# ASSESSMENT OF DRINKING WATER QUALITY AND RELATED HUSBANDRY PRACTICES IN NORTH AMERICAN ZOOS

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ABSTRACT

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Water is essential for life of all animals. However, drinking water might be of poor quality if it contains excess chemicals, nutrients or contaminants. Based on review of the scientific literature, the quality of drinking water in zoos has not been investigated. Therefore, objectives were to: 1) assess general quality of water in Association of Zoo and Aquarium zoos; 2) examine possible relationships among husbandry practices and water quality; and, 3) analyze iron concentrations in drinking water of zoos with black rhino. Forty zoos without and 10 with black rhino agreed to participate when randomly invited from a pool of 174 eligible zoos (29% response rate). Water samples were analyzed for pH, hardness, total dissolved solids, calcium, phosphorus, magnesium, potassium, sodium, iron, manganese, zinc, copper, chloride, sulfate, nitrate, total coliform, and *Escherichia coli*. A water quality index was used to rank overall quality among zoos. A questionnaire about husbandry practices and drinking water also was completed by a subsample of zoos (n = 39). Over 85% of zoos used municipal water primarily. Three of 50 zoos (above the 90$^{th}$ percentile) had poor quality water. Majority (59%) of zoos responded that drinking water quality was considered in nutritional management. However, only 18% routinely analyzed drinking water. Zoos with Black Rhino were aware of the recommendation to formulate for low dietary iron to reduce Iron Overload Disorder. However, only 2 of 8 zoos with Black Rhino routinely analyzed drinking water for iron.

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF ALGORITHMS

## KEY TO ABBREVIATIONS

BMP .....bone morphogenetic protein

BR .....black rhino

$C_i$ .....observed concentration of *i* parameter

*C* .....Horton's rating point

Ca .....calcium

$CaCO_3$ .....calcium carbonate

CT .....condensed tannin

Cu .....copper

DMB .....dry matter basis

DMT-1 .....divalent metal transporter 1

DNA .....deoxyribonucleic acid

EPA .....United States Environmental Protection Agency

Fe .....iron

$Fe^{2+}$ .....ferrous iron

$Fe^{3+}$ .....ferric iron

GI .....gastrointestinal

$H^+$ .....hydrogen

HAs .....health advisories

HCl .....hydrochloric acid

HFE .....hemochromatosis protein

HJV .....hemojuvelin

IOD .....iron overload disorder

IREs .....iron responsive elements

Ireg-1 .....ferroportin

IRP-1 .....iron responsive protein-1

K .....potassium

*M* .....Horton's coefficient

MCL .....maximum contaminant levels

Mg .....magnesium

Mn .....manganese

mRNA .....messenger ribonucleic acid

N .....nitrogen

Na .....sodium

$NH_3$ .....ammonia

$NH_{4+}$ .....ammonium

$NO_{2-}$ .....nitrite

$NO_{3-}$ .....nitrate

NRC .....National Research Council

NTU .....nephelometric turbidity unit

ORVWSC .....Ohio River Valley Water Sanitation Commission

P .....phosphorus

$q_i$ .....qualityrating scale

*QI* .....Horton's Quality Index

ROS .....reactive oxygen species

$S_i$ .....standard value of *i* parameter

$S_e$ .....selenium

SMCL .....secondary maximum contaminant levels

TC .....total coliform

TDS .....total dissolved solids

TfR-1 .....transferrin receptor-1

TfR-2 .....transferrin receptor-2

TON .....threshold odor number

$w_i$ .....relative weight

*W* .....Horton's weighting factor

WHO .....World Health Organization

WQI .....water quality index

Zn .....zinc

# CHAPTER 1

# LITERATURE REVIEW

## 1.1. Introduction

Water is an essential nutrient for life (NRC, 2005; Ross et al., 2014). Clean drinking water is essential for maintaining optimal health. For centuries, humans have known that safe, clean, drinking water is essential for avoiding disease, and maintaining health. Across the country, municipal and private water treatment facilities provide clean drinking water for millions of people, and, by living in close proximity to humans, their pets. Livestock managers and nutritionists also are aware of the importance of water quality and the problems that poor-quality drinking water can pose to livestock health and production. Research shows that providing livestock adequate access to clean drinking water can increase feed intake and can potentially increase productivity because water and feed intakes are positively correlated (NRC, 2007; Pond et al., 2005). As a result, many animal agricultural facilities make water testing and treatment a routine part of livestock management. However, based on a review of the current literature, there appears to be a lack of research into drinking water quality in zoos.

Drinking water quality can negatively impact the health of animals when certain constituents are present in large enough amounts. Even essential nutrients can be present in drinking water in great enough concentrations to contribute to excess intake leading to adverse health effects in animals if not properly monitored and corrected (NRC, 1974). Due to the lack of peer-reviewed data and information on nutritional water quality in zoos, this research project seeks to survey and obtain water samples from a random sample of Association of Zoos and Aquariums (AZA) accredited zoos across North America. The information and samples collected were analyzed and the data analyzed and summarized in order to create a resource for zoo veterinarians and nutritionists about drinking water sources, water husbandry practices, and

drinking water quality. Our goal is to assess the quality of drinking water provided to animal collections in AZA-accredited zoos; with iron concentrations in drinking water provided to black rhino being of particular interest in our study. Additionally, we seek to examine drinking water husbandry practices in zoos via a questionnaire focused on water husbandry practices provided to a subset of participating zoos.

## 1.2. Water Nutrition and Quality

### 1.2.1. Importance

Water is an essential nutrient required to sustain life. Water is crucial for metabolic processes, temperature control, fluid balance, gastrointestinal health, waste elimination, nutrient transport, and digestive function (Church, 1993; Dryden, 2008; Pond et al., 2005). Water makes up about two-thirds of the body mass in adults and more than three-quarters of the body mass of baby animals (NRC, 2005). Water also is required in every biochemical process in the body (Pond et al., 2005). Due to the great many biological processes in which water is involved in the body, most mammals cannot survive for more than a few days without an adequate supply of clean drinking water. Because water is considered the universal solvent, it is vital for the absorption and transport of water-soluble nutrients. The ability of water to act as a solvent also makes it possible for drinking water sources to contribute additional nutrients in excess of the diet to captive animals (NRC, 2005). Water also can carry pollutants, pathogens, and other constituents (Vigil, 2003).

### 1.2.2. Water Quality

Water quality is defined as determining the suitability of a body of water for a specific purpose based upon the measurement of the characteristics of the water (Johnson et al., 1997; NRC, 2007). For the scope of this study, the specific purpose of the water is drinking by various

species of zoo animals. Water is rarely ever pure; it has dissolved constituents either from nature or human activities (NRC, 1974). The water consumed by humans, livestock, and captive animals is sourced from either groundwater (aquifers) or surface water (rivers, lakes, and streams); because of this, the dissolved constituents in water can change over time (Vigil, 2003). An increase in contaminants in water can lead to decreased water intake, illness, and, in extreme cases, death of humans and animals alike (Church, 1993; DHEC, 2013; Gray, 2008).

No publications were found addressing drinking water quality in zoos. Therefore, most of the subsequent discussion and recommendations about drinking water constituents are based upon current human and (or) livestock (domestic animals) recommendations.

The criteria that define the quality of water are categorized into chemical, physical, biological, and radiological characteristics (NRC, 1974; Vigil, 2003; Gray, 2008). Chemical characteristics consist of essential minerals (iron [Fe], manganese [Mn], copper [Cu], calcium [Ca], magnesium [Mg], sodium [Na], potassium [K], zinc [Zn], and phosphorous [P]) and toxic elements and compounds (arsenic, cadmium, lead, nitrate, sulfate, and chloride) (Gray, 2008; Vigil, 2003). Physical characteristics consist of odor, taste, color, temperature, turbidity, and total dissolved solids (TDS) (NRC, 1974). Biological characteristics consist of small and microscopic living organisms; including, bacteria, protozoa, algae, and small invertebrates (Gray, 2008). Viruses are considered pathogens, so they are included as biological characteristics of water quality even though they are not truly living organisms (Alberts et al., 2013). Radiological constituents may consist of strontium-90, radium-226, tritium, and other radioactive elements and substances that end up in the water supply either via natural sources, human activities (e.g., mining), and (or) environmental spills of radioactive waste (NRC, 1974).

The typical analysis used to assess drinking water quality for livestock is called the "livestock suitability package" test, which evaluates pH, hardness, TDS, Ca, P, Mg, K, Na, Fe, Mn, Zn, Cu, chlorides, sulfates, and nitrates (CVAS, 2019). It also is possible to have water testing done to analyze and report a more limited list of water constituents. Due to the potential constant change in composition of a water source via erosion, transpiration, evaporation, precipitation, oxidation, reduction, cation and anion exchange, acid-base interactions, and microbial transformation, at least one water test per year is recommended to assess the safety of drinking water provided to animals in human care (Clauss et al., 2012; NRC, 1974).

Maximum contaminant levels (MCL) are mandatory water quality standards for drinking water contaminants established by the Environmental Protection Agency (EPA). The MCL is the maximum level allowed of a contaminant delivered to any user of a public water system (EPA, 2018). The MCLs are set as a safety precaution for contaminants that pose a possible risk to public health, but not all contaminants pose such a risk. Secondary maximum contaminant levels (SMCL) were established for 15 water contaminants (e.g., Ag, Al, $Cl^-$, Cu, Fl, Fe, Mn, pH, $SO_4$, TDS, Zn, color, corrosivity, foaming agents, and odor) that are non-mandatory water quality standards; Fe, of particular interest in this study, is included into this secondary category.

### 1.2.3. Aesthetics

Color, odor, taste, and turbidity (cloudiness) are the main factors contributing to aesthetic quality of water (Gray, 2008). Whereas these factors often do not relate to the safety of the drinking water, they can influence consumption by animals and people, as well as perceived water safety by the latter (Genther and Beede, 2013; Vigil, 2003). Color, odor, taste and turbidity characteristics can be due to a variety of different constituents including combinations of more than one. For instance, color can be affected by presence of Fe, Mn, Zn, Cu, Pb,

hydrogen sulfide, microorganisms, and other factors (DHEC, 2013; Gray, 2008). Although a change in water color does not necessarily pose an immediate health risk to consumers, it can decrease the palatability and consumption of water. The EPA guideline for color was set at 15 color units (EPA, 2018). Another visual water quality indicator is turbidity. Turbidity measures the clarity of water and is determined by shining a light through a water sample in order to determine the amount of light that is scattered by particles or materials suspended in the water sample (USGS, 2016). Turbidity is affected by particles in the water that are visible to the human eye, such as, clay, silt, soluble colored organic particles, microorganisms, and other factors. Although the turbidity of water itself likely does not pose a threat to human or animal health, it can provide an environment in which pathogens can grow or regrow after treatment (USGS, 2016). Both the color and turbidity of water are easily recognizable indicators of water quality or change to water quality. The standard for turbidity, as set by the EPA, specifies that at no time can turbidity exceed 5 Nephelometric Turbidity Units (NTU) (EPA, 2018).

Odor of water is most often related to the presence of chlorine or hydrogen sulfide (DHEC, 2013; Gray, 2008). Chlorine is used in small amounts, residual readings of 0.5mg/L or less, as a disinfecting agent in the final stage of drinking water treatment (CDC, 2014; Vigil, 2003). The presence of a "chlorine smell" can decrease water consumption of some people, but there is scant information on the effect of odor on water intake of livestock or other animals (Gray, 2008). Hydrogen sulfide when present in water imparts a "rotten egg" smell that may be offensive and decrease water consumption by humans and may contribute to decreased water intake in dairy cows, but this has not been verified with research (Beede, 2009; DHEC, 2013). Although there were studies to evaluate water preferences of livestock, primarily with cattle, it is

very difficult to attribute exactly which characteristic(s) (odor, taste, metabolic impact, or a combination of many factors) impacts animal preference the most (Lardner et al., 2013). The EPA set the SMCL for odor at 3 Threshold Odor Number (TON) (EPA, 2018).

Another aesthetic component of water, that is closely related to odor, is taste. Taste, or distaste can be affected either directly or indirectly by almost every constituent that can be present in a water sample. Metallic, bitter taste can be imparted by Fe, Mn, Zn, Cu, change in pH, high TDS, or an increase in the corrosive qualities of the water due to corrosion and release of metallic substances from pipes into the water during transport to the consumer. A salty taste can be imparted by sodium and sodium-containing compounds, chloride and chloride-containing compounds, and elevated TDS. While excess Na can alter the taste of water, it also can cause dehydration and increased thirst of animals and people. In 2018, a group of endangered black rhinoceros (*Diceros bicornis;* black rhino) were relocated within Kenya from a Lake Nakuru National Park to Tsavo East National Park in a conservation effort to boost a breeding population. Of the 11 black rhino originally relocated, 8 died due to the higher salinity in the drinking water source at their new location (Van Sant, 2018). The increased Na content of the new drinking water source caused dehydration, which increased consumption of the saline water and led to salt poisoning.

A "rotten egg" or "sulfur" taste can be imparted on water in the presence of hydrogen sulfide, bacteria, and algae (DHEC, 2013; Gray, 2008; Vigil, 2003). Whereas taste and smell are independent senses, studies in humans show a link between taste and olfaction that can enhance the experience a person has with food or drink ("Taste and Smell," 2012). Whether or not this same interaction is present in livestock and other animals is unknown, but it may be possible for

the odor and taste of water to play a combined role in the intake of water by animals; more research is needed to confirm such a connection.

### 1.2.4. Hard Water

Hard water contains increased concentrations of alkaline earth metals, such as Mg and Ca in the form of salts (e.g., calcium carbonate) (USGS, 2016a). Because these salts are alkaline earth metals, as shown on the Periodic Table, hard water is typically alkaline (basic on the pH scale). Because hard water is associated with an increased mineral content it very often has an increased concentration of TDS (DHEC, 2013). Hard water is not considered a health concern, but it can promote mineral build-up in pipes and plumbing and decreased effectiveness of soaps and cleaning agents (Vigil, 2003). The USGS defines the categories of water hardness as follows: 0-60 mg/L of calcium carbonate as considered "soft", 61-120 mg/L as "moderately hard", 121-180 mg/L as "hard", and greater than 180 mg/L calcium carbonate as "very hard" water (USGS, 2016a). Although it is not considered a health concern, a range of between 100 and 300 mg/L is considered the taste threshold for Ca ions in water for humans; with the taste threshold for Mg ions believed to be even lower (WHO, 2017). Treatment for hard water typically involves an ion exchange system that replaces the Ca and Mg in the salts with Na (DHEC, 2013). The introduction of Na as a water softening agent does increase the overall Na content of the water and may pose problems for people and animals that need a lower Na intake diet.

### 1.2.5. pH

The term pH refers to the acidic or basic (alkaline) nature of a solution, in this instance water, based upon the concentration of hydrogen ions present in the solution (Vigil, 2003). Acidic solutions have a range between 0.0 and 6.9 whereas alkaline solutions have a range

between 7.1 and 14.0 on a pH scale with 7.0 being neutral (neither acidic nor basic) (Alberts et al., 2013). Pure water, containing just molecular $H_2O$, is neutral. Any factor that affects the hydrogen ion concentration in water will change the pH of water; both beneficial and non-beneficial constituents contribute to the change in pH of water. Carbon dioxide, carbonic acid, and the rare occasion of exposure to chemicals (e.g., hydrochloric acid) will decrease the pH of water making it more acidic. Acidic water is corrosive to exposed metal in pipes and other materials used to transport and store water (DHEC, 2013). The longer acidic water is in contact with the exposed metal the more the solution will corrode the metal, releasing metal particles or ions into the water. Acidic water can be a source of Fe, Pb, Cu, and Zn in a drinking water due to the corrosion of metal pipes and joint surfaces (DHEC, 2013). Alkaline water typically has an increased presence of Ca and Mg compounds (e.g., calcium carbonate). Water pH is considered a SMCL and is generally not an issue unless the pH approaches the extreme ends of the pH scale (too acidic or too basic). The EPA set the recommended SMCL for water pH to fall within the range of 6.5 to 8.5 (EPA, 2018). As a reference, natural water bodies (e.g., lakes, rivers, and streams) typically have a pH between 6.0 and 8.5, whereas well (bore) water sources usually range in pH from 5.0 to 9.0 (DHEC, 2013; Vigil, 2003).

Adjusting the pH of water can be a tool to control or influence water quality. When a water source has increased concentrations of Fe and a pH that is not near neutral, bringing the pH as close to neutral as possible is required to utilize removal processes (DHEC, 2013). Adjusting the pH of water also can correct the undesired effects of water that is either too acidic (e.g., dissolved metals) or too basic (e.g., bitter taste). If water is acidic (corrosive) and Zn or other dissolved metals are being released from pipes, the addition of basic solutions (e.g., sodium

hydroxide) will raise the water pH to neutralize the corrosive properties of the water (Vigil, 2003). Once the corrosive properties are neutralized, running the water for adequate time will flush the metal-laden water from the lines. Acidic solutions also can be added to alkaline water to bring the pH closer to neutral. This is a common water treatment method used in industrial applications to neutralize wastewater before release back into the environment at the end of production processes (Vigil, 2003).

### 1.2.6. Temperature

The temperature of drinking water can affect water quality. Algae and bacteria grow more easily in warm water versus cold water (Vigil, 2003). Growth of algae and bacteria in drinking water decreases water quality as it introduces the possibility of pathogens, as well as, affecting color, taste, odor, and corrosive properties (WHO, 2017). Research with livestock, including horses, ponies, and dairy cows showed that the temperature of the water in relation to environmental temperature can influence water intake with the general preference being for ambient or warm water versus cold water (Huuskonen et al., 2011; NRC, 2007; Wilks et al., 1990). Research showed water intake by dairy cows and horses increases when environmental temperatures are warmer, above 81°F (NRC, 2007; Ragsdale et al., 1949 as cited by Beede, 2005). In horses, low environmental temperatures, between -8℃ to -17℃, decrease water intake by 6 to 14% (NRC, 2007). Neither the EPA nor the WHO have specific temperature value or range recommendations for water quality, but the WHO does state, “cool water is generally more palatable than warm water…” in terms of human water temperature preferences (WHO, 2017). Whereas colder water temperatures may be the more palatable choice for people, it is not necessarily the case for livestock, as stated above.

### 1.2.7. Biological Factors

Biological factors influencing water quality include bacteria, viruses, protozoa, and algae, which can lead to a wide array of diseases depending upon the specific pathogen. Bacterial agents in drinking water can lead to diseases, such as, typhoid fever, diarrhea, shigellosis, and gastroenteritis; viral agents can cause hepatitis and gastroenteritis; and protozoan agents can cause giardiasis, amoebiasis, and cryptosporidiosis (Gray, 2008). Algal agents include cyanobacteria, formerly known as "blue-green algae" that can produce toxic blooms in drinking water sources. The cyanotoxins of algae can damage nerve and liver tissue in humans, and cause disease in birds and other animals (WSDH, 2019). According to the EPA, the most common types of cyanotoxins found in U.S. waters are microcystins, cylindrospermopsin, anatoxins, and saxitoxins (EPA, 2019). Cyanotoxins can be lethal to aquatic species, and to livestock consuming contaminated water sources (WHO, 2003). A relatively recent case of cyanobacteria bloom in a moat around an exhibit in an unnamed North American zoo led to the death of several yellow-bellied sliders (*Trachemys scripta scripta*) and increased awareness and concern for cyanotoxin presence in zoo waters and the potential threat they pose for zoo animals (Doster et al., 2014). Although the EPA does not have a specific MCL for cyanobacteria, the agency published 10-day Health Advisories (HAs) for cyanotoxins in drinking water; but, these contaminants are not subject to any national primary drinking water regulation (EPA, 2019a). The 10-day HAs for cyanobacteria vary by state, cyanotoxin type, and the age of the person consuming the water. Generally speaking, 10-day HAs range from 0.3-3.0 µg/L depending upon the stipulations listed previously (EPA, 2015).

### 1.2.8. Nitrates and Nitrites

An increased concentration of nitrogen (N) in water is cause for concern as it can be an indication of increased nitrate and (or) nitrite concentrations. Nitrates ($NO_3^-$) are more stable than nitrites ($NO_2^-$) and are more common in soils (DHEC, 2013). Soil nitrates are readily dissolved in water and can travel rapidly through the environment. Both $NO_3^-$ and $NO_2^-$ are naturally occurring ions present in the N-cycle. During the N-cycle, ammonia ($NH_3$) and ammonium ($NH_4^+$) can be oxidized in aerobic conditions via ammonia-oxidizing bacteria to $NO_2^-$, which can be further oxidized to $NO_3^-$ (Stein and Klotz, 2016). Increased concentrations of $NO_3^-$ are most commonly a result of inorganic fertilizer application during crop production and (or) the presence of concentrated human or animal waste. Nitrite is less common in water sources and is most often used as a preservative in cured meats. High $NO_3^-$ concentrations can encourage the growth of bacteria and other pathogens in water. Algal and bacterial blooms cause health concerns for humans and animals consuming affected water, as discussed in the previous section. Nitrate is readily converted to $NO_2^-$ via reduction in the body and then readily converted to *N*-nitrosamines (Gray, 2008). *N*-nitrosamines are carcinogenic compounds that increase the risk of gastric cancer in experiments using mice offered both dietary and water *N*-nitrosamine sources (Tricker and Preussmann, 1991). High $NO_2^-$ intake is associated with methemoglobinemia, “blue baby syndrome”, in human infants, and other mammals (DHEC, 2013; NRC, 2007). Methemoglobinemia occurs when high concentrations of $NO_2^-$ displace the oxygen bound to Fe in hemoglobin, creating methemoglobin, decreasing the oxygen-carrying capacity of red blood cells (Gray, 2008). The decreased oxygen carrying capacity of the red blood cells leads to decreased delivery of oxygen to tissues, and in extreme cases can lead to the death of the infant or animal due to lack of oxygen.

In ruminant animals, $NO_3^-$ is rapidly reduced to $NO_2^-$ by microbes in the rumen (Church, 1993). This can become a problem for cattle and other ruminant species when high concentrations of $NO_3^-$ build up in plants used as forage. Outside factors typically contribute to the accumulation of $NO_3^-$ in forage plants, such as drought, frost damage, shading, herbicide application, and high concentrations of nitrogenous compounds in the surface and (or) groundwater where the plants are grown (Church, 1993; Costagliola et al., 2014; NRC, 2005). The increased presence of $NO_3^-$ in the forage can cause nitrite toxicity when gut microbes reduce the $NO_3^-$ to $NO_2^-$ in the rumen. The effects are identical to methemoglobinemia in humans, and if not treated can lead to the death of the animal (Church, 1993; NRC, 2005). A recent case of nitrate toxicity in dairy cattle occurred in the Campania region of Italy. A small herd of 50 dairy cows was fed waste scraps of fennel meant for human consumption that did not sell at a local market; within 48 hours of eating the fennel 15 of the cows had died. Tissue and blood samples taken during necropsies showed high levels of $NO_3^-$ present in the animals, and it was determined they had died of acute nitrate toxicity (Costagliola et al., 2014). The fennel had been grown in a contaminated region of Campania, and samples from both the green and white portions of the fennel showed high concentrations of $NO_3^-$ present in the plants (Costagliola et al., 2014).

While fennel and other novel produce are not common feedstuff for livestock in the United States, many zoo animals are provided fruit (e.g., apples, bananas, grapes, and papaya) and (or) vegetables (e.g., pumpkins, bell peppers, carrots, and leafy greens) either to help meet nutritional needs as part of the daily diet, or as a treat during enrichment and keeper interactions (Dadone et al., 2016; Shapiro et al., 2018; Shim and Dierenfeld, 2017). However, more common

forage plants (e.g., corn, straw, hay, and oats) have also been found to accumulate $NO_3^-$ in some instances (Church, 1993).

In non-ruminants the presence of increased $NO_3^-$ in feedstuffs is less of an issue, as they do not have the gut microbes required to reduce $NO_3^-$ to $NO_2^-$ that are present in ruminant animals. In order for non-ruminant species to suffer acute nitrate toxicity, an animal must drink from a nitrite-contaminated water source or directly ingest large amounts of nitrates (NRC, 2005).

The extent to which $NO_3^-$ in drinking water contribute to the detrimental effects on human and animal health is still under debate. Addiscott and Benjamin (2004) question the negative health effects associated with $NO_3^-$, and whether or not current recommendations for $NO_3^-$ in drinking water for people should be raised due to lack of convincing evidence of harm to human health. As of this writing, the EPA still considers $NO_3^-$ and $NO_2^-$ contributing factors in "blue baby syndrome" and has not changed the MCL for $NO_3^-$ nor $NO_2^-$ concentrations in drinking water (St. Clair, 2019). The current MCL for $NO_3^-$ is 10 mg/L and $NO_2^-$ is 1 mg/L in drinking water (EPA, 2018). Current drinking water recommendations for livestock are 100 mg/L and 10 mg/L for $NO_3^-$ and $NO_2^-$ respectively (NRC, 1974, 2005).

### 1.2.9. Metals

Copper, Fe, Pb, and Zn are metals that can be found in drinking water and may contribute to health issues in animals and humans. The degree to which these metals pose a risk to the health of animals consuming contaminated drinking water depends upon the type of metal present, its concentration, and the animal. Lead is recognized as a highly toxic substance even at relatively low concentrations, whereas, Fe concentrations above the EPA's SMCL recommendation of 0.3 mg/L in drinking water may or may not pose a health risk depending

upon the specie consuming the water. Each metal poses a different risk in drinking water and each has its own recommended safety concentrations, so considerations for each metal are described in detail subsequently.

#### 1.2.9.1. Copper and Molybdenum

Copper is not naturally found in drinking water supplies, but when present imparts a metallic taste. It is present in drinking water as a result of the erosion of plumbing (DHEC, 2013). It typically poses little risk to human health when present in high amounts in drinking water, because humans stop drinking the water due to the taste before ever consuming toxic concentrations (DHEC, 2013). Copper and Mo have a close interrelationship in the body. High concentrations of Mo in the diet can lead to Cu deficiency, whereas low concentrations of Mo in the diet can lead to Cu toxicity (Church, 1993). Due to this relationship, the Mo status and specie of the animal play an important role in how susceptible an individual animal will be to increased Cu in the drinking water. Like Cu, Mo is typically not naturally found in drinking water supplies. It is used for many industrial and agricultural purposes across the United States. Waste and runoff from these applications allow Mo to enter the water table, making its way into drinking water (WHO, 2011). The most common form of Mo used in industrial operations is molybdenum disulfide ($MoS_2$), which is not readily soluble in water, but is easily oxidized to more soluble and water-stable molybdates (WHO, 2011). It is recommended that the ratio of Cu-to-Mo in the diet of sheep be 6:1 or less, and not exceed 10:1, or Cu toxicosis may result (Villar et al., 2002). Whereas this is a dietary recommendation, it is important to take into account the possibility that high concentrations of Cu in drinking water can contribute to overall dietary Cu intake and an animal's Cu status. Long term or toxic exposure to excess Cu in drinking water can result in liver and kidney damage in humans, calves, and sheep with the latter

two having an increased risk of death (Church, 1993; Pond et al., 2005; EPA, 2018b). The current MCLG for Cu in drinking water for humans has an action level threshold 1.3 mg/L (EPA, 2018a). The upper limit for Cu in the drinking water of livestock and poultry is set at 0.5 mg/L (NRC, 1974). Currently, the EPA only has an HAs for Mo and it ranges from a 0.005 mg/L Reference Dose (RfD), to 0.08 mg/L 10-day HAs for children, and at the high end 0.2 mg/L Drinking Water Equivalent Level (DWEL) (EPA, 2018a).

#### 1.2.9.2. Lead

Lead is rarely found naturally in drinking water, and the primary source of Pb is the corrosion of metal pipes containing Pb due to other constituents present in the water (e.g., low pH) (DHEC, 2013). It is considered highly toxic, even when present at low concentrations in drinking water. It causes damage to many areas in the body including the red blood cells, the kidneys, the nervous system, and the brain (DHEC, 2013). All people are susceptible to the toxic effects of Pb, but unborn babies, infants, and children are at an increased risk because they are growing, and their brains are still developing. In livestock and rats, Pb in drinking water caused decreased fertility, fetal abortion, decreased growth rate, hypertension, and death (NRC, 1974). In 2015 a switch to a more corrosive water source caused Pb to be released from old pipes in Flint, Michigan and some residents had Pb concentrations of up to 0.105 mg/L in their drinking water (CNN, 2018). The increased Pb in drinking water raised concerns and increased specific testing for Pb in drinking water across the country.

The MCLG for Pb in the drinking water of humans is set at 0 mg/L, with a strictly enforced action level of 0.015 mg/L (EPA, 2018a). For cattle and other livestock, the upper limit was set at 0.10 mg/L of Pb in drinking water (Beede, 1991; NRC, 1974). When compared with the MCL for human drinking water, an upper limit of 0.10 mg/L for livestock seems high,

especially considering Pb accumulates in body tissue and can be transferred into the milk of lactating animals, which could pose a risk to dairy consumers (NRC, 1974).

