.D., 1981, Waterfowl use of a municipal sewage lagoon: *The Prairie Naturalist*, v. 13, p. 1–12.
- McKinney, R.A., and McWilliams, S.R., 2005, A new model to estimate daily energy expenditure for wintering waterfowl: *Wilson Bulletin*, v. 117, p. 44–55.
- Metro Wastewater Reclamation District, 2008, General brochure: http://www.metrowastewater.com/documents/brochure_general.pdf (accessed June 1, 2007).

- Moulton, D.W., Jensen, W.I., and Low, J.B., 1976, Avian botulism epizootiology on sewage oxidation ponds in Utah: *Journal of Wildlife Management*, v. 40, p. 735–742.
- National Climatic Data Center, 2008, Denver climatological data, December 2006 to February 2008: <http://www.ncdc.noaa.gov> (accessed Sept. 30, 2008).
- National Weather Service, 2007, Denver monthly weather forecasts: <http://www.weather.gov/climate/index.php?wfo=bou> (accessed Nov. 08, 2007).
- Nero, R.W., 1968, Wet waterbirds— a problem at sewage lagoons: *Blue Jay*, p. 2–7.
- Newton, I., 2007, Weather-related mass-mortality events in migrants: *Ibis*, v. 149, p. 453–467.
- Nudds, T.D., and Bowlby, J.N., 1984, Predator-prey size relationships in North American dabbling ducks: *Canadian Journal of Zoology*, v. 62, p. 2,002–2,008.
- Parker, B., and Barsom, G., 1970, Biological and chemical significance of surface microlayers in aquatic ecosystems: *Bioscience* v. 20, p. 87–93.
- Pienkowski, M.W., and Evans, P.R., 1985, The role of migration in the population dynamics of birds. *in* Sibly, R.M., and Smith, R.H., eds., *Behavioural ecology*: Oxford, United Kingdom, Blackwell Scientific, p. 331–352.
- ProMED-mail (listserver), 2007a, Undiagnosed die-off, waterfowl—U.S.A. (CO): <http://www.promedmail.org> (accessed Jan. 24, 2007).
- ProMED-mail (listserver), 2007b, Undiagnosed die-off, waterfowl—U.S.A. (CO): <http://www.promedmail.org> (accessed Jan. 27, 2007).
- ProMED-mail (listserver), 2007c, Undiagnosed die-off, waterfowl—U.S.A. (CO): <http://www.promedmail.org> (accessed Feb. 17, 2007).
- Rand, G.M., and Petrocelli, S.R., 1985, Introduction, *in* Rand, G.M., and Petrocelli, S.R., eds., *Fundamentals of aquatic toxicology*: Washington, D.C., Harper & Row Publishing, p. 1–28.
- Sandilands, V., Powell, K., Keeling, L., and Savory, C.J., 2004, Preen gland function in layer fowls: factors affecting preen oil fatty acid composition: *British Poultry Science*, v. 45, p. 109–115.
- Schroder, H.F., 2001, Tracing of surfactants in the biological wastewater treatment process and the identification of their metabolites by flow injection–mass spectrometry and liquid chromatography–mass spectrometry and tandem mass spectrometry: *Journal of Chromatography A*, v. 926, p. 127–150.
- Shane, B.S., 1994, Principles of ecotoxicology, *in* Cockerham, L.G., and Shane, B.S., eds., *Basic environmental toxicology*: Boca Raton, Fla., CRC Press, Inc., p. 11–47.
- Shawkey, M.D., Pillai, S.R., and Hill, G.E., 2003, Chemical warfare? Effects of uropygial oil on feather-degrading bacteria: *Journal of Avian Biology*, v. 34, p. 345–349.
- Shon, H.K., Vigneswaran, S., Snyder, S.A., 2006, Effluent organic matter (EfOM) in wastewater: Constituents, effects, and treatment: *Critical Reviews in Environmental Science and Technology*, v. 36, p. 327–374.
- Simon, E., Martin, R., and Simon-Opperman, Ch., 1981, Central nervous versus total body thermosensitivity of the duck: *International Journal of Biometeorology*, v. 25, p. 249–256.
- Soini, H.A., Schrock, S.E., Bruce, K.E., Wiesler, D., Ketterson, E.D., and Novotny, M.V., 2007, Seasonal variation in volatile compound profiles of preen gland secretions of the dark-eyed junco (*Junco hyemalis*): *Journal of Chemical Ecology*, v. 33, p. 183–198.
- Stephenson, R., 1997, Effects of oil and other surface-active organic pollutants on aquatic birds: *Environmental Conservation* v. 24, p. 121–129.
- Stephenson, R., and Andrews, C.A., 1997, The effect of water surface tension on feather wettability in aquatic birds: *Canadian Journal of Zoology*, v. 74, p. 288–294.
- Stephenson, R., Hedrick, M.S., and Jones, D.R., 1992, Cardiovascular responses to diving and involuntary submergence in the rhinoceros auklet (*Cerorhinca monocerata* Pallas): *Canadian Journal of Zoology*, v. 70, p. 2,303–2,310.

- Swanson, G.A., 1977, Diel food selection by Anatidae on a waste-stabilization system: *Journal of Wildlife Management* v. 41, p. 226–231.
- Thompson, J.D., and Baldassarre, G.A., 1991, Activity patterns of nearctic dabbling ducks wintering in Yucatan, Mexico: *Auk*, v. 108, p. 934–941.
- Vyas, N.M., Spann, J.W., Hulse, C.S., Borges, S.L., Bennett, R.S., Torres, M., Williams, B.I., and Leffel, R., 2006, Field evaluation of an avian risk assessment model: *Environmental Toxicology and Chemistry* v. 25, p. 1,762–1,771.
- Webb, G.M., and Brotherson, J.D., 1988, Time-activity budgets of drake gadwall and northern shovelers on industrial cooling ponds during late winter and early spring in central Utah: *Great Basin Naturalist* v.48. p. 280–284.
- Welty, J.C., 1982, *The life of birds*: Philadelphia, Pa., W.B. Saunders Company, 754 p.
- Will, D.H., Abel, J.H., Russell, B.E., and Levine, K.E., 1977, Colorado River desalination wild duck study: Final Project Report, June 28, 1977, 25 p.
- Wolf, B.O., and Walsberg, G.E., 2000, The role of the plumage in heat transfer processes of birds: *American Zoologist*, v. 40, p. 575–584.

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