#### 1.2.9.3. Zinc

Zinc is rarely found naturally in drinking water, even though it is prominent in rocks and soils. Like most of the other metals found in drinking water, the primary source of Zn in drinking water is corrosion of pipes, specifically galvanized pipes (DHEC, 2013). Zinc in water imparts a bitter taste that is often considered a "medicinal" taste or flavor. It is not considered a health threat in drinking water, but Zn concentrations above 3 mg/L may not be acceptable to humans (WHO, 2017). It however can cause intestinal irritation, nausea, and vomiting in humans at extremely high concentrations in water, at or above 675 mg/L, but at concentrations this high most people will refuse to drink the water due to the taste (DHEC, 2013). In livestock, decreased production and weight loss of laying hens occurred when provided high concentrations of Zn in the drinking water; mortality in rats was shown but only in the presence of high concentrations of Se and Zn (NRC, 1974).

The EPA recognizes Zn as an SMCL and has a set guideline value of 5 mg/L based on the aesthetics of human taste tolerance (EPA, 2018a). For livestock, the upper limit of Zn in drinking water is set at 25 mg/L (Beede, 1991; NRC, 1974).

#### 1.2.9.4. Manganese

Manganese is an essential nutrient required for normal development and maintenance by both birds and mammals, including humans (NRC, 2005; EPA, 2004). It is present in drinking water sources, but the major source of Mn intake for livestock and humans is from food, specifically vegetable and plant matter (EPA, 2004).

Manganese is naturally occurring but is not found in elemental form in the environment. It is instead found as a component of more than 100 minerals or salts in soil (EPA, 2004). Soil erosion, man-made products, and human activities, including, but not limited to, metal manufacturing, glass making, cleaning agents, gasoline, and fertilizers all contribute to the presence of Mn in drinking water sources (EPA, 2004; Gray, 2008; WHO, 2011a).

In water, Mn can be oxidized to its insoluble precipitate form, that can encrust pipes and slough or break-off into the water creating deposits of black particulate in holding tanks (Gray, 2008; EPA, 2004). Concentrations of Mn as low as 0.02 mg/L can cause build-up in plumbing, and in one broiler chicken study encrustation and clogging of nipple drinker systems at 0.05 mg/L Mn was reported (Batal et al., 2005; Gray, 2008). However, Batal et al. (2005) did not show a decrease in overall water intake by the broilers even at concentrations of up to 20 mg Mn/L. If drinking water systems and plumbing are not frequently cleaned and maintained when Mn is present, drinking water intake by birds and other animals could be disrupted.

In an acidic and anaerobic environment, such as ground water, Mn may stay in solution creating very high concentrations in some drinking water sources (Boyd, 2015; Gray, 2008; WHO, 2017). In the soluble form ($Mn^{2+}$), Mn is colorless and not visible to the human eye, but it does impart a bitter metallic taste to the drinking water (DHEC, 2013; Gray, 2008). This bitter taste is noticeable to the human palate at concentrations as low as 0.1 mg/L (WHO, 2017). While palatability issues arise for humans at concentrations as low as 0.1 mg/L, the Batal et al. (2005) study showed no significant decrease in water consumption by broiler chickens, across four concentrations of Mn, ranging from 0 to 20 mg Mn/L in the drinking water. Due to the palatability issues for human consumers, it is believed that drinking water containing Mn will be

unacceptable to drink and people will stop drinking the water before it is considered a health issue (Gray, 2008; WHO, 2017). The EPA set the SMCL for Mn at 0.05 mg/L, which is a more conservative standard than the WHO which set its value at 0.4 mg/L, which was set at four times the palatability threshold of 0.1 mg/L (EPA, 2018a; WHO, 2017). Specifically, for livestock there is no defined standard for Mn concentrations in drinking water (Beede, 1991; NRC, 1974).

#### 1.2.9.5. Iron

When present in water, Fe is considered a secondary constituent present in the ferrous ($Fe^{2+}$) and ferric ($Fe^{3+}$) forms (NRC, 1974). It is found naturally in rocks and soils around the world and is commonly found in well (bore) water (DHEC, 2013). It can be released from metal pipes when corrosion occurs due to other constituents present or properties of the water (e.g., low pH). Iron is not recognized by the EPA as having an adverse health effect in humans when found in drinking water (EPA, 2017). When present it typically imparts a metallic taste and a "rust" colored hue when oxidized to $Fe^{3+}$, eventually precipitating out and accumulating as deposits of rust in holding tanks and containers (DHEC, 2013). When Fe is in the $Fe^{2+}$ form and completely dissolved in water, it is colorless, odorless, tasteless, and has a greater bioavailability when ingested. This may pose an increased risk to humans and animals susceptible to Fe toxicity.

The SMCL for Fe in drinking water for humans is set at 0.3 mg/L and is based purely on the aesthetic of human taste tolerance (EPA, 2017, 2018a). In livestock, no safe upper limit was established for Fe in drinking water (Beede, 1991; NRC, 1974). An Fe concentration of 17 mg/L caused reduced growth, decreased milk production, and scouring in pastured cattle (NRC, 1974). A more recent study with dairy cows showed no observable decrease in water intake at Fe concentrations up to 4 mg/L (Genther and Beede, 2013).

Increased concentrations of Fe in water can encourage the growth of iron-loving bacteria. These bacteria are not considered a risk to human or animal health and are not known to cause any diseases (DHEC, 2013). The bacteria instead cling to the surface of pipes, oxidizing Fe from the soluble (ferrous) to insoluble (ferric) form. While attached to the surface of the pipe, generation after generation of the iron-loving bacteria build upon one another, which leads to decreased water pressure and flow through plumbing. The iron-loving bacteria can impart an unpleasant "rotten egg" taste on the water and do occasionally break loose appearing as small clumps of "rust" colored debris (DHEC, 2013). While the decrease in water pressure is a nuisance, there are no recommendations for the presence of these bacteria in drinking water. If one wanted to follow a guideline, the EPA has set a tolerance of microbial concentrations to be ideally zero, but this generally refers to disease causing pathogens discussed in **section 1.2.7.** (EPA, 2018a).

### 1.2.10. Water Quality Index (WQI)

In order for water quality to be rated on a comparative basis and to be described based on a gradation system, a method for calculating water quality as an index was developed by a sanitary engineer for the Ohio River Valley Water Sanitation Commission (ORVWSC) (Horton, 1965). Horton suggested the use of a water quality indexing system based upon a three-step process that involved the selection of quality characteristics, a rating scale for those characteristics, and finally weighting of the characteristics. The rating scale ($C$) used by Horton was arbitrary and chosen only for illustrative purposes, but his goal with a rating system was to show incremental improvements in the quality of the water in a way that one water sample could be compared with another either through time or across locations. As with the rating scale, the weighting factors ($W$) used by Horton were arbitrary and chosen for illustrative purposes in his

paper, but the objective of weighting the water characteristics used in the index was to show their relative importance. The characteristics Horton used were thought to be of greatest significance specifically for the ORVWSC, and not necessarily a set of selection characteristics applicable for every location or water use scenario (Horton, 1965). Horton also utilized coefficients (*M*) for two characteristics (temperature and "obvious pollution") that he assumed were not capable of being rated on a graduated scale like the other characteristics (e.g., dissolved oxygen, pH, coliforms, specific conductance, carbon chloroform extract, alkalinity, and chloride) used in his paper. The devised formula (**Algorithm 1.1.**) calculated a Quality Index (QI) value.

$$QI = \left\lfloor \frac{C_1W_1 + C_2W_2 \ldots + C_nW_n}{W_1 + W_2 \ldots + W_n} \right\rfloor M_1M_2 \qquad \textbf{(1.1.)}$$

**Algorithm 1.1.** Original Water Quality Index (WQI), where $\mathbf{C_n}$ is the rating scale, $\mathbf{W_n}$ is the weighting factors, and $\mathbf{M_1}$ is the coefficient for temperature, and $\mathbf{M_2}$ is the coefficient for "obvious pollution" (Horton, 1965).

A few years after Horton's original attempt to create a more universal method for calculating the quality of water with his QI formula, others attempted to improve upon his initial work (Brown et al., 1970). Utilizing the DELPHI method, Brown and his team performed three successive surveys of a single group of water quality management experts in order to gain a consensus about which characteristics should be used as parameters when gauging water quality. From the surveys, the researchers narrowed the list of characteristics from 44 down to 11, with order of importance assessed in the final survey; these eleven characteristics were labeled as "most significant parameters" (Brown et al., 1970). Once the characteristics (hereby referred to as parameters from this point forth to follow the shift in terminology in the literature) were established, the way the rating scale and weight for each parameter was calculated was re-evaluated. The rating scale (*C*) was changed to the "quality rating" ($q_i$) and calculated using the

"average curves" from the survey responses. The weighting factors ($W$) were changed to weights ($w_i$) and derived from the "significance rating" responses (Brown et al., 1970). No coefficients were used for any variables in future formulas. The modified WQI formula by Brown et al. (1970) is (**Algorithm 1.2.**):

$$WQI = \sum_{i=1}^{n} w_i q_i \quad \textbf{(1.2.)}$$

**Algorithm 1.2.** Updated Water Quality Index (WQI), where $\boldsymbol{w_i}$ is the weight of the parameter (a number between 0 to 1) and $\boldsymbol{q_i}$ is the quality of the parameter (a number between 0 to100) (Brown et al., 1970).

Further refinement occurred by other water quality experts to develop the weighted arithmetic WQI method of assessment as described below. The origins of the weighted arithmetic WQI could not be found in the literature. An article in the proceedings from the 6th International Conference on Water Pollution Research noted that Brown and colleagues were working on further improvement of the 1970 WQI formula to allow for easier understanding of water quality assessment by the general public, but no subsequent publication(s) were found (Brown et al.,1973). However, several other peer-reviewed articles show the use of the weighted arithmetic WQI formula (**Algorithm 1.5.**) as a way of assessing the quality of drinking water for human consumption (Akter et al., 2016; Tyagi et al., 2014; Yisa and Jimoh, 2010). The appeal of using the weighted arithmetic WQI, as opposed to the previous WQI formulas, is the use of the standard for each parameter, as set by a governing body (e.g., EPA, WHO, and local municipalities), to calculate the quality rating scale ($q_i$) (**Algorithm 1.4.**) and relative weight ($w_i$) (**Algorithm 1.3.**).

$$q_i = \left(\frac{C_i}{S_i}\right) \times 100 \quad \textbf{(1.3.)}$$

**Algorithm 1.3.** Formula for calculating the quality rating scale, where $C_i$ is the measured concentration of the *i*th parameter and the $S_i$ is the standard value of the *i*th parameter (Akter et al., 2016).

$$w_i = \left(\frac{1}{S_i}\right) \quad \textbf{(1.4.)}$$

**Algorithm 1.4.** Formula for calculating the relative weight (Akter et al., 2016).

$$WQI = \frac{\sum q_i w_i}{\sum w_i} \quad \textbf{(1.5.)}$$

**Algorithm 1.5.** Weighted arithmetic Water Quality Index (WQI), where $w_i$ is the relative weight of the *i*th parameter and $q_i$ is the quality rating scale of the *i*th parameter (Akter et al., 2016).

In the literature, the weighted arithmetic WQI was mainly used to assess drinking water at the surface water source, typically rivers. One study used the weighted arithmetic WQI to assess drinking water samples collected from households (Akter et al., 2016). Regardless of the water source tested, each study selected parameters relevant to assessing the quality of drinking water consumed by people and relevant to the location. The ability to choose the parameters included in the assessment calculation allows researchers to adapt the weighted arithmetic WQI to the needs of the location and to take into account the intended use of the water source being tested. This flexibility and the general ease of understanding for non-experts is why the weighted arithmetic WQI method of assessment was chosen for the use in our current study.

## 1.3 Iron in the body

### 1.3.1 Importance

Consideration of iron in drinking water is of special interest in the current study because of the potential risk of excessive iron consumption (from water or feed) to the black rhino in zoos. Iron is considered an essential nutrient due to its requirement in many metabolic processes. The ability of Fe to vary in oxidation state and redox potential makes it vital in many processes in the

body. On the other hand, this same ability allows unbound Fe to catalyze formation of reactive oxygen species (ROS), which are toxic to cells (Ross et al., 2014). One of Fe's most important functions is oxygen transport via the protein complex hemoglobin. A hemoglobin molecule consists of four heme groups and four globin protein chains. The heme groups are comprised of a porphyrin ring with a single central ferrous iron ($Fe^{2+}$) cation; this $Fe^{2+}$ cation is responsible for binding oxygen for transport through the bloodstream (Nelson and Cox, 2013; Ross et al., 2014). Iron also is a crucial cofactor or enzyme component in various metabolic pathways (e.g., the citric acid cycle). The enzymes ferredoxin, cytochrome P-450, catalase, cytochrome oxidase, and many others require Fe as a cofactor to perform their functions (Nelson and Cox, 2013).

### 1.3.2 Metabolism

Dietary Fe is typically in the ferric ($Fe^{3+}$) form. After ingestion, hydrochloric acid (HCl) in the stomach breaks down the food, releasing $Fe^{3+}$ into the stomach. The $Fe^{3+}$ binds with various small molecules within the stomach making it soluble and available for absorption when it reaches the small intestine. In the small intestine, $Fe^{3+}$ must be reduced to $Fe^{2+}$ in order to be transported into the enterocytes. Ferrireductase enzymes reduce $Fe^{3+}$ to $Fe^{2+}$, which can then be transported via divalent metal transporter 1 (DMT-1) into the cell (Ross et al., 2014). Once in the cell, the $Fe^{2+}$ can be oxidized back into the $Fe^{3+}$ state and stored as ferritin, or it can be transported out of the cell via ferroportin (Ireg-1) where it is oxidized back to $Fe^{3+}$ by the ferroxidase, hephaestin (Kaneko et al., 2008). The $Fe^{3+}$ then binds to the transporter protein transferrin, so it can safely travel through the bloodstream. Whether the Fe is stored as ferritin in the enterocyte or transported out is largely determined by the Fe status of the body. If the body has adequate Fe, the Fe remains in the enterocyte as ferritin and is excreted in the feces when the cell is sloughed off. From the bloodstream, 75% of absorbed Fe ends up in the bone marrow for

erythropoiesis, and 10 to 20% is transported to the liver where it can be stored as ferritin. The Fe transported to the liver can later be released back into the bloodstream when Fe is needed by other tissues or for erythropoiesis.

### 1.3.3 Transport

Within the body, Fe is transported via the bloodstream. In order to be transported safely through the bloodstream, Fe must be in a non-reactive form. The protein apo-transferrin binds $Fe^{3+}$ to prevent Fe-catalyzed oxidative reactions by keeping the Fe soluble while traveling through the bloodstream. This protein is commonly referred to as transferrin. Apo-transferrin is the term used to describe the protein when it is not bound to an Fe molecule. Monoferric transferrin refers to a transferrin protein bound to a single $Fe^{3+}$ molecule, leaving the second binding site empty. Diferric transferrin is the term used to describe transferrin when it is bound to two $Fe^{3+}$ molecules, both biding sites are occupied.

Whereas, transferrin can bind two molecules of $Fe^{3+}$ and is potentially able to keep two Fe molecules soluble and non-reactive during transport, this is often not the case. At normal Fe saturation levels, diferric transferrin usually only accounts for 10% of the transferrin protein within the blood plasma; apo-transferrin is the most abundant followed by monoferric transferrin (Kaneko et al., 2008). Even though monoferric transferrin may be found in large amounts in the blood plasma, diferric transferrin still accounts for the majority of the Fe delivered to tissues throughout the body.

Transferrin molecules transferring $Fe^{3+}$ through the bloodstream bind to a protein expressed on the surface of cells seeking Fe, called transferrin receptor-1 (TfR-1). The TfR-1 has an eight- to ten-fold greater binding affinity for diferric transferrin than for apo-transferrin or monoferric transferrin (Kaneko et al., 2008). This increased binding affinity is the reason

diferric transferrin delivers more Fe to body tissues than monoferric transferrin. Transferrin proteins carrying $Fe^{3+}$ bind to TfR-1 on the surface of the cell. The cell then performs endocytosis around the TfR-1 receptor, bringing it, and the bound transferrin and Fe molecules, into the cell. The vesicle created by the endocytosis, begins taking in hydrogen atoms ($H^{+}$). This influx of hydrogen lowers the pH within the vesicle releasing the $Fe^{3+}$ from the transferrin protein in the $Fe^{2+}$ form. The now apo-transferrin protein remains bound the TfR-1. The influx of hydrogen ion encourages the expression of DMT-1 within the vesicle membrane layer to counteract the drop in pH within the vesicle due to the hydrogen uptake. The DMT-1 then releases the $Fe^{2+}$ and the hydrogen atoms from the vesicle into the cell. Exocytosis then occurs releasing the apo-transferrin protein back into the bloodstream and returning the TfR-1 receptor to the cell surface.

### 1.3.4. Storage

Once the $Fe^{2+}$ is released from the vesicle via DMT-1 within a cell, it is then oxidized to $Fe^{3+}$ by catalytic sites on the surface of ferritin to be stored. Ferritin is the main storage form of Fe within the tissues of the body. A ferritin molecule consists of 24 apo-ferritin H or L monomeric subunits surrounding a central cavity that can accommodate as many as 4,000 Fe atoms in a ferric oxide core (Kaneko et al., 2008). The H subunits are associated with the catalytic sites that oxidize $Fe^{2+}$ into $Fe^{3+}$ within the cell. The L subunit does not perform oxidation of $Fe^{2+}$ to $Fe^{3+}$, but it more efficiently moves $Fe^{3+}$ to the ferric oxide core within the storage molecule. The composition of ferritin varies between cells, with L subunit ferritin molecules predominating in the liver and spleen, and H subunits predominating in erythrocytes. Binding $Fe^{3+}$ molecules in ferritin for storage is a safety measure that prevents the $Fe^{3+}$ from catalyzing damaging oxidative reactions within the tissue.

Although ferritin is the most prominent storage form of Fe, in cells suffering iron overload, hemosiderin is present. Hemosiderin is most commonly found in macrophage cells in an animal with normal Fe concentrations. Macrophage cells are responsible for the breakdown of old red blood cells within the spleen and are the most common cells to suffer iron overload even if the organism is not suffering from iron overload on a macro level. Hemosiderin consists of partially degraded ferritin molecules that form after the uptake of lysosomes (Kaneko et al., 2008). Because hemosiderin is composed of many partially degraded ferritin molecules, hemosiderin can store much larger quantities of Fe atoms than ferritin. During Iron Overload Disorder (IOD) in mammals, other cells besides macrophages can and do express the presence of hemosiderin; liver, spleen, and other tissues show increased presence of hemosiderin during IOD, and in some publications, iron overload is referred to as excess hemosiderin deposits (Pond et al., 2005).

### 1.3.5. Regulation

There are two main mechanisms of regulation for Fe uptake within the body. The first is regulation within the cytoplasm at the post-transcriptional level, and the second is a more systemic regulatory mechanism via a peptide called hepcidin.

Regulation of Fe within the cytoplasm influences the synthesis of proteins important for Fe metabolism including DMT-1, TfR-1, Ireg-1, ferrireductase proteins, and iron responsive protein-1 (IRP-1) (Kaneko et al., 2008). The IRP-1 is a protein that binds to iron responsive elements (IREs), sections of mRNA that regulate translation of proteins used in Fe metabolism. The IREs are found at both the 5' and the 3' end of mRNA. When an IRP binds to an IRE at the 3' end of the mRNA strand, the mRNA is stabilized promoting protein synthesis of TfR-1 and reducing synthesis of apo-ferritin. The opposite occurs when an IRP binds to an IRE at the 5' end, reducing synthesis of TfR-1, and promoting synthesis of apo-ferritin. Binding at the 3' end

typically occurs when cytoplasmic Fe concentrations are low, while binding at the 5' end typically occurs when cytoplasmic Fe concentrations are high. This complementary regulation of TfR-1 and apo-ferritin helps to regulate Fe concentrations within hepatocytes.

The second method of Fe regulation is through hepcidin, a small circulatory antimicrobial peptide synthesized by hepatocytes. Hepcidin works to regulate Fe in three ways: 1) absorption of Fe in the small intestine, 2) release of Fe from hepatocytes and macrophages, and 3) transport of Fe across the placenta. The ferroportin protein, Ireg-1, on the surface of enterocytes within the small intestine, allows Fe transport out of enterocytes and into the bloodstream. Hepcidin binds to Ireg-1, which causes endocytosis and breakdown of Ireg-1, reducing expression on the surface of the enterocyte. This reduced Ireg-1 expression decreases the number of channels available for Fe to leave the enterocyte, which leads to a decreased amount of Fe entering the bloodstream. This same mechanism is responsible for the regulation of Fe release from hepatocytes and macrophages, as well as, the transport of Fe across the placenta.

The production of hepcidin is modulated by the body's Fe requirements, which are affected by Fe body stores, anemia, inflammation, and the degree of erythropoiesis; the latter being the most influential on hepcidin production. The exact pathways involved in hepcidin production are still unknown, but three molecules involved in regulation of these pathways were identified: hemochromatosis (HFE) protein, transferrin receptor-2 (TfR-2), and hemojuvelin (HJV). The current hypothesis is that HFE and TfR-2 on the surface of hepatocytes regulate hepcidin in response to transferrin saturation in the bloodstream. The HFE is a protein that interacts with TfR-1 and TfR-2 on the surface of cells to help regulate the interaction between transferrin and the transferrin receptors (Ross et al., 2014). Cells express TfR-1 when diferric transferrin

saturation is high, due to the strong binding affinity present between the two molecules. The TfR-2 has a much lower binding affinity for diferric transferrin than TfR-1, so it is presumed that TfR-2 expression would be decreased when diferric transferrin concentrations are great. This would result in decreased hepcidin production and decreased release of Fe from hepatocytes and other cells during a time of high Fe concentrations in the bloodstream. Another protein believed to be involved in the regulation of hepcidin production is HJV, a membrane bound protein that is a bone morphogenetic protein (BMP) co-receptor. It was shown that certain BMPs stimulate hepcidin production via the BMP signal transduction pathway, but the exact mechanism remains unknown (Malyszko, 2009).

### 1.3.6. Iron-Manganese Interactions

Iron and Mn share common absorption mechanisms and transport proteins (Bjørklund et al., 2017; Ross et al., 2014). Both metals can be found in divalent and trivalent forms in the body, with their divalent forms binding to DMT-1, and their trivalent forms binding to transferrin. Research showed that an increased amount of Mn in the diet can lead to a decrease in Fe absorption, and an increased amount of Fe in the diet can lead to decreased Mn absorption (NRC, 2005; Ross et al., 2014). The sharing of common transport proteins may lead Fe and Mn to compete with one another for the opportunity to bind, which may cause of the inverse relationship between the two metals during the absorption process in the body.

### 1.3.7. Disorders

The two most common disorders associated with Fe metabolism are iron deficiency and iron overload. Iron deficiency comes in three stages: storage iron deficiency, iron deficient erythropoiesis, and iron deficiency anemia (anemia). The most common causes of anemia in adult domestic animals is blood loss, but it is possible to see anemia present in animals that are

fed inadequate Fe. Young and female mammals are most susceptible to anemia due to growth and menstruation, respectively. Treatment for anemia is Fe supplementation either by increasing the concentration of Fe given in the diet or supplying an Fe-containing supplement in addition to the normal diet.

Iron overload is the accumulation of Fe within the body. There are two ways to describe iron overload: hemochromatosis, the increased accumulation of Fe within cells, resulting in organ injury and failure; and hemosiderosis, the accumulation of Fe within cells without evidence of organ dysfunction. Hemosiderosis typically refers to the accumulation of Fe by macrophage cells. Hemochromatosis can further be separated into a primary and a secondary form. Primary hemochromatosis refers to inherited disorders of Fe metabolism. Secondary hemochromatosis refers to non-inherited Fe accumulation, typically due to environmental factors, such as unnecessary Fe supplementation, excess dietary Fe, repeated blood transfusions, chronic hemolytic anemia, ineffective erythropoiesis, and possibly liver disease (Kaneko et al., 2008).

The mechanism of iron overload within the body is as follows. Elevated Fe concentrations within the body increase the saturation of transferrin proteins within blood. The increased Fe concentration leads to high diferric transferrin saturation within the bloodstream, and decreased Ireg-1 expression on the surface of hepatocytes. This decreased expression of Ireg-1 may be the reason hepatocytes accumulate Fe more than macrophages or enterocytes when an animal suffers iron overload (Kaneko et al., 2008). The increased uptake of Fe by hepatocytes leads to an increased formation of ferritin within the cytoplasm, and an increased formation of hemosiderin in lysosomes (Kaneko et al., 2008). Accumulation of large amounts of Fe within the hepatocytes

can result in cell injury, increased fibrogenesis, and liver failure in severe cases. This increased uptake of Fe by hepatocytes helps to bind "free" Fe, and thus limit the production of ROS, but oxidative damage may still occur to proteins and DNA when excess Fe accumulates within cells. Other organs can accumulate excess ferritin and hemosiderin during acute and chronic cases of iron overload including in the pancreas, heart, kidneys, brain, and endocrine organs. Iron overload increases serum Fe and serum ferritin concentrations. However, due to the possibility that serum concentrations can be increased by other factors, the best way to definitively diagnose iron overload is via liver biopsy. In domestic animal species, iron overload is rare (NRC, 2005). However, IOD is a prevalent issue in captive populations of black rhino (Clauss and Paglia, 2012). Treatments for iron overload can vary by cause and species. The treatment may be phlebotomy to encourage erythropoiesis, medication to help limit Fe absorption, modification to reduce environmental factors such as the diet, or any combination of these approaches.

### 1.3.8. Dietary Recommendations for Black Rhino

When determining nutritional requirements for wildlife kept in captive settings, nutritionists and veterinarians refer to data and publications for domesticated species, as well as, look at the natural diet and feeding habits of wild animals of the same or similar species. Because black rhinoceros are large nonruminant mammals, the representative species often used to determine dietary recommendations is the domestic horse (Dierenfeld, 1996). The current dietary recommendation for Fe in domestic horses is 50 mg Fe/kg of dietary dry matter (NRC, 2007).

The current dietary Fe recommendation for captive black rhino is set at a maximum concentration of 300 mg Fe/kg body weight, or about 6 g of Fe daily, with further recommendation that the dietary concentration should be maintained within the range of 50 to

100 mg Fe/kg (Clauss et al., 2012). These dietary recommendations were determined empirically by a group of nutritionists and veterinarians at the most recent special workshop addressing IOD in captive black rhino in 2011. The basis for these recommendations comes from examining the Fe concentrations in feedstuffs consumed by wild black rhino and comparing them to the Fe concentrations of feeds and the diet provided to captive black rhino in zoos.

## 1.4. Black Rhino Biology

### 1.4.1. Wild Biology

Black rhino are critically endangered large, odd-toed ungulates from the continent of Africa. They are distinguished from white rhino by their prehensile hooked upper lip, a smaller stature, a shorter head, and smaller rounded ears (Skinner and Chimimba, 2005). Historically black rhino were found throughout sub-Saharan Africa, with the exception of the Congo Basin. Their current distribution is much more limited; today they are found in Cameroon, Kenya, Malawi, Mozambique, Ethiopia, Namibia, South Africa, Rwanda, Swaziland, Tanzania, Zimbabwe, Zambia, and Botswana with the last two states being where black rhino were re-introduced (Skinner and Chimimba, 2005; WWF, 2019). While black rhino can be found in habitats ranging from deserts to forest, they most frequently are found in transition areas between grasslands and forests. Black rhino are typically solitary animals, with the exception being a mother and her calf. Although some transitory associations have been observed between subadult rhino pairs that have been chased off by their mothers, these associations can last months or even years (Skinner and Chimimba, 2005).

In the wild, black rhino can live 30 to 35 years, and typically reach sexual maturity at 6 to 7 years for females and 7 to 8 years for males. The average calving age for a mature female (cow) is 6.5 years old, and spermatogenesis beginning in males (bulls) at 8 years old. Bulls

typically are not observed holding territory or mating until 9 years or older, unless there is an absence of older, more dominant bulls. Mating can occur during anytime of the year, with minor peaks in births twice a year in January and June to August in the Southern subregions of Africa (Skinner and Chimimba, 2005). Mature bulls and cows come together for weeks at a time during mating, as the male will begin courting the female during pro-estrus and stay through estrus mating several times with the cow (Dorst and Dandelot, 1969; Skinner and Chimimba, 2005). Gestation lasts approximately 15 months, and females give birth to one calf at a time. The calves weigh approximately 40 kg at birth and can stay with the mother for 2 to 4 years; typically, being rejected by the mother either during her next pregnancy or at the birth of her next calf (Skinner and Chimimba, 2005). The length between calving can vary from 23 months to over 3 years depending upon environmental circumstances and region. Calves begin to walk and nurse within three hours of birth and continue to nurse for up to 18 months. Starting at a few weeks old, calves will begin to browse, consuming twigs, shoots, and leaves that will become their regular diet upon weaning (Kurnit, 2009; Skinner and Chimimba, 2005).

### 1.4.2. Wild Diet and Eating Habits

Black rhino are a non-ruminant browsing species and in the wild they feed on leaves, twigs, and tree bark primarily from Acacia shrubs with a smaller proportion of the diet coming from other plant species. Feeding typically occurs overnight and can be observed at dawn and dusk, with wallowing, resting, and roaming occurring during the heat of the day (Dorst and Dandelot, 1969; Helary et al., 2012). They have been observed pushing over larger plants in order to reach edible growth (Skinner and Chimimba, 2005). Black rhino browse on a large variety of plant species within their environment, but clear preferences were observed. In a study of black rhino in Nairobi National Park in Kenya, 34 species of plants were identified as

suitable for browsing by black rhino; of the 34, two species were not consumed at all by the black rhino in the park. Of the remain 32 species, six were identified as preferential by the amount of consumption by black rhino in comparison to the other species. *Grewia similis* (most), *Hibiscus fuscus*, *Phyllanthus fischeri*, *Acacia brevispica*, *Barleria grandicalyx*, and *Lippia javanica* were the top six preferred species of browse, respectively (Muya and Oguge, 2000). There were further correlations in preference tied to the amount of crude fiber, crude protein, polyphenols, and alkaloids present in the feed items, with a preference being greater for fiber browse species with low phenol and low alkaloid presence. The selection for high fiber food items came at the expense of consuming plants with greater protein content; most high-fiber foods were naturally lower in crude protein. Black rhino in deserts and arid regions have been observed consuming the succulent pencil tree, *Euphorbia tirucalli*, at up to 70% of their diet during the dry season as a primary source of water intake (Skinner and Chimimba, 2005).

### 1.4.3. Iron in the Wild Diet

The Fe content of the wild diets consumed by black rhino varies seasonally and by region, depending upon the growth stage of the plants related to rainfall; with Fe concentrations ranging from 49.5 to 170 parts per million (ppm) dry basis. Across the same regions and seasons, the condensed tannin (CT) concentrations also were shown to vary between 1.7 to 4.3% of DM but did not vary quite as drastically as Fe content (Helary et al., 2012). The CTs are polyphenolic compounds identified as contributing to reduced bioavailability of Fe in the diet of black rhino (Lavin, 2012). The reduced bioavailability of Fe in the diet of wild black rhino may be a key factor contributing to no cases of IOD in wild black rhino (Lavin, 2012; Paglia and Dennis, 1999).

### 1.4.4. Genetics of Iron Absorption

Although the entire genome of the black rhino has not been decoded, some aspects of black rhino DNA are being looked at to determine the possible contributions genetics may have to IOD in captive black rhino. In humans IOD, primary hemochromatosis, is determined genetically. Recently, an HFE gene was identified as a possible evolutionary adaptation to the naturally low Fe diets of black rhino in the wild (Beutler et al., 2001). This evolutionary adaptation may be the cause of IOD in captive black rhino confronted with a diet much higher in more biologically available Fe than their wild counterparts or if the captive animals were in the wild. The change to the HFE gene leads to the change in the HFE protein expressed during translation of the gene sequence; the S88T allele leads to the switch of a serine-88 amino acid to a threonine within a region of the HFE protein that interacts with the transferrin receptor (Beutler et al., 2001). This change in the S88T allele was only observed in black rhino DNA when compared with the same region of DNA in white rhino and Sumatran rhino. It is theorized that the increased size of the threonine in the 88 position leads to a larger spread between neighboring amino acids that interact with the transferrin receptor. This evolutionary adaptation in black rhino causes them to be unable to properly regulate the quantities of Fe absorbed or assimilated while consuming Fe-rich diets provided in captivity.

### 1.4.5. Gut Microbiome

Recently, comparative research has been done investigating the diversity in the gut microbiomes of captive and wild individuals of the same species. One study looked at 41 different mammalian species, including black and white rhino, comparing the gut microbiome between captive and wild representatives for each species. This study found a decrease in species richness (alpha diversity) across all species examined, except black and white rhino that

appeared to have an increase in alpha diversity (McKenzie et al., 2017). When looking at the species diversity (beta diversity) between captive and wild black and white rhino populations, McKenzie et al. (2017) did find significant change in the gut microbiome make-up. A more in-depth analysis at the genus level was performed and found a significant shift in microbe species between captive and wild black rhino gut microbiomes (Gibson et al., 2019). The shift showed an increase in the amount of microbe species found in domestic livestock gut microbiomes within the captive black rhino compared to the wild black rhino sampled. More specifically, there appeared to be an increase in microbe species found in domestic ruminant animals. The Gibson study also found that 97% of the reads were unmapped, pointing to a need for a more robust database pertaining to black rhino gut microbiome (Gibson et al., 2019). Further research is needed to determine what role, if any, the shift in gut microbiome may play in the current health issues affecting captive black rhino populations.

## 1.5. Black Rhino Husbandry

### 1.5.1. Captive Diet and Eating Habits

Current diets provided to black rhino in captivity typically consist of large quantities of varying types of grass and(or) alfalfa hay, commercially produced pelleted “complete feeds”, small amounts of fruits and vegetables, and varying amounts and types of browse, if any is provided at all. The types and proportions of these feedstuffs vary by facility due to availability and cost constraints. Exact diets provided at each facility are unknown, as this is not readily shared information with the public, or even other institutions. Generic example diets were provided by the most recent IOD workshop (Clauss et al., 2012).

### 1.5.2. Iron in the Captive Diet

Current diets provided to black rhino in captivity vary greatly in Fe content depending on which feed items are provided and the quantity and quality of those feed items. Grass and alfalfa hays can vary anywhere from 10 to 2,599 ppm Fe in forage, dry basis. Other feed items, such as, corn grain, beet pulp, and soybean meal, can range anywhere from 10 to 600 ppm Fe (Clauss et al., 2012). This results in a wide range of variability and a great deal of uncertainty to just how much Fe is being provided in the diet to captive black rhino. While some facilities do evaluate the nutrient content of feed items, it may be expensive and is not always done frequently. It is suggested in the IOD workshop publication that minimal routine analysis of hay and "complete feeds" be conducted annually to keep track of overall Fe content in feeds (Clauss et al., 2012).

### 1.5.3. Iron Control Methods

As with diets provided to black rhino in captivity, methods for controlling blood Fe vary by facility. The currently recommended method is dietary control and serum testing (Clauss et al., 2012). Most feed manufacturers catering to the needs of zoos have low Fe "complete feed" diets specifically formulated for black rhino, but, due to the lack of information about diets provided across all facilities, it is hard to know how many facilities utilize the "complete feed" formulas. Another control strategy known to be utilized by at least one facility to manage blood Fe concentrations is phlebotomy (Mylniczenko et al., 2012). This black rhino facility employs the most thorough measures to combat IOD; including low Fe diets, routine testing of blood serum Fe markers, routine testing of feed items, and phlebotomy as a last resort when serum Fe markers are consistently high (Mylniczenko et al., 2012). The black rhino at this facility were trained to undergo phlebotomy awake while receiving treats and attention; they will stand and allow for up to 4 L of blood to be withdrawn in one time. The removal of blood encourages erythropoiesis to

replace the red blood cells and encourage mobilization of Fe stores from tissue and into the bloodstream (Johnson et al., 2009; Paglia and Dennis, 1999). Staff at this facility expressed success in utilizing phlebotomy to control IOD in their black rhino population, and they are looking to train staff at other facilities about how to safely and efficiently utilize this technique in other captive black rhino populations.

### 1.6. Conclusions

Water is the most essential nutrient for humans and animals alike, but it can also be a source of excess minerals and detrimental constituents if not monitored. Physical, chemical, biological, and radiological factors all contribute to the overall quality of drinking water, all of which can affect human and animal health (Gray, 2008; NRC, 1974; Vigil, 2003). The unfortunate loss of eight black rhino to salt poisoning via a drinking water source in a Kenyan national park illustrates how drinking water quality can contribute to mineral toxicity in even large mammalian species (Van Sant, 2018). All zoo animal species potentially can be affected by poor quality drinking water. Excessive Fe concentrations can be a common water quality issue for animals, as such, the presence of Fe in the drinking water provided to captive black rhino could be a contributing source of dietary Fe (Beede, 2009; Clauss et al., 2012). The Fe concentrations in water among AZA zoos might not be routinely measured and certainly is not reported in the literature.

# CHAPTER 2

# ASSESSMENT OF DRINKING WATER QUALITY AND RELATED HUSBANDRY PRACTICES IN NORTH AMERICAN ZOOS

## 2.1. Introduction

Based on a thorough review of the literature, little information was found pertaining to the monitoring, laboratory analysis, assessment, and treatment of the drinking water for animal collections in zoos. With the lack of information from studies in peer-reviewed publications, it is unknown whether or not drinking water can be a contributing factor to nutritional disorders affecting captive wild species. In general, poor quality drinking water can impact negatively the health of animals (NRC, 1974). For example, essential nutrients such as iron might be present in drinking water in great enough concentrations to contribute to health effects such as Iron Overload Disorder (IOD) in black rhinoceros, if iron concentrations are not properly monitored and corrected. Research is needed to better understand the current status of drinking water quality in zoos, and whether it may be contributing to the overall nutritional status of zoo animals. A main aim of this thesis project was to characterize the current state of drinking water quality in zoos and identify constituents in excess compared with standard concentrations per individual zoo. This information might be helpful to improve husbandry practices, and in turn, the overall health of animal collections.

In addition, it is important to understand management practices used by zoos that might affect water husbandry (e.g., sources, frequency of use, and quality testing). Knowing the source of the drinking water, at which point it enters the zoo, and who makes the decisions about drinking water practices, in addition to having drinking water quality analysis, could be beneficial to our study assessment and to zoos. Relationships found between drinking water quality analysis and husbandry practices could aid in making decisions about drinking water that may affect animal collections. In order to assess water husbandry practices, a questionnaire was

developed and distributed to a sub-sample of zoos that participated in this project.

The over-arching objective of the research pursued in this thesis project was to help fill gaps in the literature about drinking water quality in zoos. Our specific objectives were to: 1) assess the current state of drinking water quality in Association of Zoo and Aquarium (AZA) zoos across North America by analyzing drinking water samples; and, 2) administer a questionnaire to a randomly selected subset of participating zoos in order to gain information about husbandry practices related to drinking water. Subsequently we were able to assess relationships between drinking water quality and husbandry practices within participating zoos.

We shall make our findings available to all AZA zoos in an effort to increase awareness of the importance of drinking water quality and husbandry practices, and the potential impact of poor-quality drinking water on zoo animal health.

## 2.2. Materials and Methods

### 2.2.1. Sample Groups

A contact list of all zoo facilities accredited by the AZA was obtained directly from the Association. This list was used to randomly invite zoos to participate in the study. Initially the list included a total of 233 accredited facilities; some were removed from consideration or disqualified (see reasons below), and the remainder were regrouped for random selection based on purposes of the study (**Figure F.1.**). One facility was removed due to loss of AZA accreditation and two others because they were only insect collections. Another 56 were removed because they were only aquariums, with no terrestrial mammalian species. The remaining 174 zoos were then divided into two groups, those with black rhinoceros ["black rhino" (BR); n = 32] and those without BR ["non-black rhino" (Non-BR); n = 142]. Once the BR and Non-BR groups were established, a random number generator selected a sample from

each group. Zoos to be selected and studied could participate either by: 1) completing the detailed questionnaire plus providing the requested number of drinking water samples for water quality assessment (four samples per facility for BR zoos or one sample per facility for Non-BR zoos); or, 2) simply providing the requested drinking water sample(s) per group of zoo without completion of a questionnaire. There were 25 BR zoos (78% of total BR zoos) in the "questionnaire and water sample" group. The remaining seven BR zoos constituted the "water sample only" group. For the Non-BR group, 100 zoos were selected randomly for the "questionnaire and water sample" group with the remaining 42 being in the "water sample only" group. It was learned after group selection, that one BR zoo in the "questionnaire and water sample" group did not actually have black rhino in their collection. The decision was made to place this zoo in the Non-BR "questionnaire and water sample" group. This brought the BR "questionnaire and water sample" group to 24, and the Non-BR group to 101 zoos. One other BR zoo recently had moved its last black rhino to another zoo for breeding program purposes. That zoo continued to maintain the exhibit where the last black rhino had lived but housed a different rhino species; that facility remained in the BR "questionnaire and water sample" group for the study. The zoo collected the appropriate drinking water samples from the historically black rhino exhibit and completed a questionnaire while responding in reference and context to the black rhino.

Once the initial BR and Non-BR "questionnaire and water sample" and "water sample only" groups were established, all 174 eligible zoos were sent a letter of introduction with a pre-paid response postcard inviting each zoo to declare participation in our study. Zoos were provided multiple ways to respond to the invitation (e.g., the pre-paid return postcard, telephone,

or email). Three successive rounds of invitation letters and pre-paid response postcards were mailed (**Figure F.2.**). The first round of invitations resulted in 54 zoos responding to the invitation; 15 were BR zoos and 39 were Non-BR zoos. Two BR and two Non-BR zoos declined to participate; the rest agreed to participate. In the second round, another letter and postcard were sent to the 120 facilities that had not responded to the initial invitation. The second invitation resulted in 18 zoos responding with one BR zoo and 3 Non-BR zoos declining; 14 Non-BR facilities agreed to participate; no BR zoos agreed to participate in response to the second invitation. The final invitation letter and postcard were sent to the remaining 102 zoos that had not responded to the first or second invitations. Fourteen zoos responded to the final letter. Four of five BR zoos agreed to participate, and eight of nine Non-BR zoos agreed to participate. The remaining 88 zoos never responded to any one of the three invitation letters and were designated as "declined to participate". Overall, a total of 76 zoos, 17 BR and 59 Non-BR, agreed to participate in the project at this stage of the selection process.

The 76 zoos that agreed to participate in the study were sent an email asking if they required approval by a committee at their respective facilities in order to proceed and participate; 34 of 76 zoos required in-house approval. Of those 34, five committees did not approve the project and the zoos were dropped from the study. The remaining 29 committees approved their facilities' participation in the study. Twenty-three zoos did not require in-house committee approval, and three withdrew from the study for other unknown reasons. Over the course of initiation and implementation of the study another 16 were removed due to lack of response to multiple correspondence attempts after initially agreeing to participate. Later in the study, after questionnaires and water sampling kits were mailed, two zoos (one that did not require approval,

and one that received approval) also were removed due to lack of responsiveness. These two zoos did not return water sampling kits or questionnaires and stopped responding to all correspondence attempts. This brought the total number of zoos participating in the study to 50 (**Figure F.3.**).

### 2.2.2. Questionnaires

Two questionnaires were developed for the study. One questionnaire was given to Non-BR zoos randomly selected to complete the questionnaire. The second was given only to BR zoos randomly selected to complete a more extensive questionnaire.

The Non-BR questionnaire contained twelve questions. The first ten (questions 1 through 10) pertained to water husbandry practices (**Figure C.1.**), whereas the last two (questions 11 and 12) were for our recordkeeping and not included in the analysis. These Non-BR questions were general and also were included as questions 1 through 10 in the BR questionnaire. This gave a total of 10 identical questions asked to all participating zoos selected to complete a questionnaire as part of our study. In addition to the first 10 questions answered by all zoos, BR zoos also were asked questions 11 through 28 written specifically about BR husbandry and drinking water practices (**Figure C.2.**). As with the Non-BR questionnaire, the final two questions in the BR questionnaire (questions 29 and 30) were for recordkeeping and were not included in the analysis.

### 2.2.3. Water Sampling Kits

Once sample groups were established, drinking water sampling kits were created to facilitate water sample collection. To make the process as simple as possible for participating zoos, all required sampling equipment and pre-completed documents were provided with instructions in a prepaid shipping container mailed directly by the zoo to a commercial

laboratory for analysis.

Four different kits were created for the different sampling groups as follows: 1) Non-BR without questionnaire, 2) Non-BR with questionnaire, 3) BR without questionnaire, and 4) BR with questionnaire. Each kit contained drinking water sampling instructions, water sample submittal form(s) required by the laboratory, water sampling bottle(s), absorbent laboratory bench pads, 1-gallon size zip-lock bag(s), and a prepaid shipping label. For zoos completing a questionnaire, the kit also included the appropriate questionnaire and a postage prepaid envelope to return the questionnaire directly to us, separate from the water sample(s). All of the kits were packaged in a U.S. Postal Service box that could be reused as the water sample shipping container. Examples of the sampling instructions, sample submittal forms, sampling bottles, and questionnaires are in **Appendix B**.

#### 2.2.3.1. Sample Collection from Non-Black Rhino Zoos

All participating Non-BR zoos were asked to collect a single drinking water sample from the "origin" point. The "origin" is defined as the point within the zoo as close as possible to where the drinking water supply (e.g., well or off-site supply line) entered the zoo property. These samples were analyzed at a commercial laboratory [Cumberland Valley Analytical Services (CVAS), Waynesboro, Pennsylvania] using the "Livestock Suitability Package" which analyzes for a set of non-microbial factors: pH, hardness, total dissolved solids (TDS), Ca, P, Mg, K, Na, Fe, Mn, Zn, Cu, chlorides, sulfate, and nitrate (CVAS, 2019).

The Non-BR zoos that were part of the randomly selected subset also were asked to complete and return a questionnaire in addition to providing an "origin" drinking water sample.

#### 2.2.3.2. Sample Collection from Black Rhino Zoos

All participating BR zoos were asked to collect two drinking water samples from the

"origin" point, as well as, two drinking water samples from within the black rhino exhibit; a total of four drinking water samples. Two of the drinking water samples, one from the "origin" and one from within the exhibit, were analyzed by CVAS using the "Livestock Suitability Package". The remaining two drinking water samples, one from the "origin" and one from within the exhibit, were analyzed by CVAS for total coliform and *Escherichia coli* bacteria.

The BR zoos that were part of the randomly selected subset also were asked to complete and return a questionnaire in addition to providing four drinking water samples for laboratory analysis.

### 2.2.4. Water Quality Index (WQI) Calculations

The results of each drinking water quality analysis were returned from the laboratory to us and the respective zoo upon completion. The water quality analysis also was utilized to calculate four different water quality indexes (WQI) for each zoo. In general, the WQI is a mathematical algorithm derived as a method of assessing drinking water quality based upon standards of acceptability for each parameter or analyte set by government agencies (Brown et al., 1970; Horton, 1965). The different WQI calculations used in the current study were a specific set of non-microbial analytes: 1) low standard including "all analytes", 2) low standard using a set of "select analytes", 3) high standard including "all analytes", and 4) high standard using a set of "select analytes" as defined in **Table F.1**. The laboratory values for each analyte were used as the measured concentration ($C_i$) in the quality rating scale ($q_i$) calculation (**Algorithm 2.1.**).

$$q_i = \left(\frac{C_i}{S_i}\right) \times 100 \qquad \textbf{(2.1.)}$$

**Algorithm 2.1.** Formula for calculating the quality rating scale. Where $\boldsymbol{C_i}$ is the measured concentration of the *i*th parameter or analyte and the $\boldsymbol{S_i}$ is the standard value of the *i*th parameter or analyte (Akter et al., 2016).

The standard value ($S_i$) was used in the quality rating scale ($q_i$) and relative weight ($w_i$) (**Algorithm 2.2.**) calculations, being either the high or low standard value depending upon the WQI calculation performed. The standard values used for "high" and "low" standards are in **Table F.2.** with a more detailed table including the sources of the standards in **Figure D.1**. The WQI formula used for all four calculations, regardless of $S_i$ used, was the weighted arithmetic calculation (**Algorithm 2.3.**).

$$w_i = \left(\frac{1}{S_i}\right) \quad \textbf{(2.2.)}$$

**Algorithm 2.2.** Formula for calculating the relative weight (Akter et al., 2016).

$$WQI = \frac{\sum q_i w_i}{\sum w_i} \quad \textbf{(2.3.)}$$

**Algorithm 2.3.** Weighted arithmetic WQI. Where $\boldsymbol{w_i}$ is the relative weight of the *i*th parameter or analyte and $\boldsymbol{q_i}$ is the quality rating scale of the *i*th parameter or analyte (Akter et al., 2016).

Total coliform (TC) and *E. coli* values were not included as parameters in any of the WQI calculations because they have a standard value of zero by regulating bodies meaning that no amount of TC or *E. coli* in a sample is acceptable (EPA, 2018a; WHO, 2017). For the purposes of this study, any concentration of TC or *E. coli* greater than zero was automatically used to designate water not suitable for drinking by animals. In addition, K and Mg were not included in any of the four WQI calculations because no standards are found, as neither is considered a threat to human or animal health (BCME, 2017; EPA, 2018a; WHO, 2017). Also, P was not included because it is not defined as a threat to human or animal health (EPA, 2018; WHO, 2017). One standard value for P was found, but it was set in relation to algal blooms in water bodies, not for water quality related human or animal health (BCME, 2017).

### 2.2.5. Exploratory Questions Developed for Statistical Analysis of the Information

Thirteen statistical questions were developed to explore in the statistical analysis of information collected in the questionnaire. These statistical questions were not asked *per se* on the questionnaire, but rather were developed to assist in our exploratory work evaluating responses from the questionnaires:

***Question 1*** What is the current state of drinking water quality in non-black rhino zoos?

***Question 2*** What is the current state of drinking water quality within black rhino exhibits in zoos?

***Question 3*** Does having/utilizing a nutritionist or nutrition consultant affect whether or not the drinking water is tested for quality in zoos (non-black rhino and black rhino)?

***Question 4*** Does having/utilizing a nutritionist or nutrition consultant affect whether or not the drinking water provided to the black rhino is tested specifically for iron concentration?

***Question 5*** Are zoos with nutritionists/nutrition consultants more likely to be aware of the Nutrition Advisory Group (NAG) black rhino recommendations?

***Question 6*** If zoos are aware of the Nutrition Advisory Group (NAG) black rhino recommendations do they formulate the black rhino diets based upon them?

***Question 7*** Is there a significant difference between the quality of the origin point drinking water and the quality of the black rhino exhibit drinking water?

***Question 8*** Does the age of the zoo affect the difference in drinking water quality between the origin point and black rhino exhibit?

***Question 9*** Does the water source (e.g., municipal, well [bore], or river) affect the drinking water quality in zoos?

***Question 10*** Does the size of the zoo have any effect on drinking water quality?

***Question 11*** Does replacing pipes within the zoo affect the difference in drinking water quality between the origin point and the black rhino exhibit?

***Question 12*** Does the frequency of cleaning of the drinking water receptacle have any effect on drinking water quality within the black rhino exhibit?

***Question 13*** Do zoos that test their drinking water for quality have better overall drinking water quality than zoos that do not test their drinking water?

### 2.2.6. Statistical Analysis

Measures of central tendency and percentiles were used to evaluate questions 1 and 2 listed above. Cross-tabulation and Pearson Chi-square tests were used for questions 3 through 6. Because the data were not normally distributed, nonparametric analysis was used for the remainder of the questions (7 through 13; Linebach, Tesch, and Kovacsiss, 2014). A Sign test was used for question 7, Kruskal-Wallis H tests were used for questions 8 and 10, and Mann-Whitney U tests were used for questions 9 and 11 through 13 (Laerd Statistics, 2015). While not equivalent, a Sign test can be used for comparison of differences between paired observation data as an alternative analysis to a paired-samples t-test when the data are not normally distributed or Wilcoxon signed-ranked test when the data distribution is not symmetrical (Laerd Statistics, 2015). A Kruskal-Wallis H test can be considered a one-way ANOVA for non-parametric data, whereas the Mann-Whitney U test is an alternative to the independent-samples t-test for non-parametric data; although neither are equivalent (Laerd Statistics, 2015). All effort was made to provide effect size values where possible (Tomczak and Tomczak, 2014). Descriptive statistics also were used to describe drinking water quality based upon calculated WQI values. All statistical analysis was performed using SPSS 25.0 software (IBM Corp., 2017). Differences were declared at a significance level of $P < 0.05$.

## 2.3. Results and Discussion

The following presentation of results and discussion is divided into three parts: 1) questionnaire responses; 2) results of WQI analysis; and, 3) examination of statistical questions utilizing WQI and questionnaire responses.

### 2.3.1. Questionnaire Responses

Questions 1 through 10 were asked to all Non-BR and BR zoos completing questionnaires (**Figure C.1.**). In addition to the first 10 questions answered by all zoos, the BR zoos also were asked questions specifically about BR husbandry and drinking water practices. These questions were 11 through 28 in the BR questionnaire (**Figure C.2.**).

#### 2.3.1.1. General Questionnaire Responses (Questions 1 through 10)

*Water Quality and Sources.* When asked if drinking water quality was ever considered in the nutritional management of their animal collections, 23 of 39 (59%) zoos indicated "yes". Surprisingly, only seven (18%) of the same 39 zoos routinely analyzed the quality of their drinking water by laboratory analysis. Of the seven zoos that routinely analyzed drinking water, the majority (4 of 7) did so once per year, followed by monthly (2 of 7) and weekly (1 of 7). Eight of 39 (21%) had concerns about the quality of the drinking water in the zoo in the last 5 years.

Of the 39 zoos surveyed, the majority (92%) knew where the drinking water source entered the zoo property. Five of 39 (13%) treated the drinking water using one or more treatment methods (e.g., water softener, ion exchange, reverse osmosis, physical filtration, water recycling, or chlorination). Of these five, one zoo that treated the drinking water did not routinely analyze the water to monitor quality, and one other outsourced the monitoring and treatment of their drinking water to a contracted company.

A breakdown of the primary drinking water sources used by all 39 zoos that completed questionnaires is in **Figure F.4**. The majority of zoos (85%) used municipal (city) water as the primary drinking water source. Well (bore) water was a distant second (8%). The remaining facilities utilized a combination of drinking water sources from municipal and well (bore) water (5%), or municipal and river water (3%).

Although the majority (59%) of zoos indicated that drinking water quality was considered in the management of their animal collections, further questionnaire responses do not strongly support these claims. A fraction of the zoos (7 of 39) said they routinely analyze the drinking water provided to their animals to monitor the quality. With such a large portion (85%) of zoos getting their drinking water from municipalities, it may be that they are concerned about drinking water quality, but assumed that the municipality is monitoring and treating the drinking water to a sufficient water quality standard for humans, which is, thus, acceptable for zoo animals.

While it should be acceptable to trust the municipality to monitor and treat drinking water to a sufficient standard, recent events (e.g., high concentrations of lead in the drinking water in Flint, Michigan [CNN, 2018]) might leave this to question. Contaminants and analytes may enter the drinking water via leaching or corrosion of the pipes carrying the drinking water from the municipal treatment facility to the zoo. Testing of the drinking water within the zoo at least annually is recommended in order to monitor and, if necessary, treat the water before it is consumed by zoo animals.

*Diet Formulation and Length of Facility Operation.* Questions not pertaining specifically to drinking water were asked to gain more perspectives about the 39 zoos. They were asked about diet formulation and who was responsible for the task. The majority (31%) of zoos worked

with an outside nutrition consultant to formulate diets. Some zoos formulated diets in-house either employing a full-time nutritionist (20%) or having a veterinarian(s) (26%) responsible for diet formulation. The remainder (23%) did not employ a nutritionist, consult a nutritionist, nor did they have a veterinarian handle the diet formulation. It is unknown how or if these zoos had diet formulation and (or) monitoring of the diets for the animals in their care.

The last two questions asked about the age and the size (measured in number of species kept) of the facilities. The majority of zoos had been in operation between 51 and 100 years (38%), followed by 36% for 50 years or less, and then 26% for over 100 years. Thirty-nine percent of zoos had over 201 species in their collections, followed by 33% with 101 to 200 species, and 28% with 100 species or less at the time of the study.

Most of the zoos in this study are large and old; with the majority (72%) having 101 species or more and 64% being more than 50 years old. The older the facility the greater the likelihood that plumbing for the drinking water also is old. Older pipes may corrode and contribute contaminants or analytes to the drinking water after the water enters the zoo. In a similar fashion, the more species a zoo has, the larger the zoo is likely to be spatially, and drinking water may have to travel through more plumbing within the zoo to reach the exhibits. Due to this, both zoo age and zoo size might contribute to overall drinking water quality. Along the same lines, individuals tasked specifically with diet formulation might take drinking water quality into consideration and might monitor drinking water provided to the animals in their care. These relationships were investigated in this project.

#### 2.3.1.2. Questionnaire Responses of Zoos with Black Rhino (Questions 11 through 28)

In addition to the questions all zoos answered, BR zoos also responded to questions more specifically aimed at BR husbandry and drinking water quality.

*Drinking Water Quality and Receptacles.* Of the eight BR zoos that answered questionnaires, only two tested the drinking water for overall quality, and those same two specifically tested the drinking water for iron (Fe) concentration.

Receptacles used to provide drinking water to BR within the eight zoos included troughs exclusively (3 of 8) or automatic waterers (3 of 8). The remaining zoos (2 of 8) used trough waterers, but also provided a pool of water for recreation and drinking. The majority (5 of 8) of water receptacles consisted entirely of concrete. The remainder were made of metal (1 of 8) or multiple materials (2 of 8). Five of the eight facilities cleaned the drinking water receptacles daily and the remaining three cleaned less frequently than daily.

*Recreational Water Provided to the BR.* Enrichment ice blocks were used by seven of eight zoos at least once a year. Three zoos provided ice block enrichment monthly; the rest provided ice blocks either yearly (2 of 7) or twice per year (2 of 7). All eight zoos provided their BR with at least one other source of recreational water. All provided wallows for the BR for cooling. The other forms of recreational water provided in conjunction with a wallow by some zoos included pools, hoses, and misters. The recreational water sources were available through spring, summer, and fall in all eight zoos. Four of eight zoos also continued to allow access to the recreational water through winter at their locations.

The zoos were asked if they knew the type of substrate used to form the wallow, in addition to the water, two of eight zoos used red clay, one zoo used regular topsoil, and the remaining five zoos did not know what type of substrate was used in the wallow. For the type of substrate in the exterior area of the BR exhibit, all eight zoos had multiple types of substrate, and all contained some grass. In the interior of the BR exhibit, half of the eight BR zoos used bare

concrete with no other surface substrate, three of eight used concrete with some sort of bedding, and one zoo used a product called “Sydney Flooring Solutions”. According to the product website, Sydney Flooring Solutions (China Spring, Texas) is a type of multiple layered durable rubber flooring for use over concrete in livestock barns and zoos (Sydney Flooring Solutions, 2020). The type of substrate and (or) flooring provided in interior, exterior, and recreational areas may not seem important to drinking water quality but, depending upon the type of substrate used, unknown contaminants might get into the drinking water provided the rhino. Unaccounted for water contaminants might negatively affect drinking water quality and may contribute additional nutrients to the overall diet of the rhino (e.g., added iron in the water from the red clay substrate in the wallow).

*Nutrition and Husbandry Practices in BR Zoos.* In addition to drinking water and housing questions, zoos with BR also were asked about overall nutrition and husbandry. In order to gain an understanding of how nutrition is monitored, we asked these zoos how often diets are analyzed for nutrient content. Half of the zoos analyzed feed ingredients yearly, one facility only analyzed new hay shipments, and another routinely analyzed dietary ingredients monthly, as well as with every new hay shipment. Two zoos indicated they analyzed feed items "as needed”.

The majority (6 of 8) BR zoos were aware of the Nutrition Advisory Group (NAG) 2011 Workshop and its BR dietary recommendations for iron. Of the six zoos that were aware of the NAG recommendations, all six formulated BR diets based on the recommendations to control and (or) limit iron intake. In addition, a majority (6 of 8) of the zoos also had their BR diets tested specifically for iron concentration. The majority (6 of 8) of BR zoos indicated they would be willing to share their current formulated diet composition and (or) nutrient analysis with

researchers.

The last two questions asked of BR zoos pertained to BR body weight and body condition scoring (BCS) practices. Half (4 of 8) zoos weighed their BR monthly, two weighed twice a year, one weighed daily, and one never weighed their animals. To assess BCS, two of eight zoos assessed their animals daily, and two never assessed BCS. The remaining zoos assessed the BCS either monthly (1 of 8), quarterly (1 of 8), twice per year (1 of 8), or yearly (1 of 8).

### 2.3.2. Results of Water Quality Index Analysis

For background and perspective, based upon the nature of the WQI algorithm, the closer the calculated WQI value is to zero, the better the quality of the drinking water being evaluated relative to a particular set of analyte standards defined by the person doing the calculation. Also, the WQI value being closer to zero, indicating better quality water, is true regardless of the particular set of analytes used in the calculation. During our investigation, it was found that WQI calculations using higher standard values in the formula, that is higher (but acceptable in terms of water quality) analyte concentrations, were likely to have a greater WQI value in the end calculation in some cases, compared with the same calculation using lower drinking water standards (lower analyte concentrations) for the same data from laboratory analysis of a zoo sample. All calculated WQI values for origin point sampling for Non-BR and BR zoos are in **Table F.3.** All WQI values for BR exhibit sampling points are in **Table F.4.** For BR zoos, the WQI difference was calculated by subtracting the BR exhibit WQI value from the origin WQI value (**Table F.5**).

Due to the nature of the WQI calculation, as explained in **section 2.2.4.** above, it appears a higher standard value leads to a smaller weight ($w$) and quality rating scale ($q$). The final WQI

formula uses a summation of the weights as the denominator in the formula (**Algorithm 2.3.**), with a high standard resulting in a smaller denominator than a low standard, leading to a higher WQI value that may or may not necessarily reflect the severity of the water quality in a drinking water sample. Further refinement of the WQI formula may be needed in future studies. The seemingly skewed WQI values are clearly seen in **Table F.3.**, looking specifically at zoo identification numbers 1007, 1026, 1038, and 2004. While these four zoos are good examples of the WQI calculation discrepancy, they are by no means the only zoos in this study to exhibit this apparent bias between WQI for a particular water sample when low (low analyte concentrations) or high (high analyte concentrations) standard values are used.

**Table F.3.** presents the WQI values at the origin sampling point for all zoos (Non-BR and BR). The WQI values are ranked from highest to lowest using the low: all analytes formula. The $50_{th}$ and $90_{th}$ percentile values, 2.0 and 13.2 respectively, across all four analyte formulas were chosen to highlight values in the table to gain a general understanding of where each zoo ranked in terms of drinking water quality within the overall sampling group. Three zoos had higher WQI values (**Table F.3.**) and will be highlighted and discussed in further detail.

*Zoo 1101*. The WQI values for zoo 1101 were by far the highest of any zoo in the study indicating this zoo had poor overall drinking water quality. The high WQI values were influenced heavily by manganese (Mn), which was exceptionally high at 2.24 mg/L from the drinking water sample analysis; roughly 45 times the low standard of 0.05 mg/L and 5.6 times the high standard of 0.4 mg/L. Such a high concentration of Mn was the main contributing factor to the high WQI value. Zoo 1101 reported its primary drinking water source was well (bore) water, which may be a contributing factor to the high concentration of Mn in the drinking water

sample provided, as Mn is sometimes found at higher concentrations in ground water (Boyd, 2015; Gray, 2008; WHO, 2017).

*Zoo 1012*. The next highest WQI values were for zoo 1012. Manganese again was high in concentration (0.12 mg/L) which was 2.4 times the low standard, but only 0.3 times the high standard. Looking at other analytes, hardness also was present in a concentration above both standards at 312 mg/L, with the low and high standards being 100 mg/L and 300 mg/L respectively. These two variables contributed to the higher WQI values for zoo 1012. Again, this zoo exclusively used well (bore) water as the primary drinking water source.

*Zoo 1038*. While the WQI values for zoo 1038 were not quite as high as the previous two zoos, all four of its WQI values were outside of the 90$^{th}$ percentile, similar to zoos 1101 and 1012. Looking at the laboratory analysis for zoo 1038, the measured iron (Fe) concentration (0.48 mg/L) was above the low and high standards, 0.2 mg/L and 0.3 mg/L respectively. Even though zoo 1028 reported using municipal water exclusively as the primary water source, the Fe concentration greater than standard values led to the zoo having the third highest WQI values in the sample group.

Only two zoos (1101 and 1026) had Mn present in the drinking water regardless of sampling point. With no other participating zoos having Mn in the drinking water sample analysis, all other zoos figuratively had one less variable in the WQI analysis compared with zoos 1101 and 1026. This influences zoos 1101 and 1026 to have higher overall WQI values than any other zoo in the study.

A similar trend was identified with Fe, as five of the six zoos with the highest WQI values at the origin sampling point had Fe present in the drinking water; 1101 being the

exception. Of the five zoos with Fe present in the origin point drinking water sample, only zoo 1038 had Fe present at concentrations greater than the standards. The lack of Fe in the drinking water in other zoo origin point samples, again, figuratively gave them less variables in their WQI analysis calculation, which lead to lower WQI values.

**Table F.4.** shows the WQI values for the BR exhibit sampling point within BR zoos. The WQI values are ranked from highest to lowest using the Low: All Analytes formula. The 50th and 90th percentile values, 2.0 and 10.2 respectively, across all four analyte formulas were chosen to highlight the WQI results within the table to gain a general understanding of where each zoo ranked.

For the WQI for the BR exhibit, no Mn was present in any samples and only one zoo (2020) had Fe present in the exhibit drinking water sample. Consequently, zoo 2020 also had the highest WQI values. Although the measured Fe concentration (0.05 mg/L) was well below the standards, the presence of Fe in the WQI calculation influenced putting zoo 2020 at the top of the list. Zoo 2020 also was the only zoo with a WQI value not within the 90th percentile at the BR exhibit sampling point.

Any change in rank order of WQI values among BR zoos between **Table F.3.** and **Table F.4.** can be attributed to a change in analytes present within the water samples between the origin and BR exhibit sampling points. For example, zoo 2004 is above zoo 2020 in **Table F.3.** but below it in **Table F.4.**; this change can be attributed to the change in Fe presence between sampling points. Zoo 2004 had Fe present in the origin point sample but not in the BR exhibit sample, and zoo 2020 had no Fe present in the origin point sample but had Fe in the BR exhibit sample. Smaller changes in rank order can be contributed to slight changes in analyte

concentrations at the two different sampling points.

While not included in WQI calculation or the water quality analysis, as stated above in **section 2.2.4.**, one BR zoo had total coliform (TC) present. Zoo 2012 had TC present at 73.8 colonies per 100 mL in the BR exhibit water sample, but not at the origin sampling point. The reason for this contamination is unknown. The water should be sampled and analyzed again to verify the results. Additional water samples should be taken at the same sampling points within some zoos to verify the initial laboratory results. Once verified, further action to correct the high analyte concentrations may need to be taken.

**Table F.5.** shows the difference in WQI values between the origin and exhibit sampling points in BR zoos. A negative difference represents a decrease in drinking water quality from the origin point to the BR exhibit, a positive difference represents an increase in drinking water quality, and a zero difference suggests no change in drinking water quality. A negative zero (-0.0) value is the result of rounding minor changes between water quality (e.g., -0.0067 rounded to -0.0).

Zoo 2004 had a difference of 5.6 with a greater WQI value at the origin sampling point then the BR exhibit sampling point (**Table F.5.**). This change in water quality was due to the presence of Fe in the origin point sample and the absence of Fe in the BR exhibit sample.

The opposite was presented for zoo 2020 with a WQI difference of -4.7 with a greater WQI in the BR exhibit than at the origin sampling point. Again, this is likely due to the varied presence of Fe in the samples with no Fe in the origin point sample but Fe present in the BR exhibit water sample. Additional water analysis would be recommended at both sampling points to verify the difference in Fe concentration.

#### 2.3.2.1. WQI of Non-Black Rhino Zoos

For all Non-BR zoos, the drinking water samples were taken at the origin point for WQI calculations. Because the results across all four standard WQI calculations had some exceptionally high values, the median, mode, minimum, and maximum values are reported in addition to the mean and standard deviation (**Table F.6.**). Percentiles were calculated, including the 10th, 50th, and 90th percentiles, for the WQI values at the origin sampling point in all Non-BR zoos. (**Table F.7.**). At the 90$_{th}$ percentile, for the "Low: All Analytes" WQI calculation in **Table F.7.**, 90% of all WQI values for that category were at or below a value of 7.4.

Descriptive statistics were used and provided to give a better overview of the WQI results for Non-BR zoos. The mean, median, mode, minimum, and maximum in **Table F.6.** and the percentiles in **Table F.7.** provide a way for Non-BR zoos to compare the WQI value for their facility to that rest of the sampled group of Non-BR zoos. Allowing individual Non-BR zoos to identify which percentile their zoo categorized into, how close to the mean and median they were, and if their WQI value occurred frequently across their sampled group. The standard deviation values provided in **Table F.6.** indicate the data are spread out across a wide range, indicating a wide variability in drinking water quality across the sampled Non-BR zoos.

#### 2.3.2.2. WQI of Black Rhino Zoos

For all BR zoos, drinking water samples were taken at the origin point and within the BR exhibit. A WQI calculation was performed based on the laboratory water analysis at each sampling location. One sample from one BR zoo had a measurable concentration of Total Coliforms in the laboratory analysis and was deemed not suitable for drinking. Even though the WQI values for both the origin (**Table F.8.**) and BR exhibit (**Table F.9.**) sampling points had fewer and less extreme high values than in the non-BR zoos, for consistency the median, mode,

minimum, and maximum values are still reported in addition to the mean and standard deviation.

In addition to measures of central tendency, percentiles also were calculated, including the 10th, 50th, and 90th percentiles, for the WQI values for both the origin (**Table F.10.**) and BR exhibit (**Table F.11.**) sampling points within BR zoos.

Again, the mean, median, mode, minimum, and maximum in **Table F.8.** and **Table F.9.** as well as, the percentiles in **Table F.10.** and **Table F.11.** provide a way for BR zoos that participated in the study to compare the WQI value at both the origin and BR exhibit sampling point for their facility to that rest of the sampled group of BR zoos. Allowing individual BR zoos to identify which percentile their zoo categorized into, how close to the mean and median they were, and if their WQI value occurred frequently across their sampled group. The standard deviation values provided in **Table F.8.** and **Table F.9.** show the data for both the origin point and BR exhibit sampling points to be less spread out than was the case in Non-BR zoo origin point results, indicating less variability in drinking water quality across the sampled BR zoos at both sampling locations.

### 2.3.3. Examination of Statistical Questions Utilizing WQI and Questionnaire Responses

The questions addressed in the statistical analysis were previously listed in the Material and Methods 2.3. Analysis of the key questions and interpretation of answers are addressed below.

***Question 3.*** *Does having/utilizing a nutritionist or nutrition consultant affect whether or not the drinking water is tested for quality in zoos (non-black rhino and black rhino)?*

Due to having a small sample size and the data failing the third assumption (all cells should have expected counts greater than five) required for a Chi-square test of association, the Fisher's Exact Test was instead used to analyze the data.

Of the 40 participating Non-BR zoos, 31 were from the randomly selected group of zoos invited to complete a questionnaire; the remaining 9 only submitted drinking water samples. Of the group of 31 zoos, only four employed a full-time nutritionist while 27 did not. Of the zoos that did not employ a nutritionist, only four (15%) routinely analyzed drinking water. For zoos that did employ a nutritionist, surprisingly, none routinely analyzed the drinking water. There was no association between a Non-BR zoo employing a full-time nutritionist and that zoo routinely analyzing the quality of drinking water as assessed by a Fisher's exact test, ($P = 1.00$).

From the same group of 31 Non-BR zoos that completed a questionnaire, 11 employed a nutrition consultant while 20 did not. Of the zoos that did not employ a nutrition consultant, only two (10%) routinely analyzed their drinking water. For zoos that did employ a nutrition consultant, surprisingly again, only two (18%) routinely analyzed the drinking water. There was no association between a zoo employing a nutrition consultant and the zoo routinely analyzing the drinking water for quality as assessed by a Fisher's exact test, ($P = 0.601$).

Of the 10 BR zoos, eight were from the randomly selected group of zoos invited to complete a questionnaire. Of the eight black rhino zoos, four employed a full-time nutritionist and four did not. Of the zoos that did not employ a nutritionist, two routinely analyzed the drinking water for quality. For zoos that did employ a nutritionist, one routinely analyzed the drinking water. There was no association between a BR zoo employing a full-time nutritionist and the zoo routinely analyzing the drinking water for quality as assessed by a Fisher's exact test, ($P = 1.00$).

From the same group of eight BR zoos that completed a questionnaire, one employed a nutrition consultant and seven did not. Of the zoos that did not employ a nutrition consultant,

three routinely analyzed the drinking water. For the zoo that did employ a nutrition consultant, it did not routinely analyze the drinking water. There was no association between a zoo employing a nutrition consultant and the zoo routinely analyzing the drinking water as assessed by a Fisher's exact test, ($P$ = 1.00).

***Question* 4.** *Does having/utilizing a nutritionist or nutrition consultant affect whether or not the drinking water provided to the black rhino is tested specifically for iron concentration?*

Of the four BR zoos that did not employ a full-time nutritionist, one had analyzed the drinking water for iron concentration. For the four zoos that did employ a nutritionist, again, one analyzed the drinking water specifically for iron concentration. There was no association between a zoo employing a nutritionist and the zoo having ever analyzed the drinking water provided to the black rhino specifically for iron concentration as assessed by a Fisher's exact test, ($P$ = 1.00).

Of the seven BR zoos that did not employ a nutrition consultant, two analyzed the drinking water specifically for iron concentration. For the one zoo that did employ a nutrition consultant, again, it had never analyzed the drinking water for iron concentration. There was no association between a zoo employing a nutrition consultant and the zoo having ever analyzed the drinking water for iron concentration as assessed by a Fisher's exact test, ($P$ = 1.00).

***Question 5.*** *Are zoos with nutritionists/nutrition consultants more likely to be aware of the Nutrition Advisory Group (NAG) black rhino recommendations?*

From the same group of eight BR zoos invited to complete the questionnaire, four employed a full-time nutritionist and four zoos did not. All four of the zoos that did not employ a nutritionist were aware of the NAG nutritional recommendations for formulating black rhino

diets. For the zoos that did employ a nutritionist, two were aware of the NAG dietary recommendations. There was no association between a zoo employing a nutritionist and whether or not that zoo was more likely to be aware of the NAG dietary recommendations for black rhino when assessed by a Fisher's exact test, ($P = 0.429$).

Again, seven BR zoos did not, and one did employ a nutrition consultant. Of the zoos that did not employ a nutrition consultant, five were aware of the NAG dietary recommendations for formulating black rhino diets. The one zoo that did employ a nutrition consultant also was aware of the NAG dietary recommendations. There was no association between a zoo employing a nutrition consultant and whether or not that zoo was more likely to be aware of the NAG dietary recommendations for black rhino as assessed by a Fisher's exact test, ($P = 1.00$).

***Question 6.*** *If zoos are aware of the Nutrition Advisory Group (NAG) black rhino recommendations do they formulate the black rhino diets based on them?*

Of the eight BR zoos randomly selected to complete a questionnaire, six were aware of the NAG dietary recommendations, and all six formulated the BR diets based upon the NAG recommendations. The other two zoos that completed the questionnaire were not aware of the NAG recommendations, and therefore, the question related to formulating the black rhino diets based upon the recommendations did not apply. There was an association between a zoo being aware of the NAG dietary recommendations and formulating black rhino diets based upon those recommendations as assessed by a Fisher's exact test, ($P = 0.036$).

***Question 7.*** *Is there a significant difference between the origin point drinking water quality and the black rhino exhibit drinking water quality?*

Due to the distribution of the data being neither normally nor symmetrically distributed, an

exact Sign test was used to determine if there was a difference in drinking water quality between the origin point and within the BR exhibit across the four different analyte formulas to compute the WQI in the 10 BR zoos. The exact Sign test compares the change in sign when the difference is calculated. Results are presented as medians unless otherwise stated. There was no median change in drinking water quality between the origin point and within the BR exhibit across the four different analyte formulas (**Table F.12.**).

As noted above, the exact Sign test compares the change in sign when the difference is calculated. In this case WQI within the BR exhibit is subtracted from the WQI at the origin point. The change in sign indicates whether the water quality is worse at the origin point (+), worse within the BR exhibit (-), or whether there was no change in water quality as signified by the respective WQI between the two sampling points (**Table F.13.**).

Based upon the results, there was no change in drinking water quality between the two sampling points regardless of set of analytes used to calculate the WQI value. No change between the two sampling points suggests that the pipes carrying the water between the locations were not contributing any additional analytes to the drinking water. For example, had there been a significant increase in the WQI value from the origin point to the BR exhibit it would be advisable to examine, and possibly replace, the pipes for corrosion, damage, or contamination. Continued sampling at both points, and comparison between the results, would help zoo personnel monitor and respond to any changes in drinking water pipe status in the future.

***Question 8.*** *Does the age of the zoo affect the difference in drinking water quality between the origin point and the water source within black rhino exhibit?*

Due to the non-linear relationship between the age of the BR zoos and the difference in WQI

value between the two sampling points, linear regression could not be used to analyze the data; instead a Kruskal-Wallis H test was used in this analysis (**Table F.14.**). Zoo age categories were in three groups: “less than 50 years old” (n = 2), “51 to 100 years old” (n = 2), and “greater than 100 years old” (n = 4). The difference in drinking water quality between the origin and BR exhibit was calculated by subtracting the WQI: BR Exhibit from the WQI: Origin, creating a new variable “WQI: Difference”. Values are mean ranks unless otherwise stated. Distributions of WQI: Difference were not similar for all zoo age groups, as assessed by visual inspection of box plots (**Figures F.5., F.6., F.7., and F.8.**) for each WQI calculation (low: all analytes, low: select analytes, high: all analytes, and high: select analytes). The mean rank of WQI: Difference was not different between zoo age groups across all four analyte formulas. While that is the case, the change in drinking water quality between the origin point and BR exhibit decreased as the mean rank value increased; meaning the quality of the drinking water was lower as the mean rank increased.

Based on the *P*-values in **Table F.14.**, the age of the zoo had no effect on the difference in drinking water quality between the origin and BR exhibit sampling points. The change in mean rank does indicate that the lowest quality drinking water is in the age category “less than or equal to 50 years old” (6.00), then “greater than 100 years old” (4.13), and the highest quality drinking water is in the age category “51-100 years old” (3.75), supporting the *P*-value results.

The idea behind this analysis was older zoos might have older plumbing and pipe systems, which might affect drinking water quality. Monitoring drinking water quality is still important regardless of the age of the zoo, as shown here, with younger zoos having the lowest quality drinking water across zoo age categories.

***Question 9.*** *Does the water source (e.g., municipal, well [bore], or river) affect the drinking water quality in zoos?*

A Mann-Whitney U test, using an exact sampling distribution for U (Dineen and Blakesley, 1973), was used to determine whether there were differences in drinking water quality (WQI) at the origin point among zoos using different drinking water sources (municipal, well [bore], or river) across all four WQI computations.

Pyramid chart distributions for the WQI at the origin point for zoos that did and zoos that did not use municipal water as a drinking water source were not similar (**Figures F.9.**, **F.10.**, **F.11.**, and **F.12.**). Because of this, inferences cannot be made about the difference in medians between groups. Instead we looked at the differences in the distributions and mean ranks. Recall that a WQI value closer to zero indicates better drinking water quality. The WQI values at the origin point for zoos that did not use municipal water were greater than for zoos that did use municipal water as a drinking water source for three out of the four analyte formulas (**Table F.15.**). Only the "high: all analytes" calculation method did not have a difference in drinking water quality among zoos that did and zoos that did not use municipal drinking water as their primary water source. Overall, the drinking water quality at the origin point for zoos that used municipal water was better than that of zoos that did not use municipal water as their primary drinking source as shown by the change in mean rank value, with a smaller mean rank indicating better drinking water quality.

Pyramid chart distributions for the WQI at the origin point for zoos that did and zoos that did not use well (bore) water as a drinking water source were not similar (**Figures F.13.**, **F.14.**, **F.15.**, and **F.16.**). Because of this, inferences cannot be made about the difference in medians

between groups. Instead we looked at the differences in the distributions and mean ranks. Median WQI values at the origin point for zoos that did use well (bore) water as a drinking water source compared with zoos that did not use well (bore) water were not different across all four analyte formulas or WQI computations (**Table F.16.**). Therefore, there was no difference in drinking water quality between zoos that did and zoos that did not use well (bore) water as the primary drinking water source.

Pyramid chart distributions for the WQI at the origin point for zoos that did or did not use river water as a drinking water source were not similar (**Figures F.17.**, **F.18.**, **F.19.**, and **F.20.**). Because of this, inferences cannot be made about the difference in medians between groups. Instead we looked at the differences in the distributions and mean ranks. Median WQI value at the origin point for zoos that did use river water as a drinking water source compared with zoos that did not use river water as a drinking water source was not different across all four analyte formulas (**Table F.17.**). Again, there was no difference in drinking water quality between zoos that did and zoos that did not use river water as the primary drinking water source.

***Question 10.*** *Does the size of the zoo have any effect on drinking water quality?*

A Kruskal-Wallis H test was used to determine if the size of zoos affects the quality of the drinking water at the origin point. The zoo size groups were: "less than or equal to 100 animal species" (n = 11), "101 to 200 species" (n = 13), and "greater than 200 species" (n = 15). Values are mean ranks unless otherwise stated. Distributions of WQI: Difference were not similar for all "zoo size" groups, as assessed by visual inspection of box plots (**Figures F.21., F.22., F.23.,** and **F.24.**) for each calculation (low: all analytes, low: select analytes, high: all analytes, and high: select analytes) of WQI. Drinking water quality at the origin point decreased as the mean

rank value increased meaning that the quality of the drinking water is lower as the median rank increases (**Table F.18.**). Therefore, the size of the zoo had no effect on overall origin point drinking water quality.

***Question 11.*** *Does replacing pipes within the zoo affect the difference in drinking water quality between the origin point and the black rhino exhibit?*

Of the zoos that responded to the questionnaire, nine had replaced and 39 had not replaced any drinking water pipes within the last 5 years. A Mann-Whitney U test was run to determine if there was a difference in drinking water quality between the origin and the BR exhibit sampling points for zoos that had replaced their drinking water pipes versus zoos that had not replaced their drinking water pipes within the last 5 years across all four WQI analyte formulas. For all four formulas, pyramid chart distributions for zoos that had replaced their drinking water pipes compared with zoos that had not replaced pipes were not similarly shaped (**Figure F.25.**, **F.26.**, **F.27.**, and **F.28.**). There was no difference in the drinking water quality for zoos that had or had not replaced pipes within the last five years between the origin point and black rhino exhibit, regardless of analyte formula (WQI) used (**Table F.19.**).

***Question 12.*** *Does the frequency of cleaning of the drinking water receptacle have any effect on drinking water quality within the black rhino exhibit?*

A Mann-Whitney U test was used to determine if the frequency at which the drinking water receptacles are cleaned has an effect on the drinking water quality within the BR exhibit. The cleaning frequency of the drinking water receptacles was broken into two categories; "daily" (n = 5) and "less than daily" (n = 3). Distributions for cleaning frequency were not similar, as assessed by visual inspection of the pyramid chart (**Figure F.29.**, **F.30.**, **F.31.**, and **F.32.**). There

was no difference in the WQI value within the BR exhibit in relation to the drinking water receptacle cleaning frequency, regardless of formula used (**Table F.20.**). Although the data showed the frequency in which drinking water receptacles are cleaned does not have an effect on drinking water quality, it is worth noting the water samples taken for analysis in this study were all taken at one point in time.

***Question 13.*** *Do zoos that test their drinking water for quality have better overall drinking water quality than zoos that do not test their drinking water?*

Only 7 of 39 zoos in the study routinely analyzed the drinking water for quality. A Mann-Whitney U test was used to determine if routine analysis of the quality of the drinking water affects overall drinking water quality as measured at the origin sampling point across all four analyte formulas and WQI computations. Pyramid chart distributions for the routine analysis of the drinking water were not similarly shaped (**Figures F.33.**, **F.34.**, **F.35.**, and **F.36.**). Because of this, inferences cannot be made about the difference in medians among groups. Instead we looked at the differences in the distributions and mean ranks. There was no difference in the quality of the drinking water at the origin point as indicated by the WQI values in relation to whether or not the facility routinely analyzed the drinking water for quality, regardless of analyte formula used (**Table F.21.**). Based upon the results of the Mann-Whitney U test, routine analysis of the quality of the drinking water does not affect the actual drinking water quality at the sampling origin point.

**2.4. Conclusions.**

Using the weighted arithmetic WQI algorithm with two different sets of analyte standards and two different groups of analyte selections (analyte concentrations), this study calculated four

WQI values for each zoo allowing for a thorough assessment of the drinking water quality in each zoo. Half of the zoos had a WQI at or below 2.0, with the majority (90%) below 13.2 across all four analyte formulas. This was the first known use of a WQI to assess drinking water quality in zoos and suggests the possibility for further use of WQI calculations in assessing water quality by zoo staff and future researchers. This study also sought to find possible associations among drinking water quality husbandry practices and zoo characteristics.

The questionnaire responses were interesting. The majority (59%) of zoos responded that drinking water quality was taken into consideration in the nutritional management of their animal collections, yet surprisingly few (18%) routinely analyzed the drinking water and even fewer (13%) used some form of water treatment. Yet, 21% of zoos that responded had concerns about the quality of their drinking water within the last 5 years. All zoos should be routinely analyzing the drinking water for basic quality at the minimum once per year and more frequently if necessary. This will allow zoos to know the quality of their drinking water, as well as, allow them to monitor, and correct, any changes that might occur.

When it came specifically to monitoring drinking water provided to the black rhino populations within participating zoos, only two out of eight zoos routinely analyze the water to monitor iron concentration. Considering the recommendation from the leading workshop on Iron Overload Disorder (IOD) in black rhino suggests testing the drinking water for iron concentration at least once per year, this is a lower number in our study than anticipated (Clauss et al., 2012). When asked, six of the eight BR zoos that responded to questionnaires responded that they were aware of the workshop recommendations, and all six that were aware said they formulate the black rhino diets based upon the recommendations. With six of eight zoos

adhering to the dietary recommendations offered in the workshop publication, it is surprising these zoos do not also follow the drinking water testing recommendations, as well. Again, in addition to routine analysis of general drinking water quality zoos with black rhino should follow the advice of the IOD Workshop and monitor iron concentration in the drinking water provided to their black rhino collections.

Only three zoos in our study sample (n = 50) had WQI values at the origin point above the 90th percentile cutoff value of 13.2. Of the three zoos above the 90th percentile, two exclusively used well (bore) water as the primary drinking water source. It might be advisable for zoos using well (bore) water to monitor the drinking water quality more than once per year to more vigilantly monitor changes in the drinking water quality so action can be taken to correct high analyte concentrations when necessary. In the case of the two zoos (1101 and 1012 in **Table F.3.**) with the highest WQI values, manganese (Mn) was the problem analyte that should be monitored.

Comparing questionnaire responses to water quality values expressed as WQI found very little relationship between zoo husbandry practices, size, age, or drinking water source with one exception. Based upon the analysis, zoos that used municipal water as their drinking water source had lower WQI values. The lower the WQI the better the drinking water quality, which means zoos that used municipal water had better drinking water quality than zoos that used a different source for drinking water.

The use of the weighted arithmetic WQI formula in this project, to compare drinking water quality using both a high and a low standard, also led to the discovery of an unusual characteristic in how the WQI formula works. Use of a high drinking water standard results in a

smaller denominator, which in turn leads to a larger WQI value than if a low drinking water standard was used on the same data points, as shown in **Table F.3.** Further research is needed to confirm this characteristic and, if necessary, derive an alternative mathematical representation for the algorithm. Otherwise, a very sound understanding of the behavior of the algorithm and the resulting WQI values is needed for interpretation of WQI from field and research samples.

Future research into drinking water quality in zoos needs to be done, preferably with an even larger sample size than was used in this study. Frankly, the direct impact on health of zoo animals, except the information known about iron on black rhino, of any of the analytes measured in this study is largely unknown and simply surmised or inferred from knowledge in other animal species. Furthermore, the impact of high concentrations of other analytes and other drinking water contaminants not measured in this study (e.g., lead) on zoo animal health is unknown. There is a wide variety of species kept in zoos around the world. These species vary greatly in size, dietary needs, and health requirements. More research into relationships between drinking water quality and animal health is needed.

# APPENDICES

# APPENDIX A

## RANDOMIZED NUMBER GENERATOR OUTPUT


**Figure A.1.** Random number generator output to select the Black Rhino Zoo subsample invited to complete a questionnaire and submit drinking water samples. Twenty-five in the outline are included within the questionnaire subsample, and the remaining BR zoos only submitted a drinking water sample.

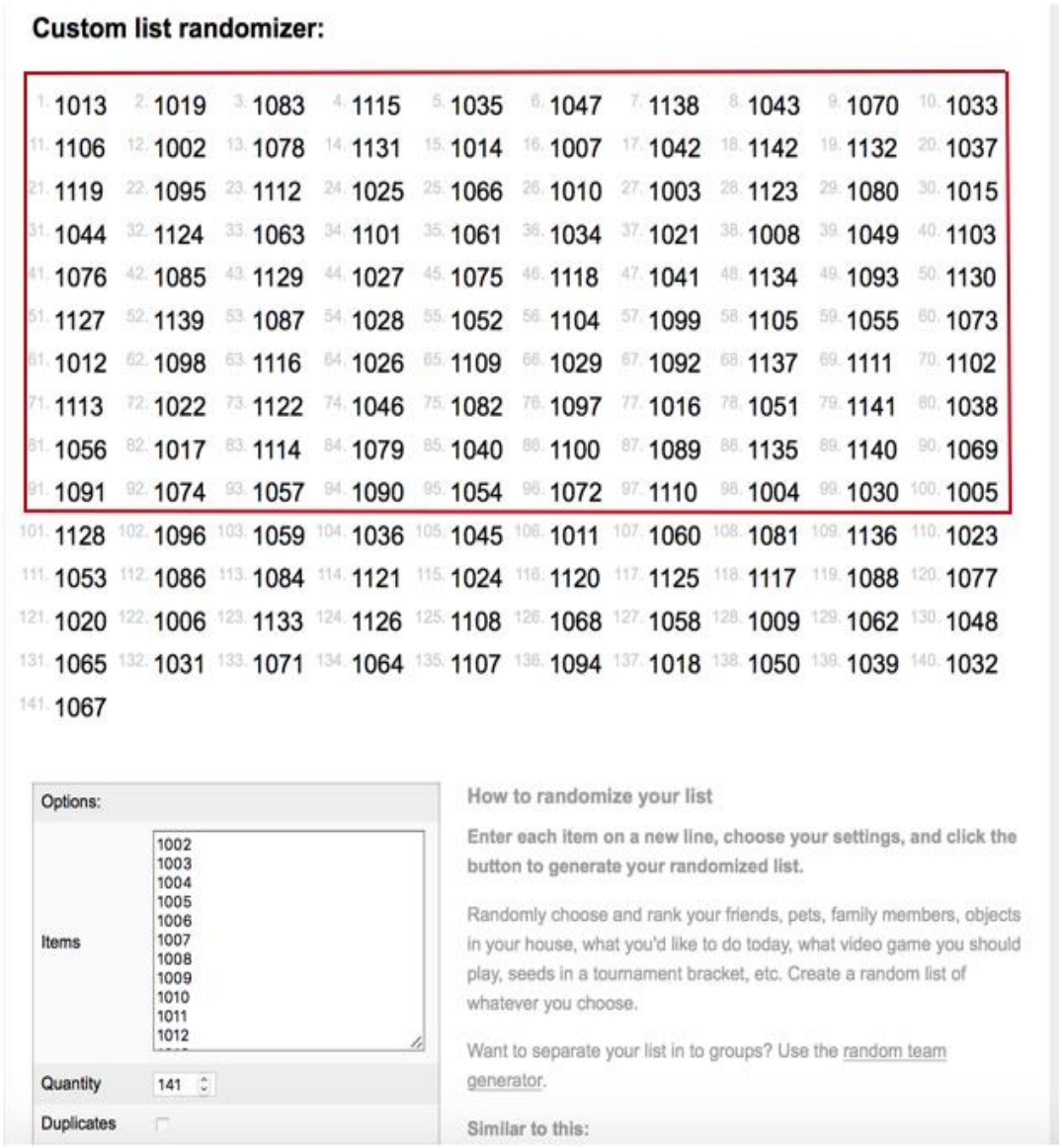


**Figure A.2.** Random number generator output to select the Non-Black Rhino zoo subsample invited to complete a questionnaire and submit drinking water samples. One hundred in the outline are included within the questionnaire subsample, and the remaining Non-Black Rhino zoos only submitted a drinking water sample.

# APPENDIX B

## ZOO SAMPLING KIT DOCUMENTS

**MSU Zoo Drinking Water Project**

**INSTRUCTIONS – PAGE 1**

**Thanks for agreeing to participate in this project!**

**Task 1: Water Sample Collection**

**A.** **Please review and follow the accompanying water sampling instructions.**

**B.** **Use the white Priority Mail shipping box that you received with the empty bottle to send the filled bottle to the laboratory (shipping label accompanying).**

**C.** **Complete the green highlighted sections of the Water Sample submittal form(s) and insert the form(s) into the box with the full water bottle(s).**

**D.** **Cross out "United States Postal Service and PRIORITY MAIL" and Affix the UPS Next Day Air label over the top of your address on the box, please seal the box with shipping tape (not scotch tape), and send immediately via UPS on Monday, Tuesday, or Wednesday.**

**Thanks!**

**Figure B.1.** Cover page for all zoo water sample only sampling kits.

**MSU Zoo Drinking Water Project**

**INSTRUCTIONS – PAGE 1**

**Thanks for agreeing to participate in this project!**

**Two tasks need to be performed as soon as possible.**

**Task 1: Water Sample Collection**

**A.** **Please review and follow the accompanying water sampling instructions.**

**B.** **Use the white Priority Mail shipping box that you received with the empty bottle to send the filled bottle to the laboratory (shipping label accompanying).**

**C.** **Complete the green highlighted sections of the Water Sample submittal form(s) and insert the form(s) into the box with the full water bottle(s).**

**D.** **Cross out "United States Postal Service and PRIORITY MAIL" and Affix the UPS Next Day Air label over the top of your address on the box, please seal the box with shipping tape (not scotch tape), and send immediately via UPS on Monday, Tuesday, or Wednesday.**

**Task 2: Complete the Questionnaire ASAP!**

**A.** **Appropriate zoo staff (e.g., curator, nutritionist, veterinarian, animal keepers) please complete questionnaire within 1 week.**

**B.** **Return it in the accompanying postage paid envelope to Christine Homminga.**

**Thanks!**

**Figure B.2.** Cover page for all zoo water sample and questionnaire subsample sampling kits.

**Water Sample Collection:**

Please collect a drinking water sample using the water sample collection instructions on the back of this page.

- One livestock suitability as close as possible to the **origin** (e.g., well or off-site supply line) of the drinking water source.

If you have any questions or need any clarification of the water sampling instructions; please email Christine at homming2@msu.edu

**Water Sampling Instructions: Livestock Suitability**

**Sample Container:**


- Sample from the **cold-water** faucet/hose/spigot. **Do not take sample from standing water**
- Run cold water for 2-3 minutes.
- Rinse bottle 5 times, by filling to the top and dumping the water out.
- After the 5th rinse; fill the sample bottle to the shoulder.
- Tighten the lid completely to avoid spillage during shipping.
- Wrap water sample with one of the blue absorbent pads provided.
- Place water sample in Ziploc bag and seal.

**Figure B.3.** Origin point water sample collection instruction sheet for Non-Black Rhino zoo sampling kits.

**Figure B.3.** (cont'd)

- Place water sample into re-used white shipping box; using the other blue absorbent pad as cushioning.
- Fill out highlighted sections on the water sample submittal form, and place in shipping box.
- Affix provided shipping label over top old address and postage mark, and mail within 24 hours of collection Monday through Wednesday

**Make sure to package water bottles so that there is adequate cushioning to prevent breakage during shipment. Do not send out samples on Thursdays, Fridays, Saturdays or Holidays because we will not receive them in time to be analyzed.**

**Samples need to be received within 24 hours of collection for testing. Do not freeze samples. If possible, refrigerate samples prior to shipping.**

**Water Sample Collection:**

Please collect four drinking water samples total using the attached water sample collection instructions.

- Two (one livestock suitability and one bacterial) as close as possible to the origin (e.g., well or off-site supply line) of the drinking water source.

- Two (one livestock suitability and one bacterial) from within the black rhino exhibit, as close as possible to the location the drinking water enters the exhibit.

**Water Sampling Instructions: Livestock Suitability**

**Sample Container:**


- Sample from the **cold-water** faucet/hose/spigot. **Do not take sample from standing water**
- Run cold water for 2-3 minutes.
- Rinse bottle 5 times, by filling to the top and dumping the water out.
- After the 5th rinse; fill the sample bottle to the shoulder.
- Tighten the lid completely to avoid spillage during shipping.
- Wrap water sample with one of the blue absorbent pads provided.

**Figure B.4.** Origin point and Exhibit water sample collection instruction sheet for Black Rhino zoo sampling kits.

**Figure B.4.** (cont'd)

- Place water sample in Ziploc bag and seal.
- Place water sample into re-used white shipping box; using the other blue absorbent pad as cushioning.
- Fill out highlighted sections on the water sample submittal form, and place in shipping box.
- Affix provided shipping label over top old address and postage mark, and mail within 24 hours of collection Monday through Wednesday

**Make sure to package water bottles so that there is adequate cushioning to prevent breakage during shipment. Do not send out samples on Thursdays, Fridays, Saturdays or Holidays because we will not receive them in time to be analyzed.**

**Samples need to be received within 24 hours of collection for testing. Do not freeze samples. If possible, refrigerate samples prior to shipping.**

**Water Sampling Instructions: Bacterial**

Sample Container:


Do not rinse bottle prior to collection because this will remove the sodium thiosulfate (used to remove residual chlorine). These are sterile, sealed containers. To avoid contamination, do not touch the inside of the bottle, cap, or threads.

**Figure B.4.** (cont'd)

- Sample from the cold-water faucet/hose/spigot. **Do not take sample from standing water source (trough, bucket, etc.)**
- Run cold water for 2-3 minutes.
- **DO NOT RINSE!**
- Fill the sample bottle to just above the 100ml line.
- Tighten the lid completely to avoid spillage during shipping.
- Wrap water sample with one of the blue absorbent pads provided.
- Place water sample in Ziploc bag and seal.
- Place water sample into re-used white shipping box; using the other blue absorbent pad as cushioning.
- Fill out highlighted sections on the water sample submittal form, and place in shipping box.
- Affix provided shipping label over top old address and postage mark, and mail within 24 hours of collection Monday through Wednesday

**Make sure to package water bottles so that there is adequate cushioning to prevent breakage during shipment. Do not send out samples on Thursdays, Fridays, Saturdays or Holidays because we will not receive them in time to be analyzed.**

**Samples need to be received within 24 hours of collection for testing. Do not freeze samples. If possible, refrigerate samples prior to shipping.**


**Cumberland Valley Analytical Services, Inc.**
800-282-7522
301-790-1980
www.foragelab.com
mail@foragelab.com

*Mailing & UPS/FedEx Address:*
4999 Zane A. Miller Drive
Waynesboro, PA 17268

**Lab Use**

Date Received _____

Time Received_____

Sample ID_____

Mail Charge _____

Total to Bill _____ Date Billed_____

## Water Sample Submittal Form

Use one form per sample
*Please clearly provide complete contact information*

| **Party to Bill** | | **Preferred Reporting Method (s)** |
|---|---|---|
| Account # | "Zoo" | Mail |
| Name | Dr. David K. Beede | Fax |
| Street | 474 S. Shaw Ln Room 2265K | ■ Email |
| City | East Lansing | Internet |
| State | Michigan Zip 48824 | |
| Phone | 517-432-5400 | |
| Fax | 517-432-0147 | |
| Email | beede@msu.edu | |

| **Copy 1** | | **Preferred Reporting Method (s)** |
|---|---|---|
| Account # | "Zoo" | ■ Mail |
| Name | Christine Homminga | Fax |
| Street | 474 S. Shaw Ln Room 2280 | ■ Email |
| City | East Lansing | Internet |
| State | Michigan Zip 48824 | |
| Phone | 517-706-9345 | |
| Fax | 517-432-0147 | |
| Email | homming2@msu.edu | |

| **Copy 2** | | **Preferred Reporting Method (s)** |
|---|---|---|
| Account # | "Zoo" | Mail |
| Name | | Fax |
| Street | | Email |
| City | | Internet |
| State | Zip | |
| Phone | | |
| Fax | | |
| Email | | |

| **Farm/Client:** |
|---|
| |
| **Sample Description/Source:** |
| Origin-Livestock Suitability |
| **Date and Time Collected:** |
| |

| | |
|---|---|
| ■ Water Suitability Package | $37.25 |
| *pH, Hardness, Total Dissolved Solids, Ca, Mg, K, Na, Fe, P, Mn, Zn, Cu, Chlorides, Sulfates, Nitrates* **(need 500ml bottle)** | |

| **Other Charges:** | |
|---|---|
| US Mail Priority Fee (*at US mail Rates)* | $5.60+ |
| UPS Ground Service | $8.50 |
| UPS Next Day Service | $32.00 |

Included are sample bottles and shipping labels for US Mail Priority Mail Service, UPS Ground service, UPS Second Day Service, and UPS Next Day (overnight, Red) Service. You may choose the service that best meets your needs, or not use the bill-back labels provided. Shipping charges using our UPS/US Mail labels are billed back at the rates listed. **Water samples should arrive at the lab within 24 hours. 100ml sterile bottles are needed for the bacterial analyses and 500ml bottles are needed for all other water analyses. Refrigerate samples and keep cool during transit to the lab. Please follow water sampling instructions when collecting your sample to avoid contamination.**

**Figure B.5.** Laboratory water sample submittal forms required for each water sample submitted by a zoo.

# APPENDIX C

# QUESTIONNAIRE

1. Is drinking water quality ever considered in the nutritional management of your animal collection?
   Yes No

2. Does your facility routinely analyze the drinking water provided to your animal collection for quality?
   Yes No
   a. If yes, how frequently?

3. What is the zoo's primary source of drinking water? (check all that apply)
   City (Municipal) Well (bore) Recycled Other (please explain):

4. Has your facility had any concerns about drinking water quality during the last 5 years?
   Yes No
   a. If yes, please explain briefly.

5. Do you or some other employee know the location where the main drinking water source comes into the zoo?
   Yes No

6. Has your facility replaced any or all of the drinking water pipes within the last 5 years?
   Yes No

7. Does your facility treat the drinking water to improve quality?
   Yes No
   a. If yes, what type of drinking water treatment is your facility currently employing?

8. Does your facility employ a full-time nutritionist or a nutrition consultant to formulate zoo animal diets?

9. How old is the original portion of your facility?

10. How many species does your facility have in its collection?
    a. If greater than 500, please specify total number of species below:

**Figure C.1.** Questions 1 through 10 provided to all zoos (Black Rhino and Non-Black Rhino) in the questionnaire subsample group.

11. Has your facility ever tested the drinking water provided to black rhino for water quality?
    Yes No
12. Has your facility ever tested the drinking water provided to the black rhino specifically for iron concentration?
    Yes No
13. How many drinking water receptacles are provided in the black rhino exhibit and holding area(s)?

14. What type of drinking water receptacles are provided in the black rhino exhibit and holding area(s)?

15. What type of material is/are the drinking water receptacle(s) made of?

16. How frequently is/are the drinking water receptacle(s) cleaned in the black rhino exhibit and holding area(s)?

17. Does your facility provide ice block enrichment to your black rhino at any time throughout the year?
    Yes No
    a. If yes, how often are the black rhino offered ice block enrichment?

    b. What is the water source used to create the ice blocks, if known?

18. Do the black rhino at your facility have access to a recreational water source (e.g., wallow, pool, hose, sprinkler/mister, etc.)?
    Yes No
    a. If yes, what type of recreational water source is provided to the black rhino?
    a. If yes, what is the water source used to supply the recreational water, if known?

    b. If yes, what type of clay/soil is in your wallow, if known?

    c. If yes, in which seasons do the black rhino have access to the recreational water source? (check all that apply):
       Spring Summer Fall Winter
19. What type of ground cover is currently in the **exterior area** of the black rhino exhibit?

**Figure C.2.** Questions 11 through 19 of 28 provided only to Black Rhino zoos in the questionnaire subsample group.

**Figure C.2.** (cont'd)

20. What type of ground cover is currently in the **interior area** of the black rhino exhibit and holding area(s)?

21. How frequently does your facility analyze the nutrient contents of diets (e.g., crude protein, fiber, and minerals)?

22. Are the persons responsible for your facility's black rhino diets aware of the nutritional recommendations reported by the 2011 Nutrition Advisory Group workshop (published in the Journal of Zoo and Wildlife Medicine)?
    Yes No
    a. If yes, are the black rhino diets formulated according to the recommendations published in the workshop report?
    Yes No
23. Has your facility ever tested the diet provided to the black rhino specifically for iron content?
    Yes No
24. Would your facility be willing to share the current rhino diet formulations and/or analyses?
    Yes No
25. How many **male** black rhino are currently at your facility? (please circle correct number):
    0 1 2 3 4 5 6 7 8

26. How many **female** black rhino are currently at your facility? (please circle correct number):
    0 1 2 3 4 5 6 7 8

27. How frequently are the black rhino at your facility weighed?

28. How frequently does your facility assess body condition score on the black rhino?

# APPENDIX D

# ANALYTE STANDARDS

Analyte Standards Chart

| Analytes | EPA[1b]; *MCL*[10b] | EPA[1b]; *MCLG*[11b] | EPA[1b]; *SDWR*[12b] | EPA[1b] *DWA*[13b]; *Health-Based Value* | WHO[2c]; *GV*[14d] | WHO[2c]; *AA*[14d] | EU[3e]; *PV*[16e] | BCME[4e]; *MAC*[17a] | BCME[4e]; *AO*[18a] | Equine NRC[5f] 6th Edition | CVAS[6g]; *Possible Problem Level for Cattle*[19g] | Lowest Threshold Standard Value | Highest Threshold Standard Value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| pH | – | – | 6.5-8.5 | – | Not of health concern at levels found in drinking-water | 6.5-8.5 | – | – | – | – | <5.5 or >8.5 | 6.5 | 8.5 |
| Nitrate as Nitrogen (mg/L) | 10 | 10 | – | – | – | – | – | 10 | – | – | 23 | 10 | 10 |
| Nitrate as NO3 (mg/L) | – | – | – | – | 50 | – | 50 | 45 | – | 100 | 100 | 45 | 50 |
| Hardness (mg/L) | – | – | – | – | Not of health concern at levels found in drinking-water | 100-300 | – | – | – | – | – | 100 | 300 |
| Total Dissolved Solids (mg/L) | – | – | 500 | – | Not of health concern at levels found in drinking-water | 600 | – | – | – | <1,000 | 3000 | 500 | 600 |
| Chloride (mg/L) | – | – | 250 | – | Not of health concern at levels found in drinking-water | 250 | 250 | – | 250 | – | 300 | 250 | 250 |
| Sulfate (mg/L) | – | – | 250 | 500 | Not of health concern at levels found in drinking-water | <500 | 250 | – | 500 | – | 500 | 250 | 500 |
| Calcium (mg/L) | – | – | – | – | – | 100-300 | – | – | – | – | 150 | 100 | 300 |
| Phosphorus (mg/L) | – | – | – | – | – | – | – | – | 0.01 | – | 0.7 | 0.01 | 0.01 |
| Magnesium (mg/L) | – | – | – | – | – | – | – | – | – | – | 100 | – | – |
| Potassium (mg/L) | – | – | – | – | Occurs in drinking-water at concentrations well below those of health concern | – | – | – | – | – | 20 | – | – |
| Sodium (mg/L) | – | – | – | 20 | Not of health concern at levels found in drinking-water | 200 | 200 | – | – | 350-1,390 | 300 | 20 | 200 |
| Iron (mg/L) | – | – | 0.3 | – | Not of health concern at levels found in drinking-water | 0.3 | 0.2 | – | 0.3 | – | 0.4 (taste) | 0.2 | 0.3 |
| Manganese (mg/L) | – | – | 0.05 | – | 0.4 | 0.1 | 0.05 | – | 0.05 | – | 0.05 (taste) | 0.05 | 0.4 |
| Zinc (mg/L) | – | – | 5.0 | – | Not of health concern at levels found in drinking-water | 3.0 | – | – | 5.0 | 25 | 25 | 3.0 | 5.0 |
| Copper (mg/L) | TT | 1.3 | 1.0 | – | 2.0 | 2.0 | 2.0 | – | 1.0 | 0.5 | 0.6 | 1.0 | 2.0 |
| Total Coliform | 5% positive samples per month | 0% positive samples per month | – | – | Must not be detectable in any 100mL sample | | 0 per 100mL | less than 10 coliforms/100mL; 90th percentile (min. of 5 samples) | – | 100 thermotolerant coliforms/100mL | 15 colonies per 100mL | 0 | 0 |

***Definitions:***

1. **EPA:** United States Environmental Protection Agency
2. **WHO:** World Health Organization
3. **EU:** European Union
4. **BCME:** British Columbia Ministry of Environment
5. **NRC:** National Research Council
6. **CVAS:** Cumberland Valley Analytical Services
7. **WQI:** Water Quality Index
8. **Treatment Technique (TT):** Defined by the EPA as a required process intended to reduce a contaminant in drinking water.
9. **Thermotolerant coliforms:** a term used to describe fecal coliform more accurately, and can grow in temperature range of 44-45°C.
10. **Maximum Contaminant Level (MCL):** The highest concentration of a contaminant allowed in drinking water, and are standards enforceable by law.
11. **Maximum Contaminant Level Goal (MCLG):** A contaminant concentration goal set at a value for which no known adverse health effects occur. MCLGs are non-enforceable health based standards.
12. **Secondary Drinking Water Regulations (SDWR):** Federal guidelines for drinking water that cover aesthetic (taste, odor, or color) and cosmetic (tooth or skin discoloration) effects, and are non-enforceable.
13. **Drinking Water Advisory (DWA):** A contaminant concentration in drinking water that is likely to have no adverse effects on aesthetics and health for the period which it is derived, and are non-regulatory.
14. **Guideline Value (GV):** A contaminant concentration which poses no significant risk to health over a lifetime of consumption. Considered to be a health-based guideline value.
15. **Acceptability Aspects (AA):** A contaminant concentration in drinking water which may cause color/appearance, odor, or taste to occur in water that some consumers may find unacceptable, but may not pose a risk to health. Can be used as an indicator for further water testing.
16. **Parametric Value (PV):** A metric of measure used by the EU in defining contaminant concentrations in drinking water. The PV is based closely upon the WHO's GV, and as such, is defined the same.
17. **Maximum Acceptable Concentration (MAC):** A concentration established for certain contaminants that are known to, or may cause, adverse health effects.
18. **Aesthetic Objective (AO):** A concentration for contaminants that do not cause adverse health effects, but may effect taste, odor, or color/appearance of drinking water.
19. **Possible Problem Level for Cattle:** Concentration values based upon research literature and field experiences provided by the CVAS lab on each drinking water quality analysis. These values are based upon the work of Mike T. Socha, et al., as well as, Richard S. Adams and William Sharpe.

***Sources:***

a. British Columbia Ministry of Environment. 2017. *B.C. Source Drinking Water Quality Guidelines: Guideline Summary.* Water Quality Guideline Series, WQG-01. Prov. B.C., Victoria, B.C.
b. United States Environmental Protection Agency. 2018. *2018 Edition of the Drinking Water Standards and Health Advisories Tables.* EPA 822-F-18-001. Washington, D.C.
c. World Health Organization. 2017. *Guidelines for drinking-water quality: fourth edition incorporating the first addendum.* License: CC BY-NC-SA 3.0 IGO. Geneva, CH.
d. World Health Organization. 2009. *WHO Guidelines for Drinking-water Quality Policies and Procedures used in updating the WHO Guidelines for Drinking-water Quality.* Geneva, CH.
e. World Health Organization: Regional Office for Europe. 2017. *Drinking Water Parameter Cooperation Project: Support to the revision of Annex I Council Directive 98/83/EC on the Quality of Water Intended for Human Consumption (Drinking Water Directive).*
f. National Research Council. 2007. *Nutrient Requirements of Horses: Sixth Revised Edition.* Washington, DC: The National Academies Press.
g. Cumberland Valley Analytical Services. 2018. *Water Analysis Report.* https://www.foragelab.com/Media/WaterAnalysisReport.pdf.

**Figure D.1.** Standard values and sources used for both the low and high standard Water Quality Index (WQI) calculations.

# APPENDIX E

## QUESTIONS USED FOR STATISTICAL ANALYSIS

***Question 1*** What is the current state of drinking water quality in non-black rhino zoos?

***Question 2*** What is the current state of drinking water quality within black rhino exhibits in zoos?

***Question 3*** Does having/utilizing a nutritionist or nutrition consultant affect whether or not the drinking water is tested for quality in zoos (non-black rhino and black rhino)?

***Question 4*** Does having/utilizing a nutritionist or nutrition consultant affect whether or not the drinking water provided to the black rhino is tested specifically for iron concentration?

***Question 5*** Are zoos with nutritionists/nutrition consultants more likely to be aware of the Nutrition Advisory Group (NAG) black rhino recommendations?

***Question 6*** If zoos are aware of the Nutrition Advisory Group (NAG) black rhino recommendations do they formulate the black rhino diets based upon them?

***Question 7*** Is there a significant difference between the quality of the origin point drinking water and the quality of the black rhino exhibit drinking water?

***Question 8*** Does the age of the zoo affect the difference in drinking water quality between the origin point and black rhino exhibit?

***Question 9*** Does the water source (e.g., municipal, well [bore], or river) affect the drinking water quality in zoos?

***Question 10*** Does the size of the zoo have any effect on drinking water quality?

***Question 11*** Does replacing pipes within the zoo affect the difference in drinking water quality between the origin point and the black rhino exhibit?

***Question 12*** Does the frequency of cleaning of the drinking water receptacle have any effect on drinking water quality within the black rhino exhibit?

***Question 13*** Do zoos that test their drinking water for quality have better overall drinking water quality than zoos that do not test their drinking water?

# APPENDIX F

# FIGURES AND TABLES

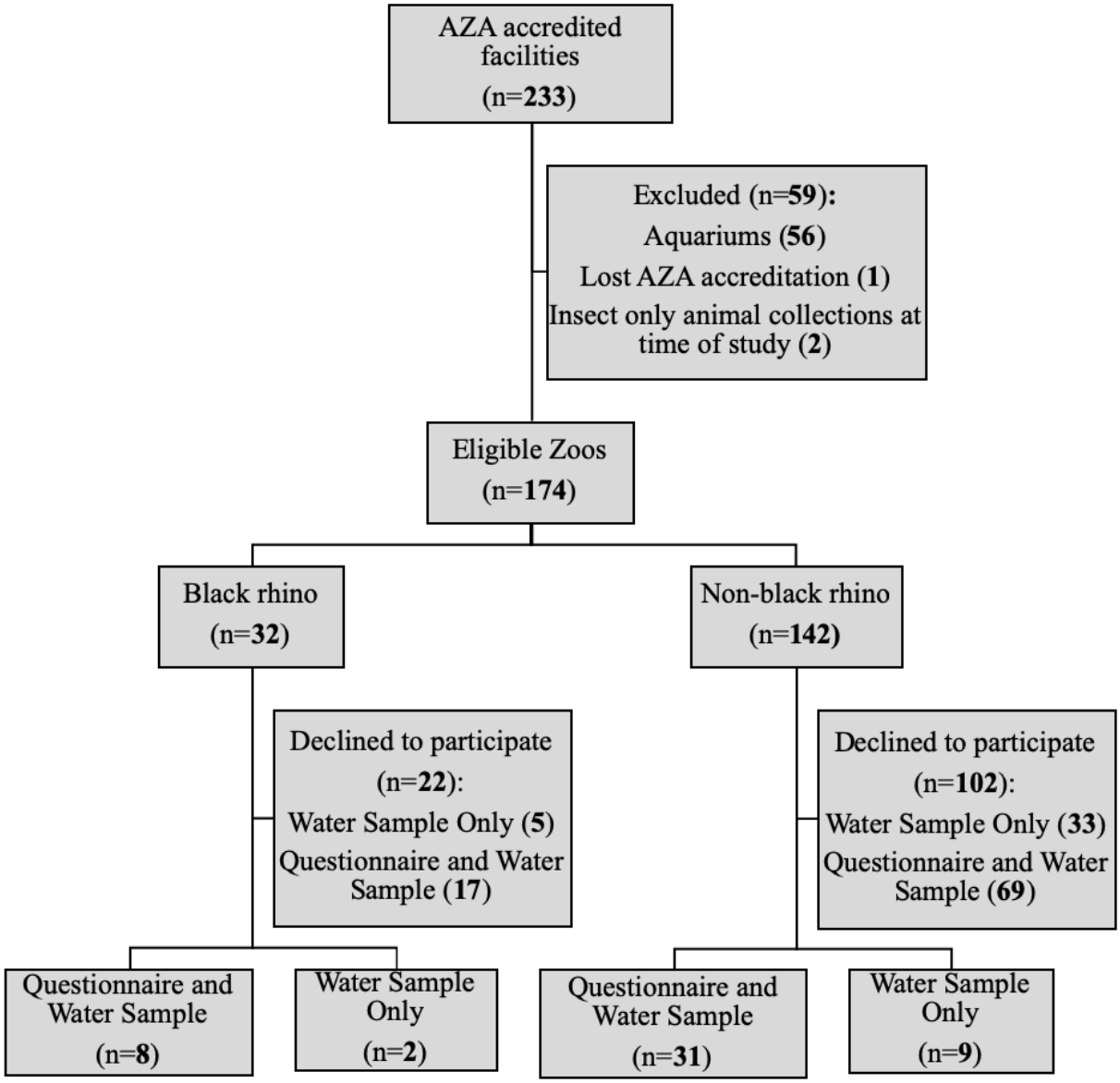


**Figure F.1.** Organizational chart showing definition and partitioning of candidate zoos in the study design for participation.

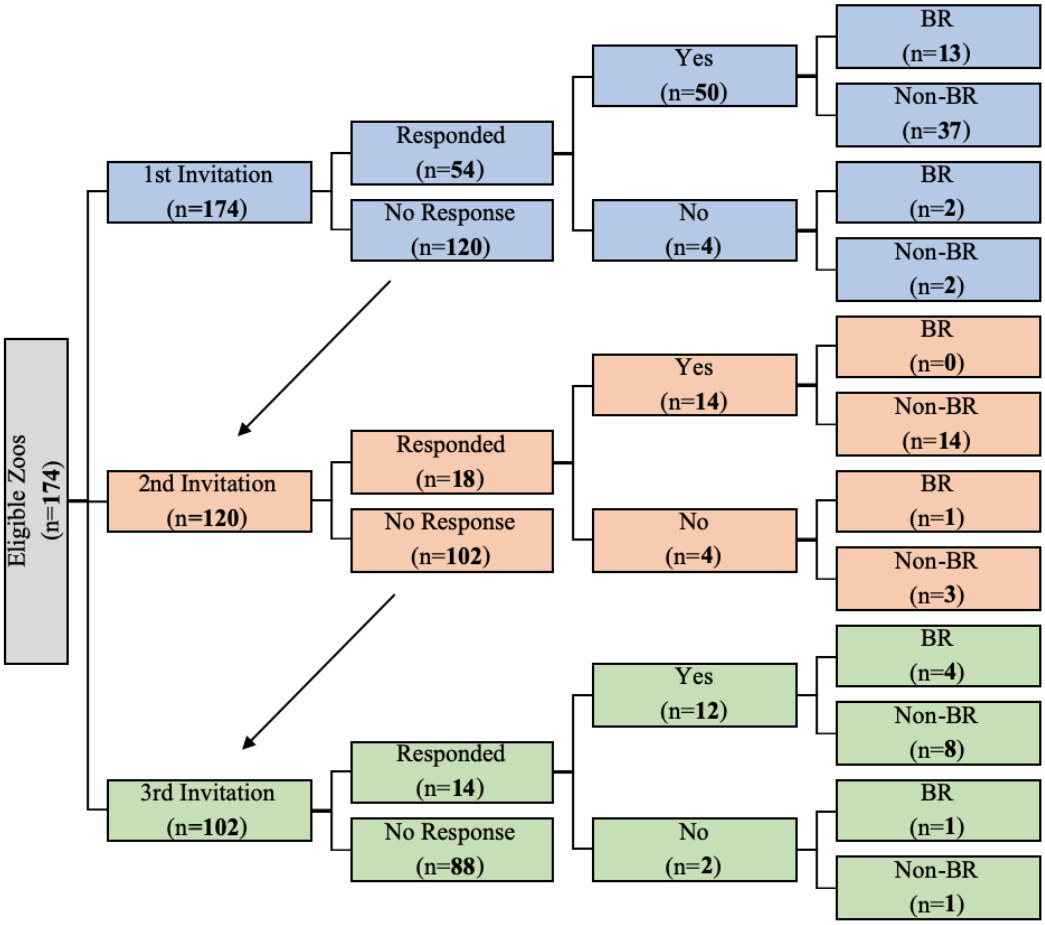


**Figure F.2.** Organizational flow chart of study invitation responses.

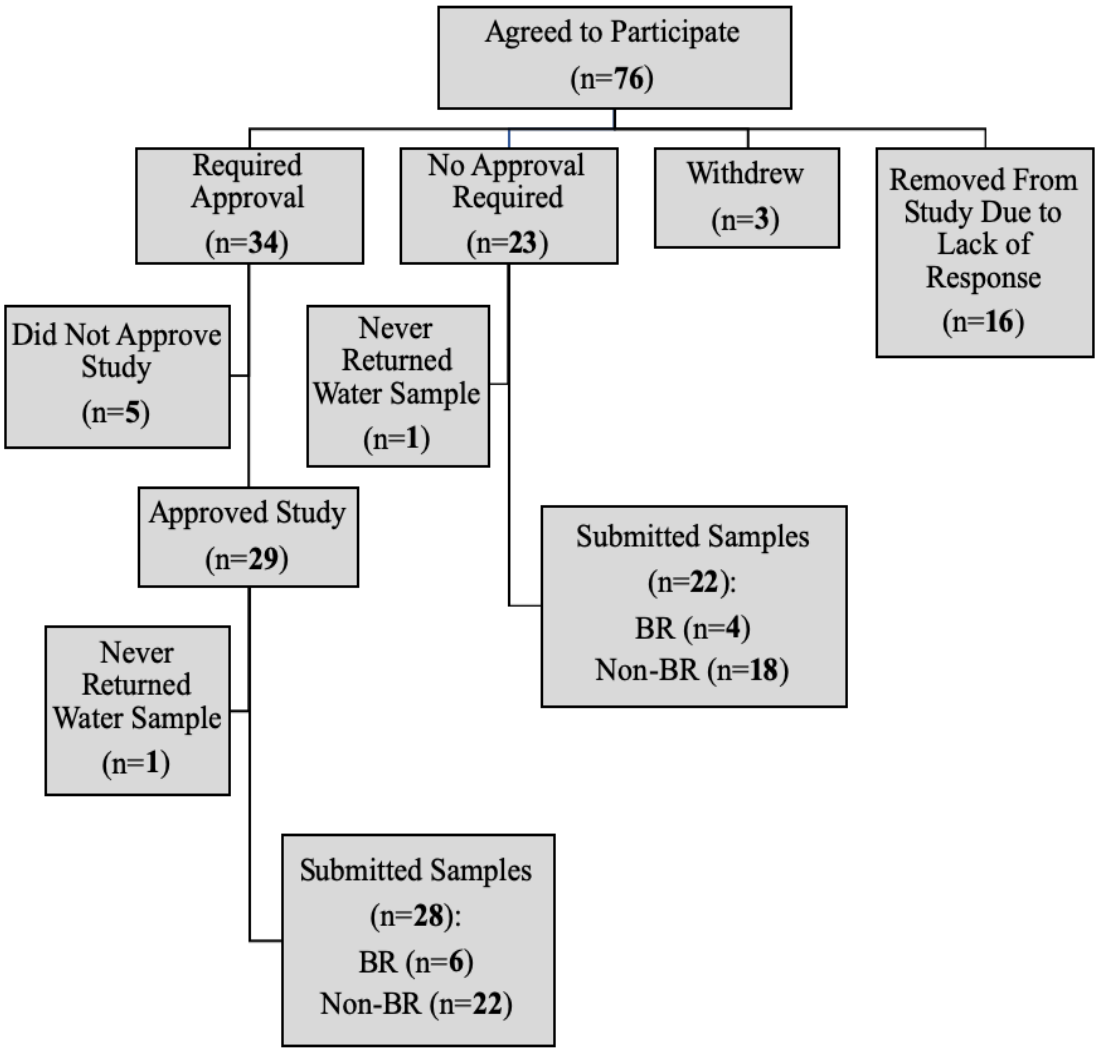


**Figure F.3.** Organizational chart showing final disposition and fate of zoos initially agreeing to participate based on confirmation to one of the three invitations. Fifty total zoos participated in the study (10 in the Black Rhino [BR] group and 40 in the Non-Black Rhino [Non-BR] group).

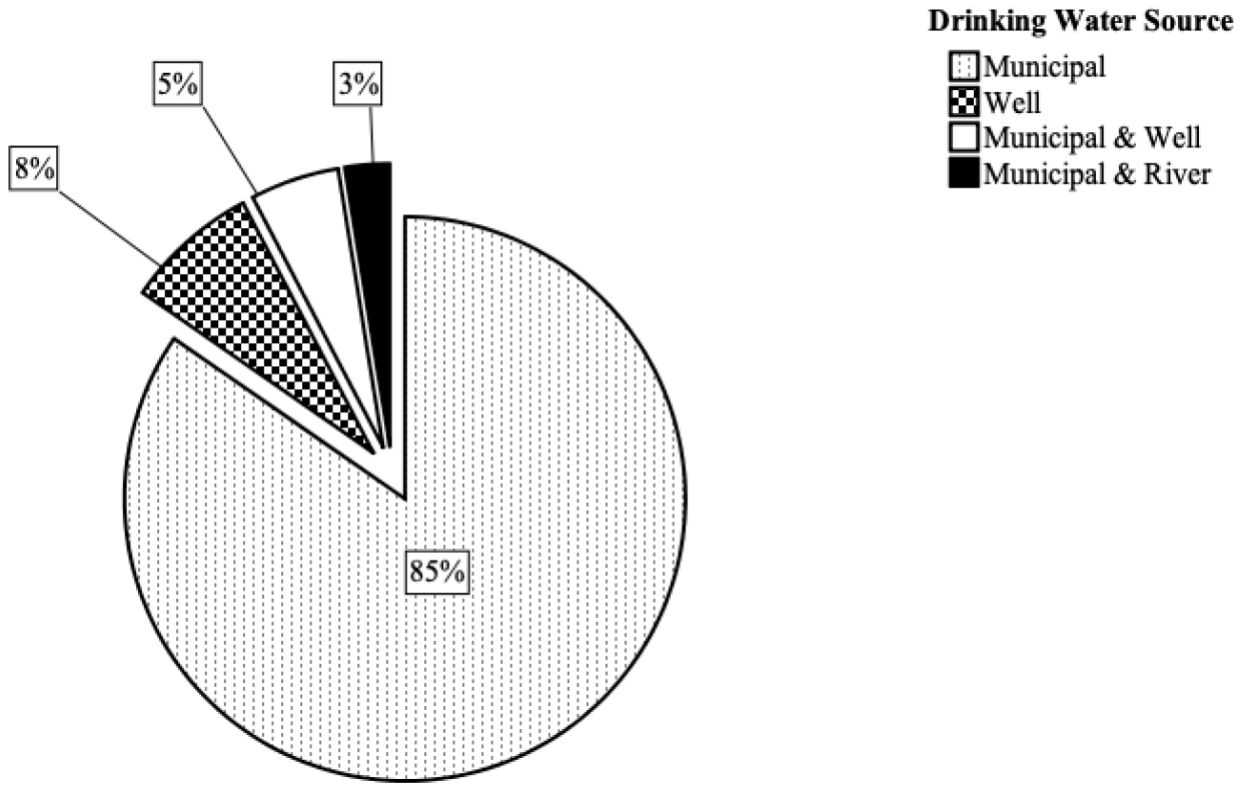


**Figure F.4.** Pie chart showing the overall breakdown of primary drinking water sources used by all 39 zoos to complete questionnaires, including both Non-Black Rhino and Black Rhino facilities.

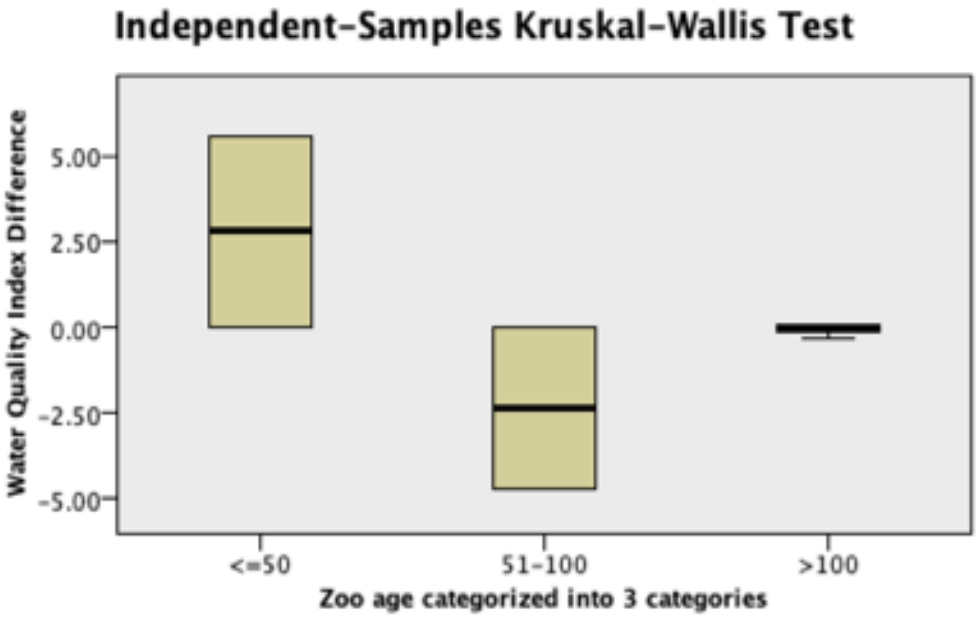


**Figure F.5.** Box plot chart showing the distributions for zoo age across Black Rhino zoo Water Quality Index (WQI) difference for the "Low: All Analytes" calculation. The plots show the distributions between zoo age group were not similar in shape. Due to the distributions not being similarly shaped, the *p*-value presented was an asymptotic value and not an exact *p*-value.

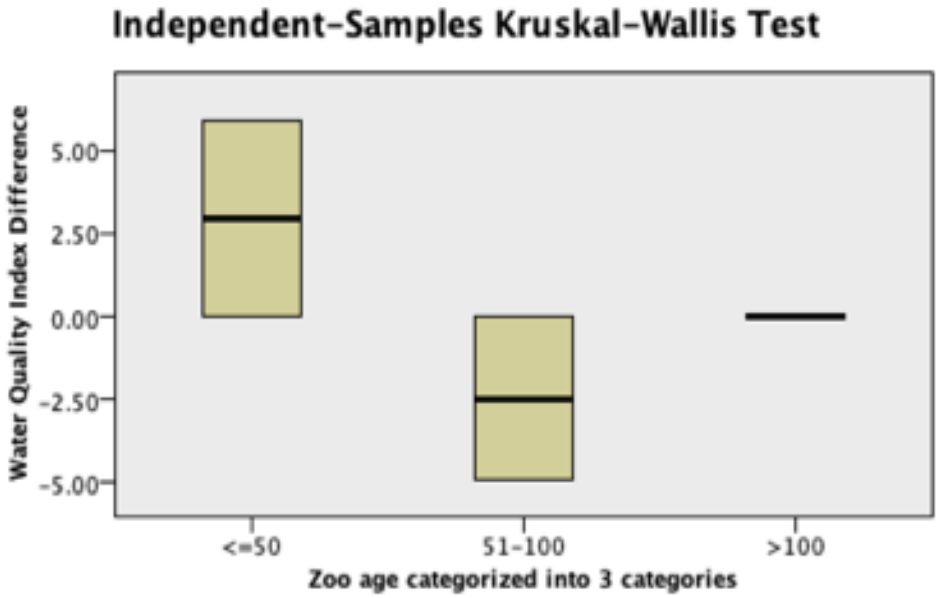


**Figure F.6.** Box plot chart showing the distributions for zoo age across Black Rhino zoo Water Quality Index (WQI) difference for the "Low: Select Analytes" calculation. The plots show the distributions between zoo age group were not similar in shape. Due to the distributions not being similarly shaped, the *p*-value presented was an asymptotic value and not an exact *p*-value.

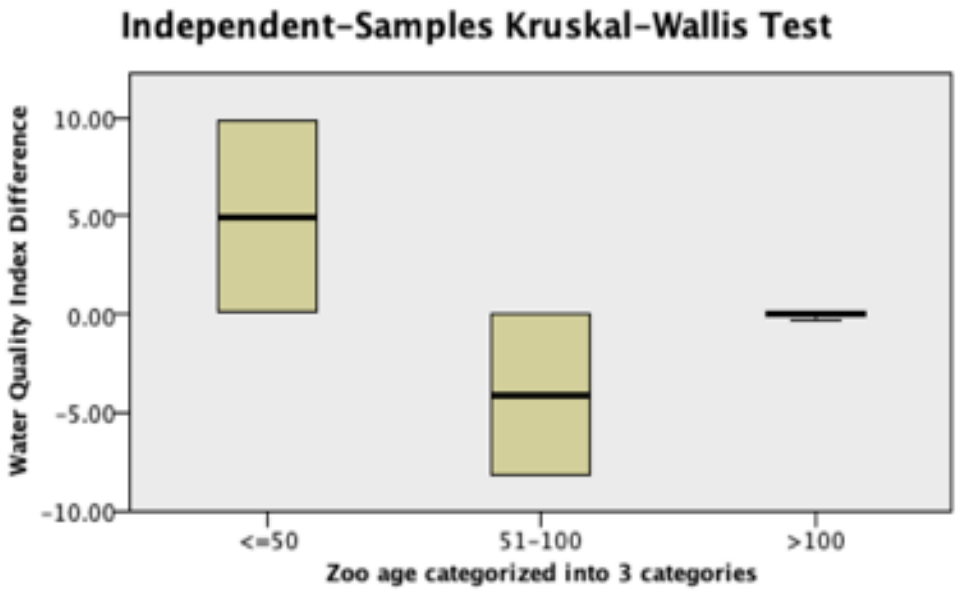


**Figure F.7.** Box plot chart showing the distributions for zoo age across Black Rhino zoo Water Quality Index (WQI) difference for the "High: All Analytes" calculation. The plots show the distributions between zoo age group were not similar in shape. Due to the distributions not being similarly shaped, the *p*-value presented was an asymptotic value and not an exact *p*-value.

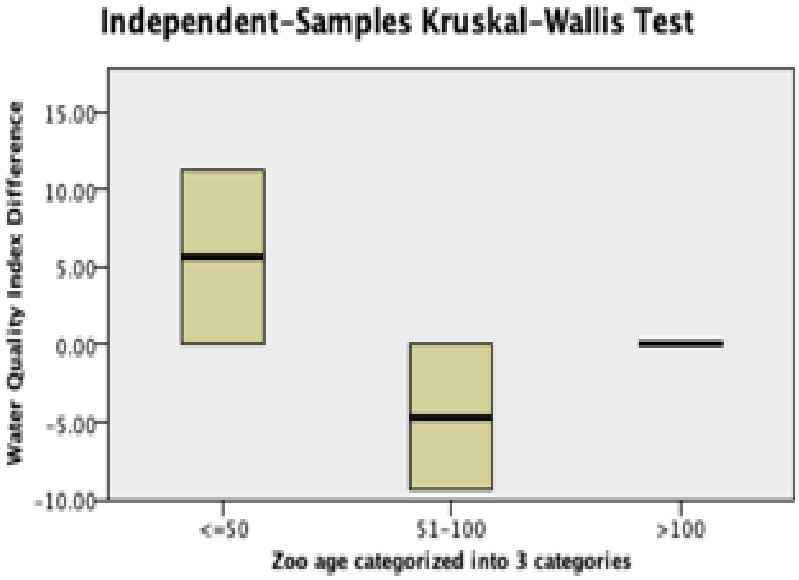


**Figure F.8.** Box plot chart showing the distributions for zoo age across Black Rhino zoo Water Quality Index (WQI) difference for the "High: Select Analytes" calculation. The plots show the distributions between zoo age group were not similar in shape. Due to the distributions not being similarly shaped, the p-value presented was an asymptotic value and not an exact *p*-value.

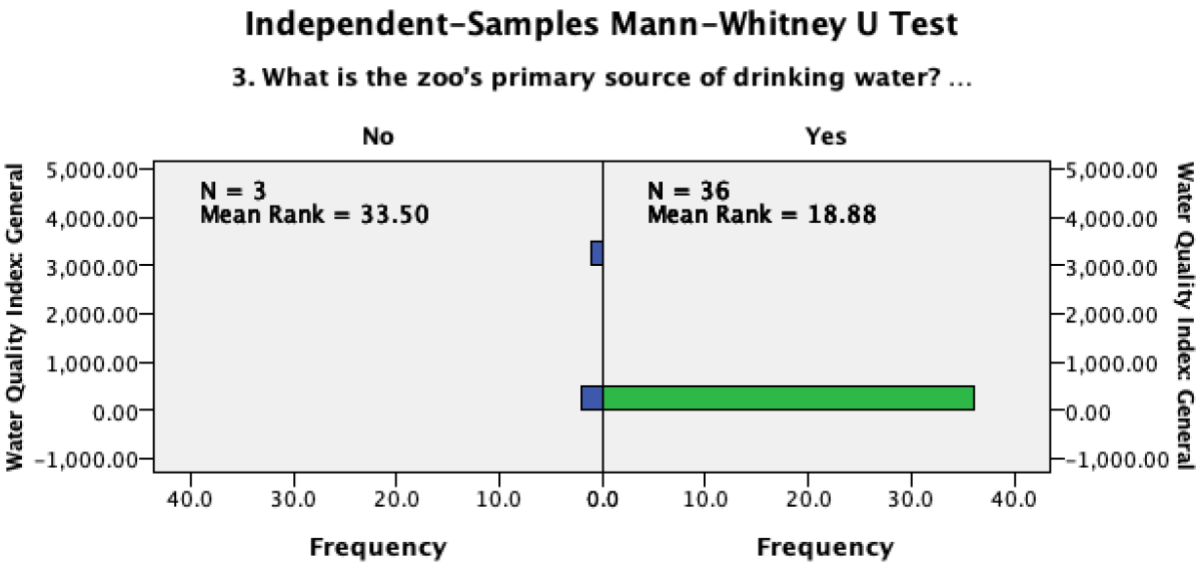


**Figure F.9.** Pyramid chart showing the distribution of zoos that did and did not use municipal water as their primary drinking water source. All Water Quality Index (WQI) values are from the origin sampling point and were calculated using the "Low: All Analytes" formula. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping. Due to the distributions not being similarly shaped, the p-value presented was an asymptotic value and not an exact *p*-value.

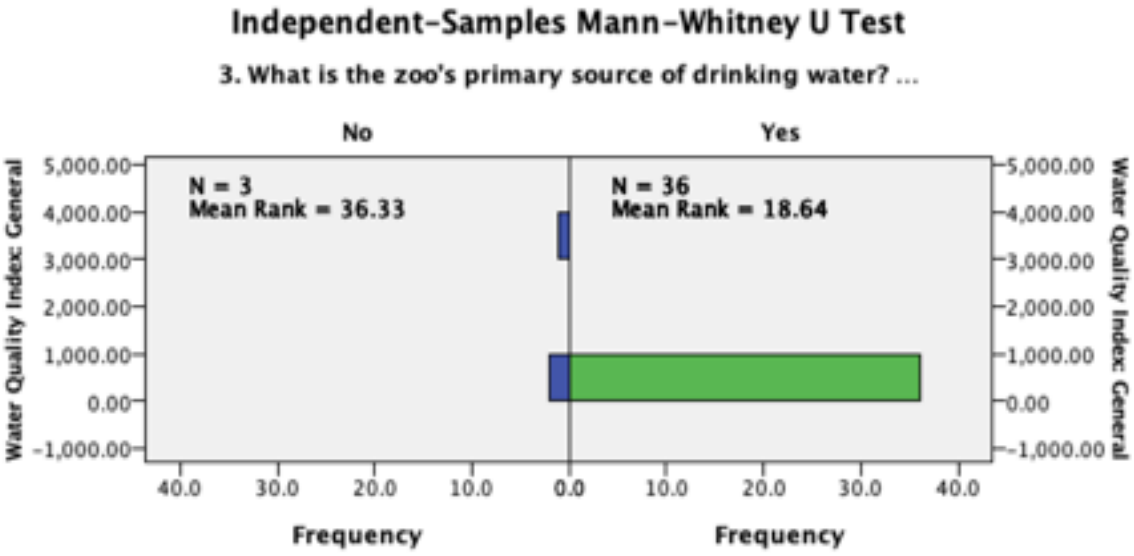


**Figure F.10.** Pyramid chart showing the distribution of zoos that did and did not use municipal water as their primary drinking water source. All Water Quality Index (WQI) values are from the origin sampling point and were calculated using the "Low: Select Analytes" formula. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping. Due to the distributions not being similarly shaped, the p-value presented was an asymptotic value and not an exact *p*-value.

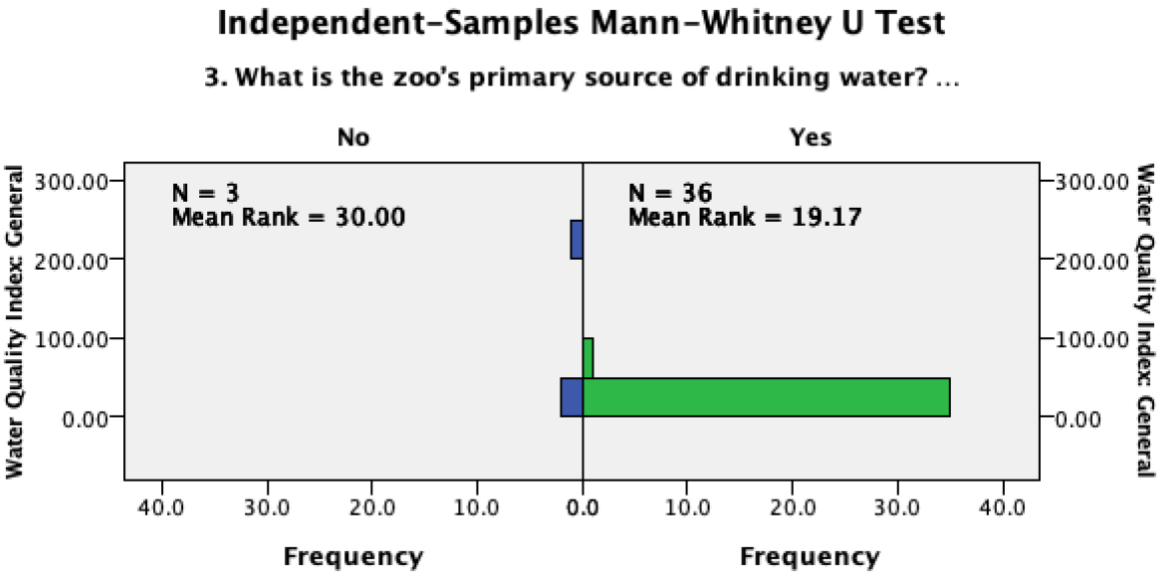


**Figure F.11.** Pyramid chart showing the distribution of zoos that did and did not use municipal water as their primary drinking water source. All Water Quality Index (WQI) values are from the origin sampling point and were calculated using the "High: All Analytes" formula. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping. Due to the distributions not being similarly shaped, the p-value presented was an asymptotic value and not an exact *p*-value.

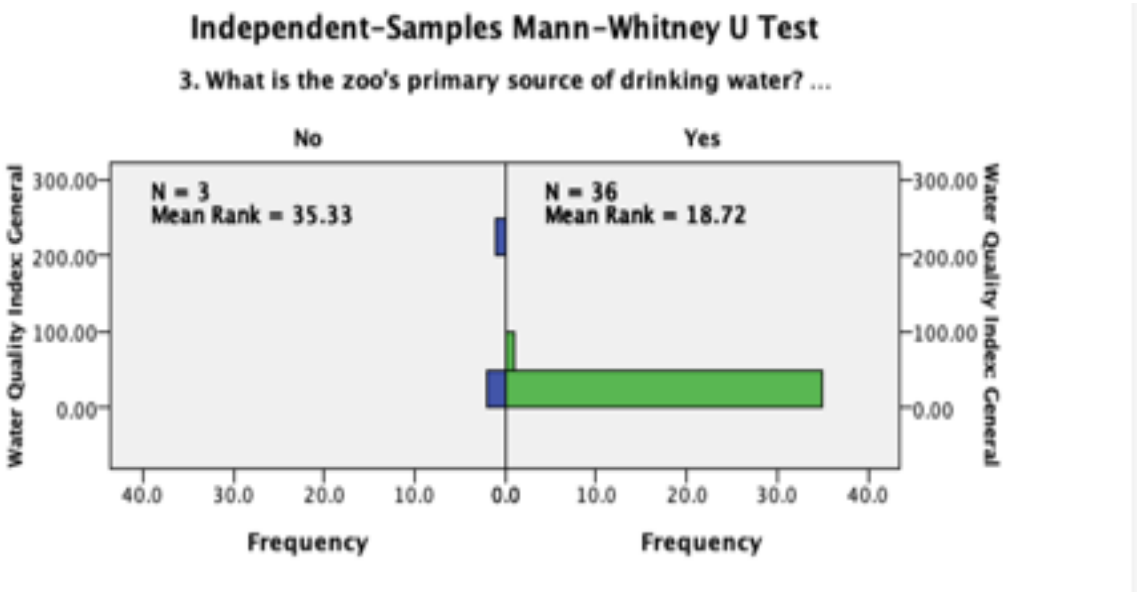


**Figure F.12.** Pyramid chart showing the distribution of zoos that did and did not use municipal water as their primary drinking water source. All Water Quality Index (WQI) values are from the origin sampling point and were calculated using the "High: Select Analytes" formula. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping. Due to the distributions not being similarly shaped, the p-value presented was an asymptotic value and not an exact *p*-value.

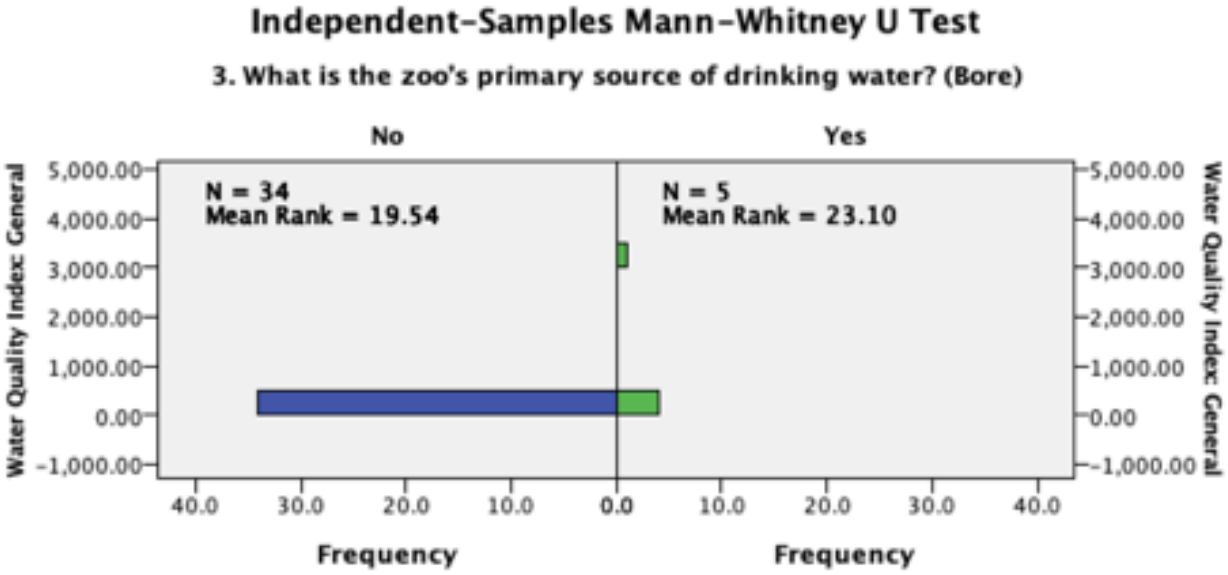


**Figure F.13.** Pyramid chart showing the distribution of zoos that did and did not use well(bore) water as their primary drinking water source. All Water Quality Index (WQI) values are from the origin sampling point and were calculated using the "Low: All Analytes" formula. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping. Due to the distributions not being similarly shaped, the p-value presented was an asymptotic value and not an exact *p*-value.

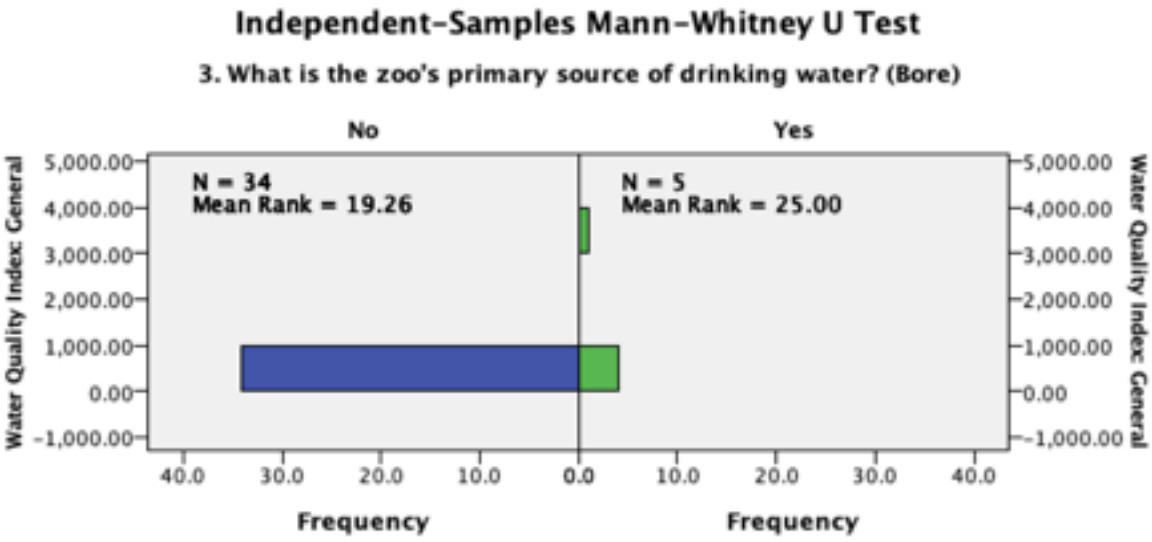


**Figure F.14.** Pyramid chart showing the distribution of zoos that did and did not use well(bore) water as their primary drinking water source. All Water Quality Index (WQI) values are from the origin sampling point and were calculated using the "Low: Select Analytes" formula. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping. Due to the distributions not being similarly shaped, the p-value presented was an asymptotic value and not an exact *p*-value.

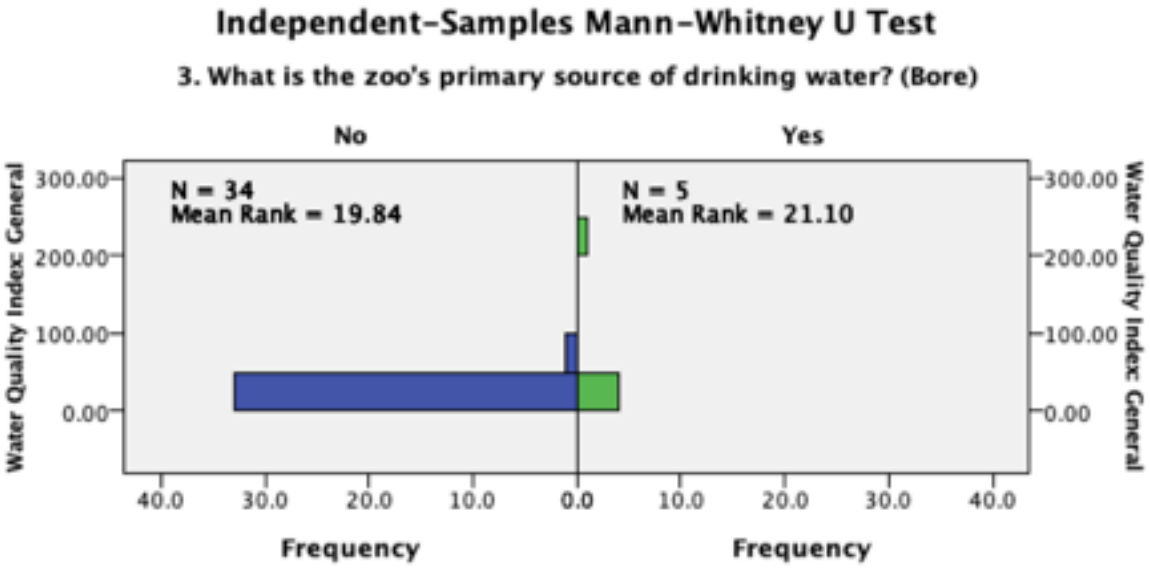


**Figure F.15.** Pyramid chart showing the distribution of zoos that did and did not use well(bore) water as their primary drinking water source. All Water Quality Index (WQI) values are from the origin sampling point and were calculated using the "High: All Analytes" formula. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping. Due to the distributions not being similarly shaped, the p-value presented was an asymptotic value and not an exact *p*-value.

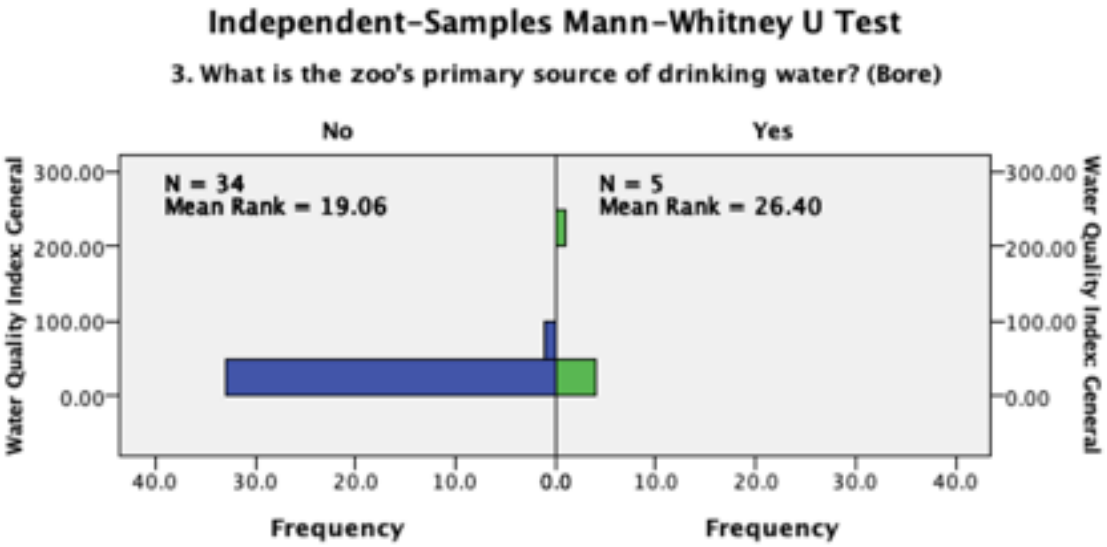


**Figure F.16.** Pyramid chart showing the distribution of zoos that did and did not use well(bore) water as their primary drinking water source. All Water Quality Index (WQI) values are from the origin sampling point and were calculated using the "High: Select Analytes" formula. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping. Due to the distributions not being similarly shaped, the p-value presented was an asymptotic value and not an exact *p*-value.

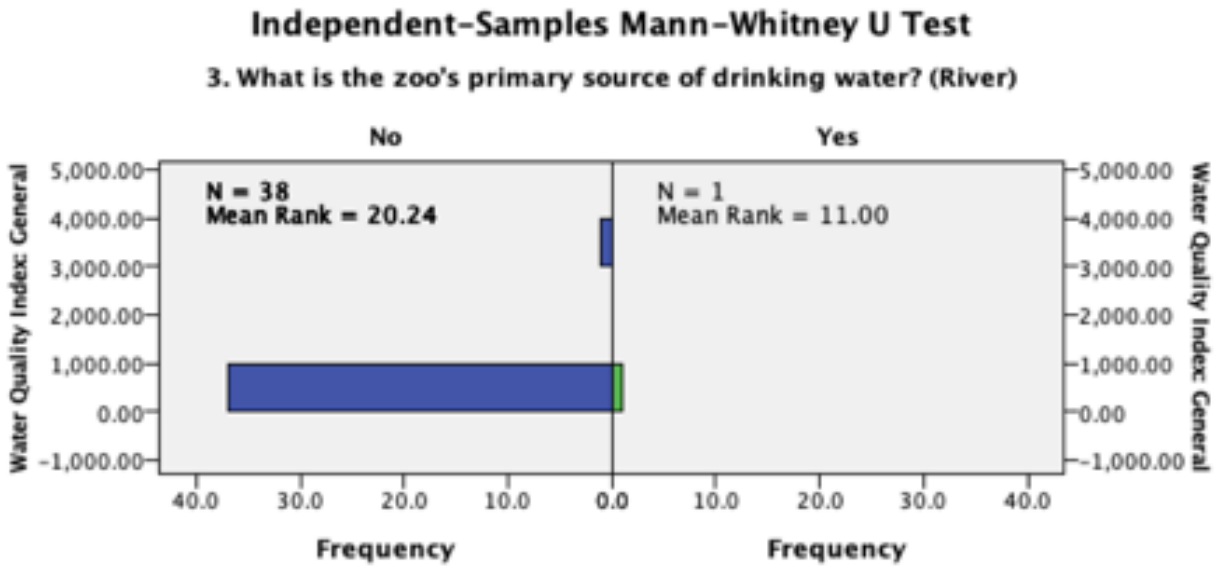


**Figure F.17.** Pyramid chart showing the distribution of zoos that did and did not use river water as their primary drinking water source. All Water Quality Index (WQI) values are from the origin sampling point and were calculated using the "Low: All Analytes" formula. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping. Due to the distributions not being similarly shaped, the p-value presented was an asymptotic value and not an exact *p*-value.

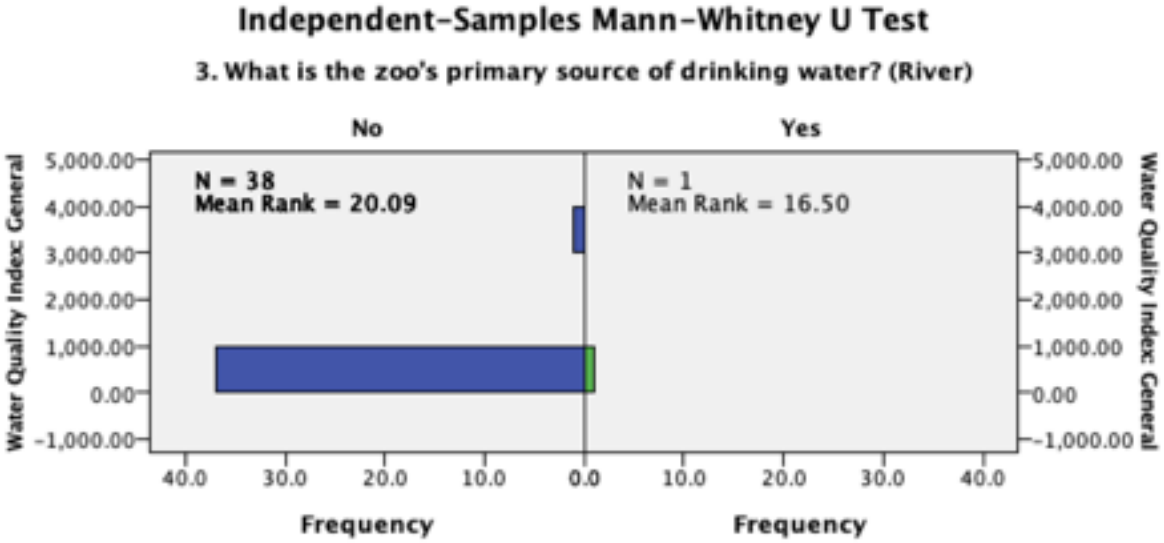


**Figure F.18.** Pyramid chart showing the distribution of zoos that did and did not use river water as their primary drinking water source. All Water Quality Index (WQI) values are from the origin sampling point and were calculated using the "Low: Select Analytes" formula. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping. Due to the distributions not being similarly shaped, the *p*-value presented was an asymptotic value and not an exact p-value.

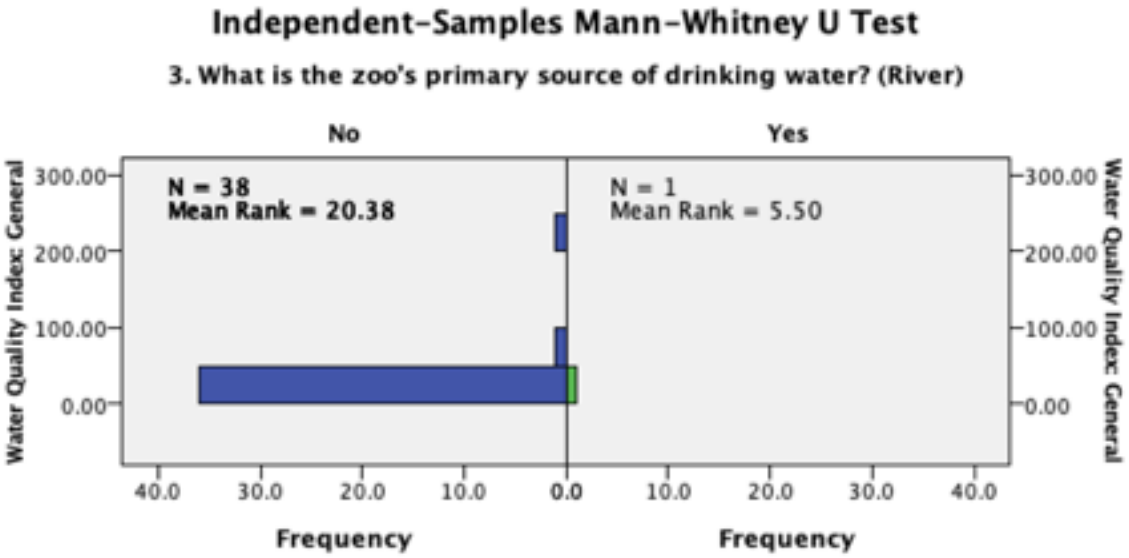


**Figure F.19.** Pyramid chart showing the distribution of zoos that did and did not use river water as their primary drinking water source. All Water Quality Index (WQI) values are from the origin sampling point and were calculated using the "High: All Analytes" formula. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping. Due to the distributions not being similarly shaped, the *p*-value presented was an asymptotic value and not an exact p-value.

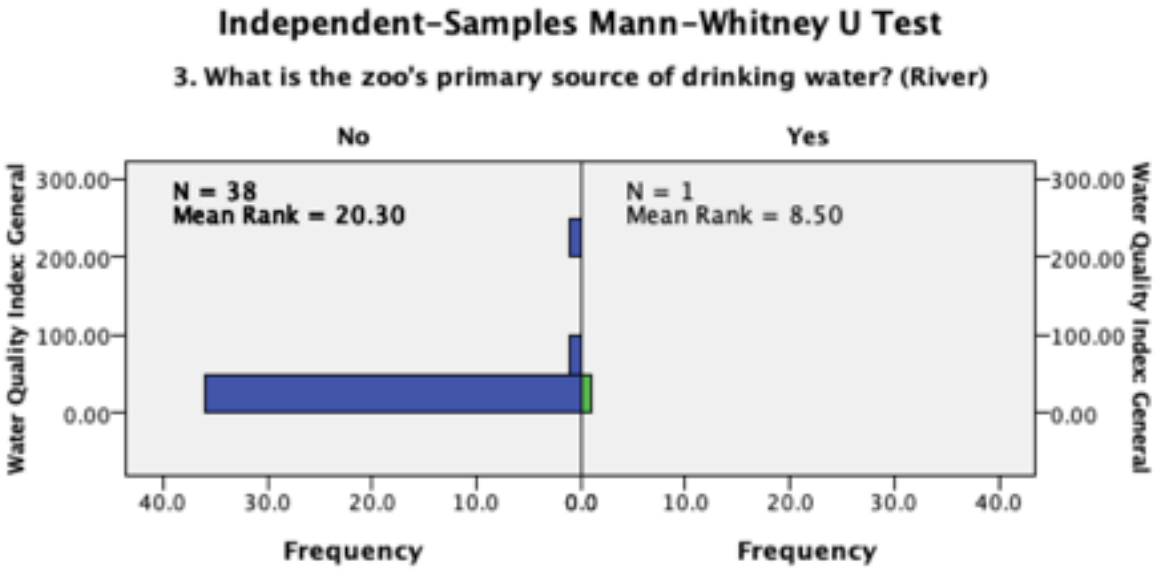


**Figure F.20.** Pyramid chart showing the distribution of zoos that did and did not use river water as their primary drinking water source. All Water Quality Index (WQI) values are from the origin sampling point and were calculated using the "High: Select Analytes" formula. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping. Due to the distributions not being similarly shaped, the *p*-value presented was an asymptotic value and not an exact p-value.

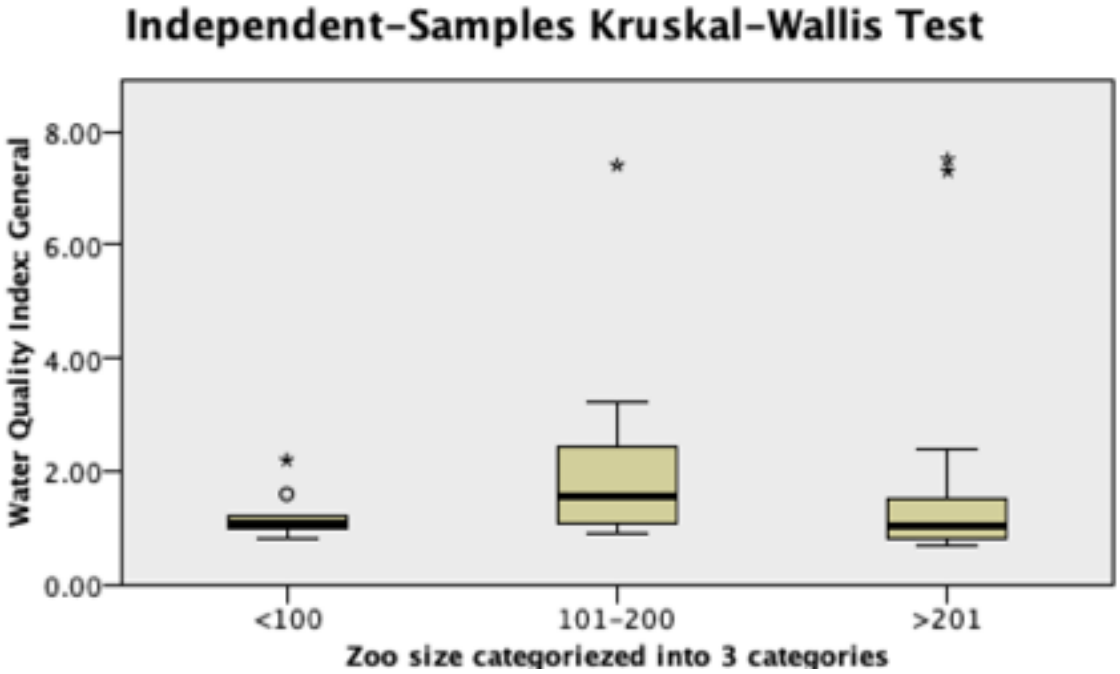


**Figure F.21.** Box plot showing the distribution of zoo size across categories in number of species. All Water Quality Index (WQI) values are from the origin sampling point and were calculated using the "Low: All Analytes" formula. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping. Due to the distributions not being similarly shaped, the p-value presented was an asymptotic value and not an exact *p*-value. Due to the few exceptionally high WQI values making it difficult to see whether or not the box plots are similarly shaped, these values were removed in order to better assess the box plot distributions. All analysis was performed, and all results are presented with the exceptionally high values included in the data set.

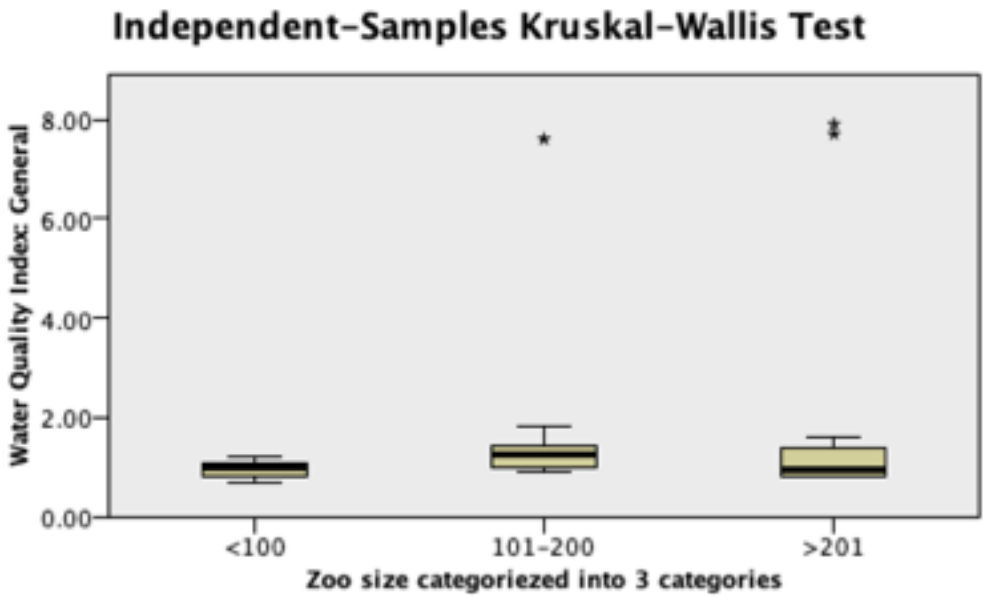


**Figure F.22.** Box plot showing the distribution of zoo size across categories in number of species. All Water Quality Index (WQI) values are from the origin sampling point and were calculated using the "Low: Select Analytes" formula. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping. Due to the distributions not being similarly shaped, the p-value presented was an asymptotic value and not an exact *p*-value. Due to the few exceptionally high WQI values making it difficult to see whether or not the box plots are similarly shaped, these values were removed in order to better assess the box plot distributions. All analysis was performed, and all results are presented with the exceptionally high values included in the data set.

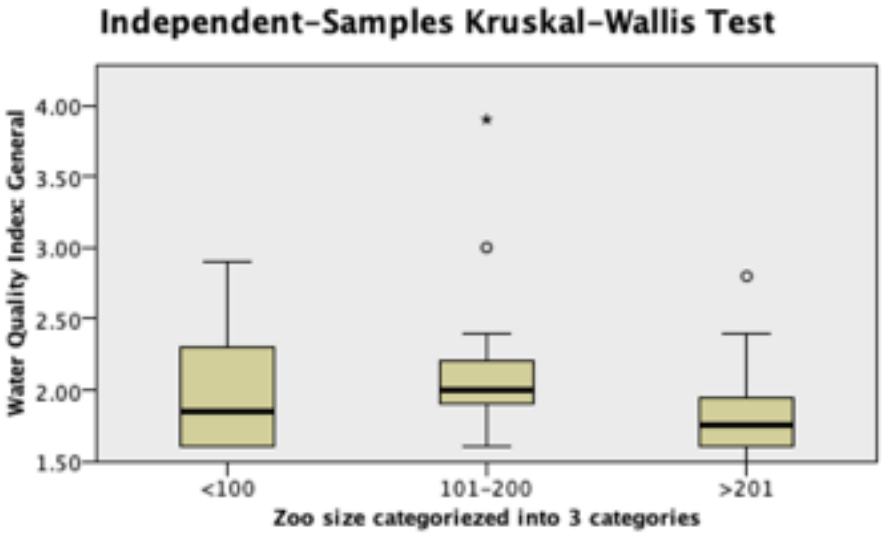


**Figure F.23.** Box plot showing the distribution of zoo size across categories in number of species. All Water Quality Index (WQI) values are from the origin sampling point and were calculated using the "High: All Analytes" formula. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping. Due to the distributions not being similarly shaped, the p-value presented was an asymptotic value and not an exact *p*-value. Due to the few exceptionally high WQI values making it difficult to see whether or not the box plots are similarly shaped, these values were removed in order to better assess the box plot distributions. All analysis was performed, and all results are presented with the exceptionally high values included in the data set.

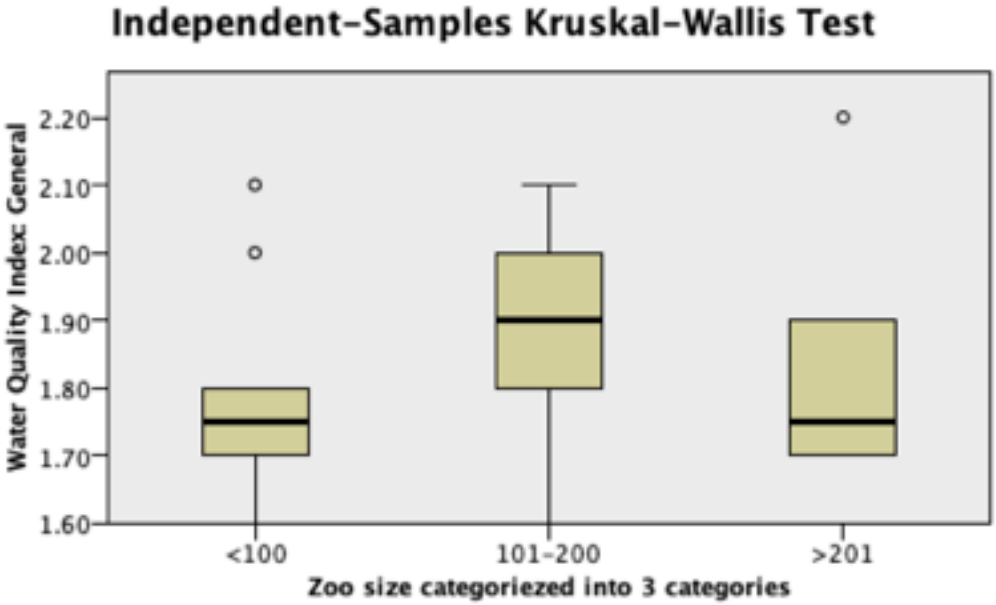


**Figure F.24.** Box plot showing the distribution of zoo size across categories in number of species. All Water Quality Index (WQI) values are from the origin sampling point and were calculated using the "High: Select Analytes" formula. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping. Due to the distributions not being similarly shaped, the p-value presented was an asymptotic value and not an exact *p*-value. Due to the few exceptionally high WQI values making it difficult to see whether or not the box plots are similarly shaped, these values were removed in order to better assess the box plot distributions. All analysis was performed, and all results are presented with the exceptionally high values included in the data set.

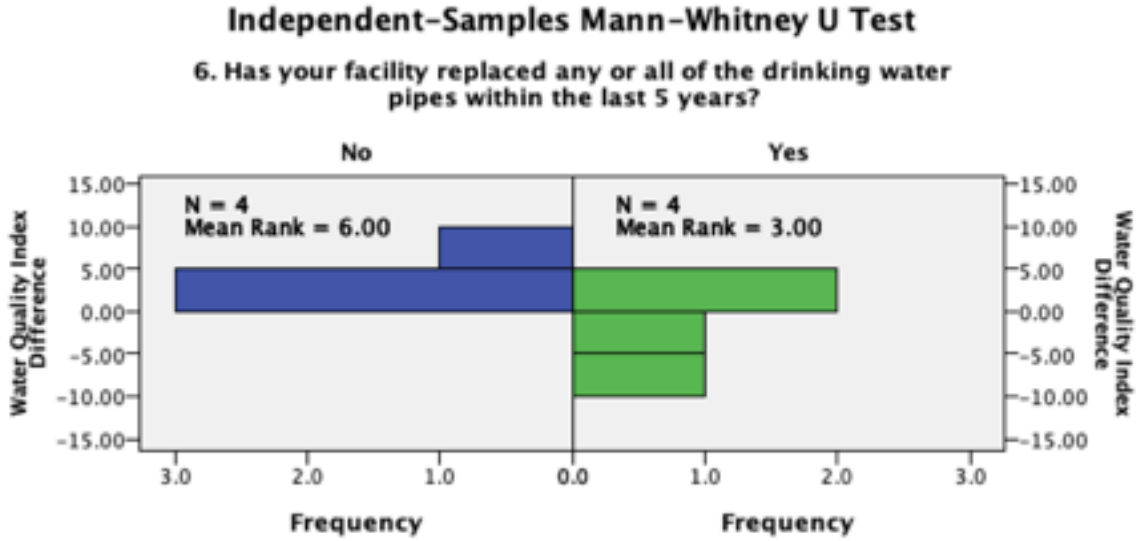


**Figure F.25.** Pyramid chart showing the distribution of zoos that had and had not replaced drinking water pipes within the last 5 years. All Water Quality Index (WQI) values are from the origin sampling point and were calculated using the "Low: All Analytes" formula. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping. Due to the distributions not being similarly shaped, the p-value presented was an asymptotic value and not an exact *p*-value.

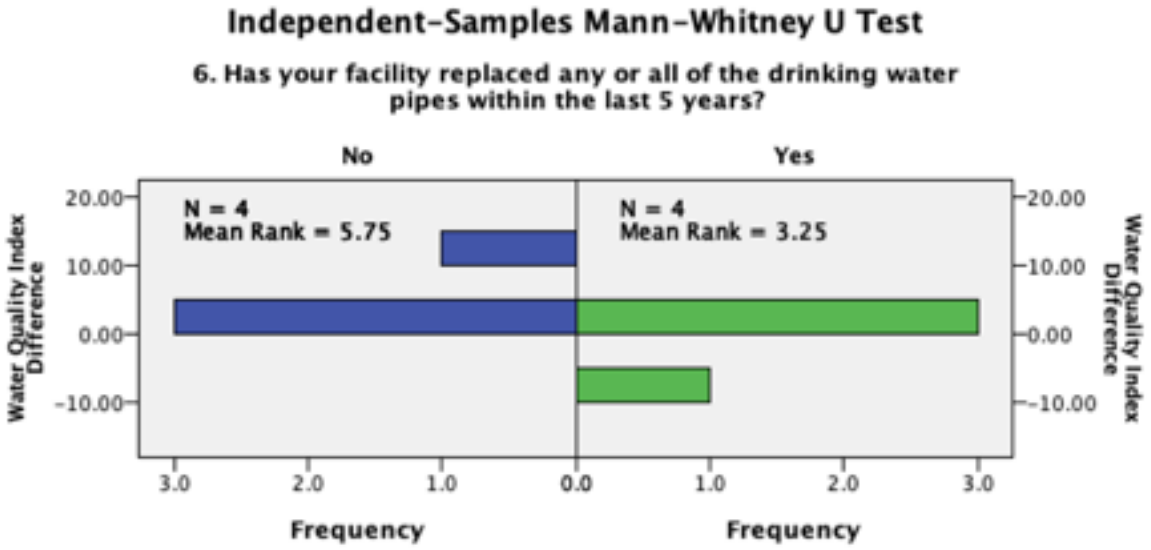


**Figure F.26.** Pyramid chart showing the distribution of zoos that had and had not replaced drinking water pipes within the last 5 years. All Water Quality Index (WQI) values are from the origin sampling point and were calculated using the "Low: Select Analytes" formula. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping. Due to the distributions not being similarly shaped, the p-value presented was an asymptotic value and not an exact *p*-value.

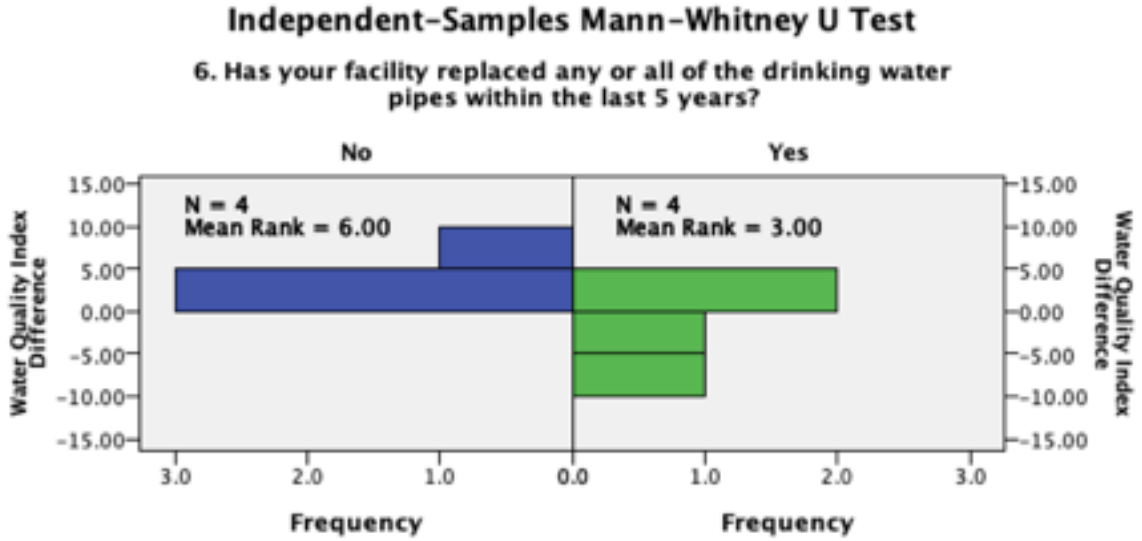


**Figure F.27.** Pyramid chart showing the distribution of zoos that had and had not replaced drinking water pipes within the last 5 years. All Water Quality Index (WQI) values are from the origin sampling point and were calculated using the "High: All Analytes" formula. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping. Due to the distributions not being similarly shaped, the p-value presented was an asymptotic value and not an exact *p*-value.

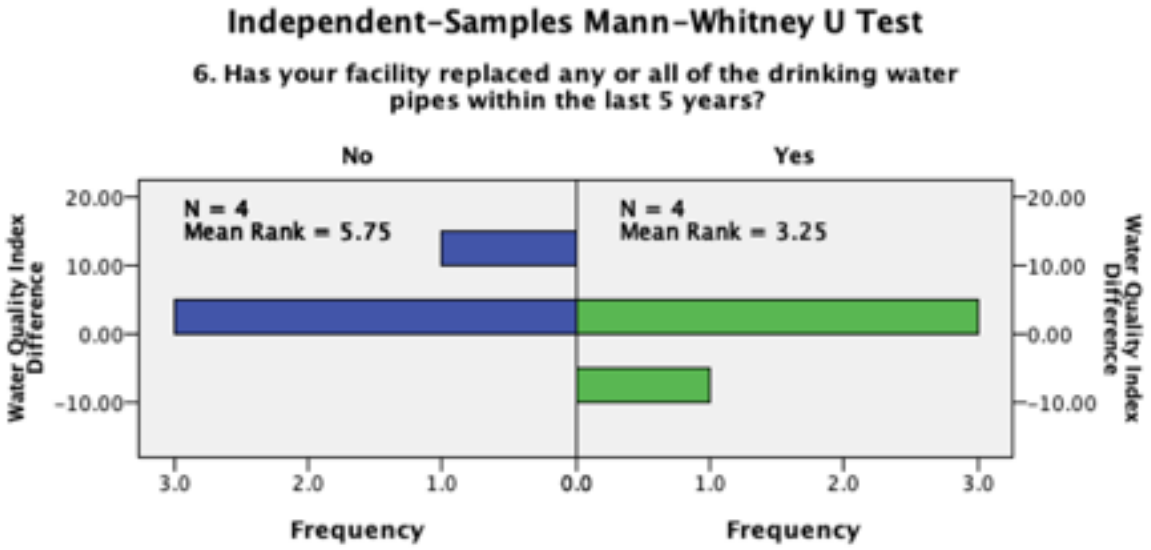


**Figure F.28.** Pyramid chart showing the distribution of zoos that had and had not replaced drinking water pipes within the last 5 years. All Water Quality Index (WQI) values are from the origin sampling point and were calculated using the "High: Select Analytes" formula. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping. Due to the distributions not being similarly shaped, the p-value presented was an asymptotic value and not an exact *p*-value.

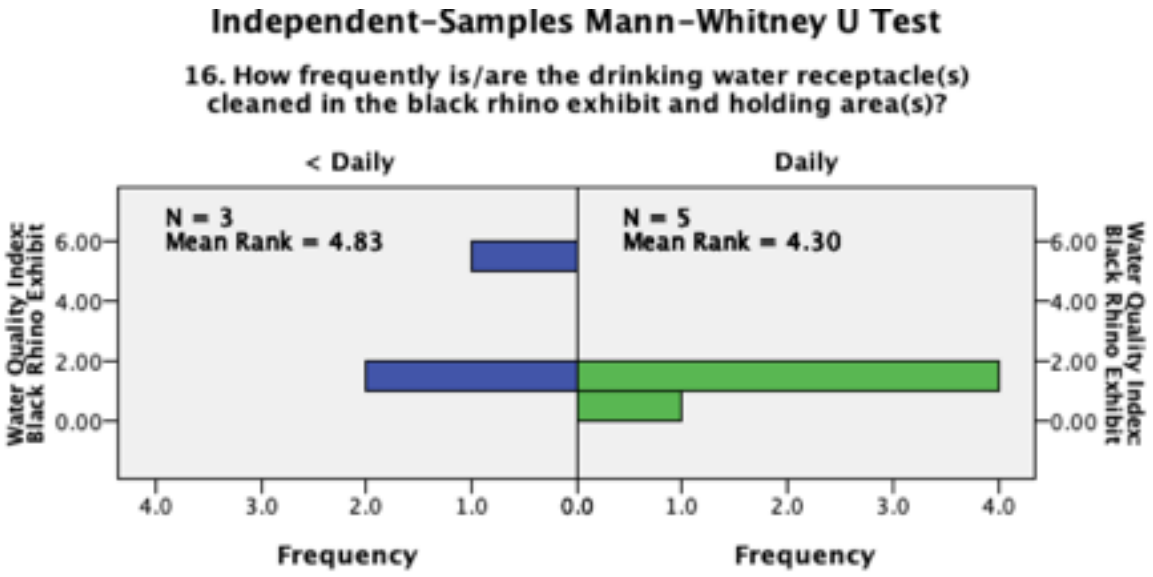


**Figure F.29.** Pyramid chart showing the distribution of zoos that cleaned the drinking water receptacles provided to the black rhino daily or less than daily. All Water Quality Index (WQI) values are from the Black Rhino exhibit sampling point and were calculated using the "Low: All Analytes" formula. Only Black Rhino zoos are included in this WQI grouping. Due to the distributions not being similarly shaped, the p-value presented was an asymptotic value and not an exact *p*-value.

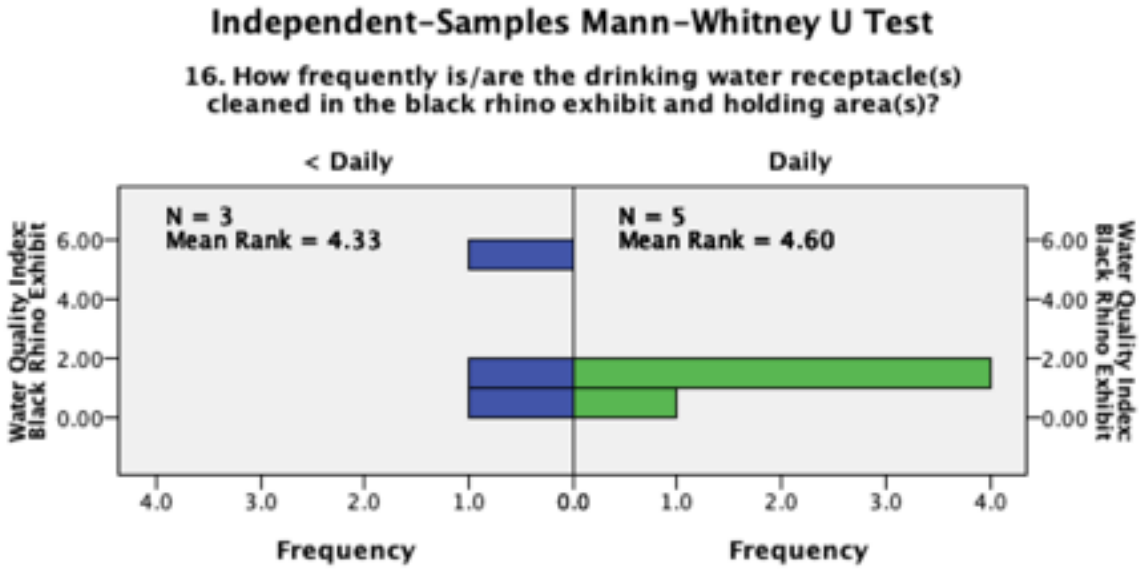


**Figure F.30.** Pyramid chart showing the distribution of zoos that cleaned the drinking water receptacles provided to the black rhino daily or less than daily. All Water Quality Index (WQI) values are from the Black Rhino exhibit sampling point and were calculated using the "Low: Select Analytes" formula. Only Black Rhino zoos are included in this WQI grouping. Due to the distributions not being similarly shaped, the p-value presented was an asymptotic value and not an exact *p*-value.

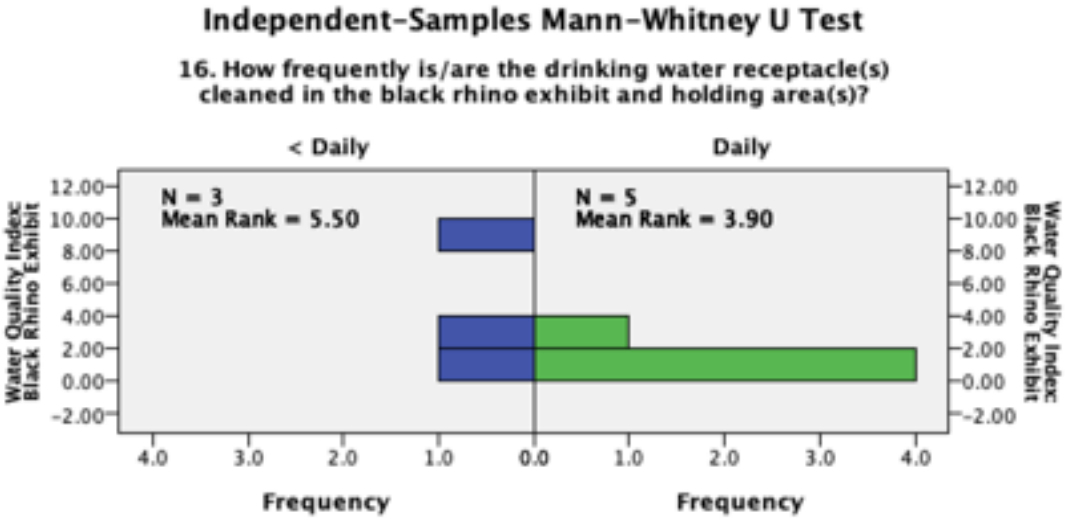


**Figure F.31.** Pyramid chart showing the distribution of zoos that cleaned the drinking water receptacles provided to the black rhino daily or less than daily. All Water Quality Index (WQI) values are from the Black Rhino exhibit sampling point and were calculated using the "High: All Analytes" formula. Only Black Rhino zoos are included in this WQI grouping. Due to the distributions not being similarly shaped, the p-value presented was an asymptotic value and not an exact *p*-value.

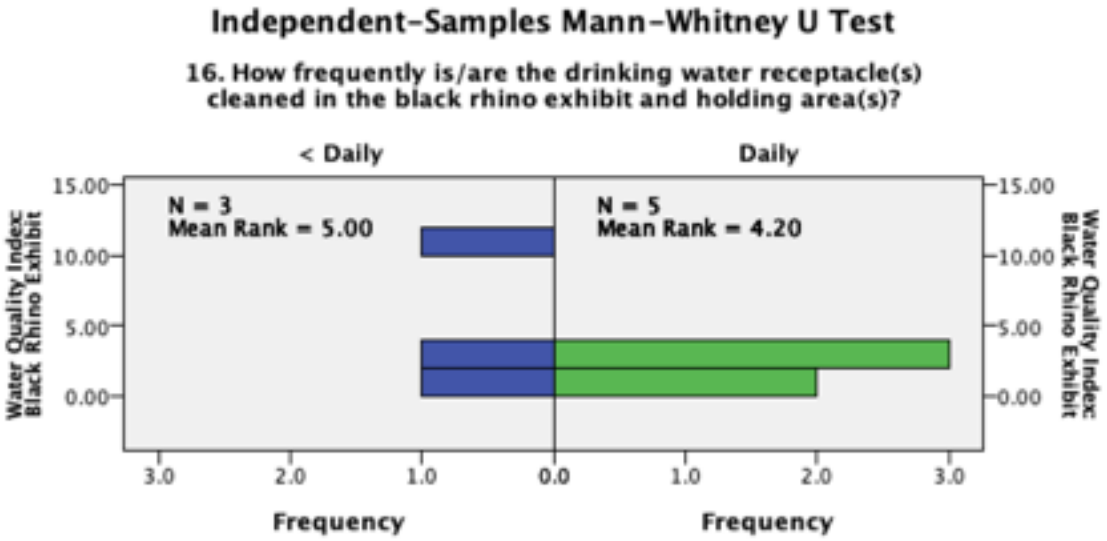


**Figure F.32.** Pyramid chart showing the distribution of zoos that cleaned the drinking water receptacles provided to the black rhino daily or less than daily. All Water Quality Index (WQI) values are from the Black Rhino exhibit sampling point and were calculated using the "High: Select Analytes" formula. Only Black Rhino zoos are included in this WQI grouping. Due to the distributions not being similarly shaped, the p-value presented was an asymptotic value and not an exact *p*-value.

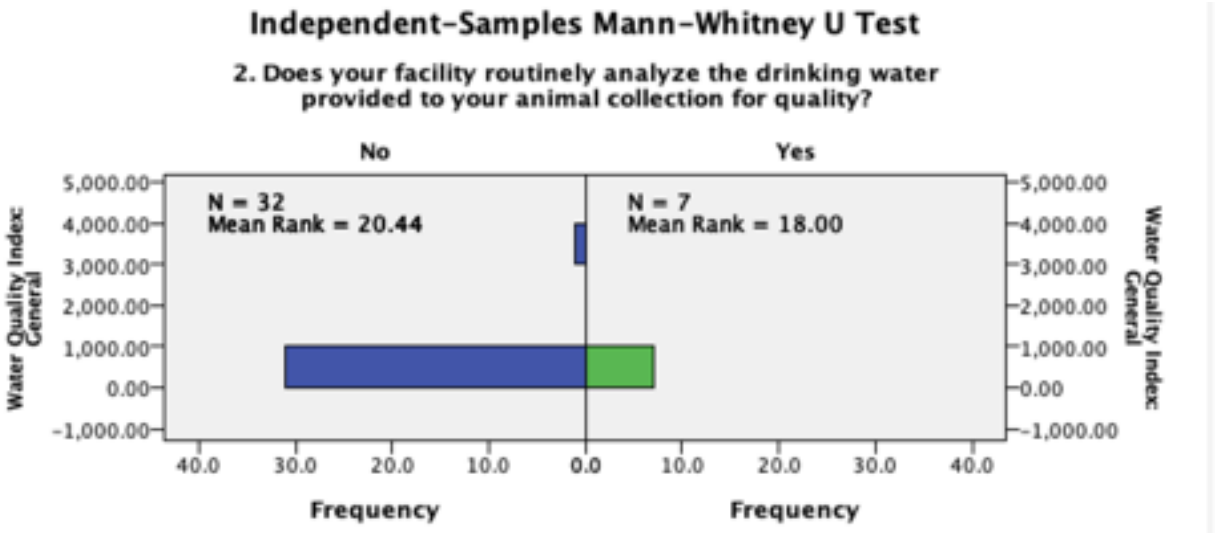


**Figure F.33.** Pyramid chart showing the distribution of zoos that did and did not routinely analyze the drinking water provided to their animal collections. All Water Quality Index (WQI) values are from the origin sampling point and were calculated using the "Low: All Analytes" formula. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping. Due to the distributions not being similarly shaped, the p-value presented was an asymptotic value and not an exact *p*-value.

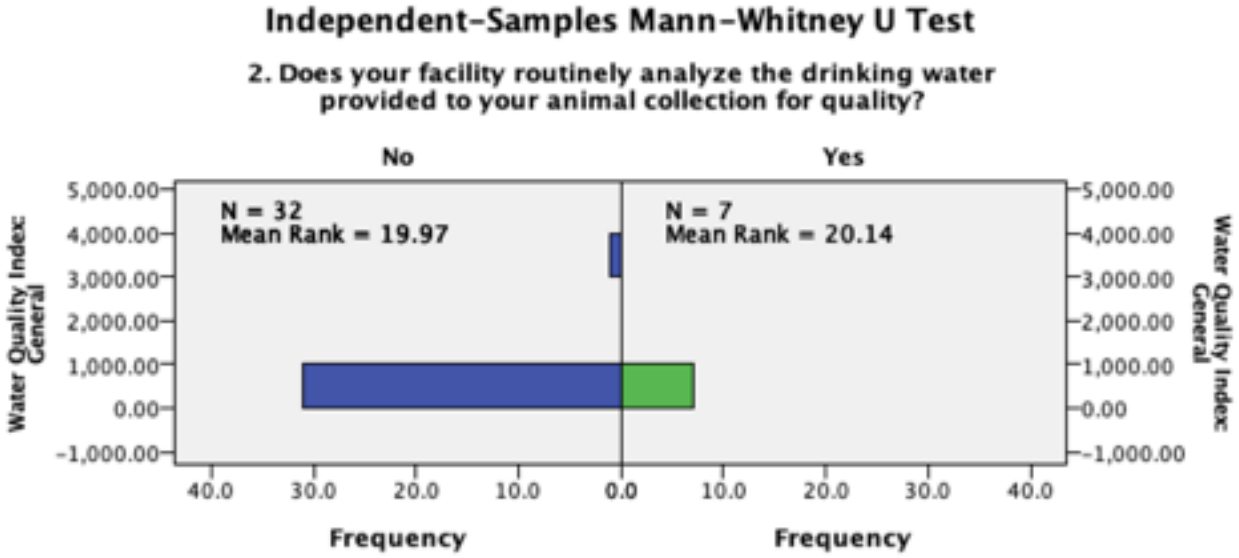


**Figure F.34.** Pyramid chart showing the distribution of zoos that did and did not routinely analyze the drinking water provided to their animal collections. All Water Quality Index (WQI) values are from the origin sampling point and were calculated using the "Low: Select Analytes" formula. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping. Due to the distributions not being similarly shaped, the p-value presented was an asymptotic value and not an exact *p*-value.

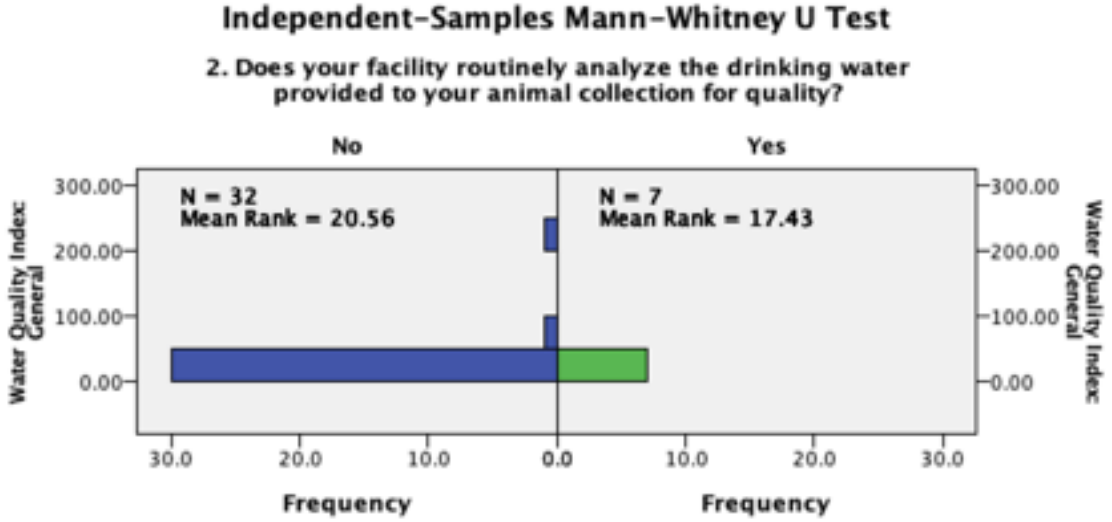


**Figure F.35.** Pyramid chart showing the distribution of zoos that did and did not routinely analyze the drinking water provided to their animal collections. All Water Quality Index (WQI) values are from the origin sampling point and were calculated using the "High: All Analytes" formula. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping. Due to the distributions not being similarly shaped, the p-value presented was an asymptotic value and not an exact *p*-value.

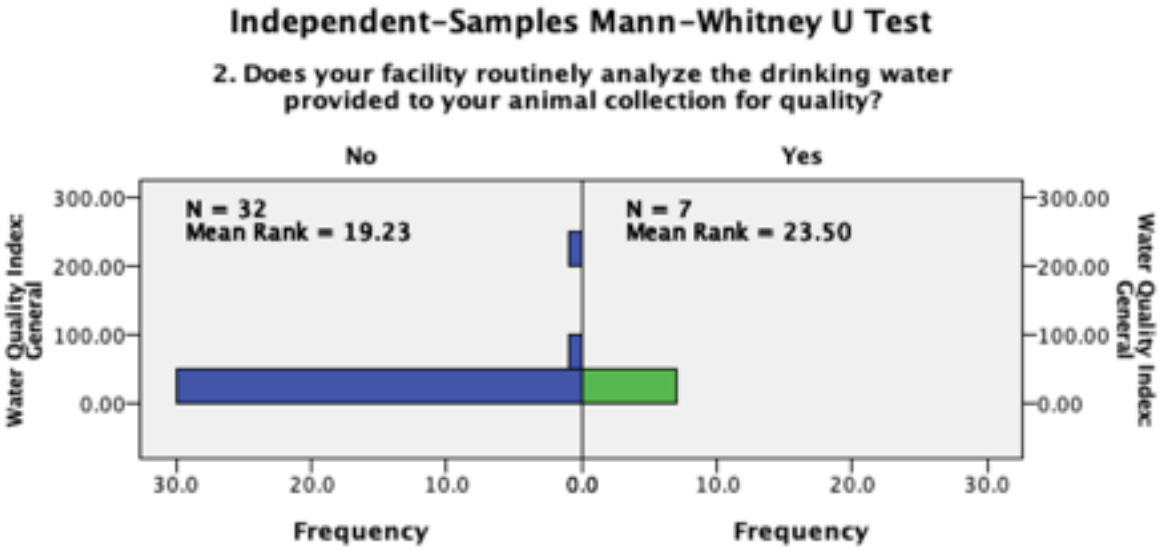


**Figure F.36.** Pyramid chart showing the distribution of zoos that did and did not routinely analyze the drinking water provided to their animal collections. All Water Quality Index (WQI) values are from the origin sampling point and were calculated using the "High: Select Analytes" formula. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping. Due to the distributions not being similarly shaped, the p-value presented was an asymptotic value and not an exact *p*-value.

**Table F.1**. Analytes included in each of the four Water Quality Index (WQI) calculations performed for each participating zoo. The four WQI calculations were as follows 1) Low all analytes, 2) Low select analyte, 3) High all analytes, and 4) High select analytes.

| WQI Calculations | | | |
|---|---|---|---|
| Low | | High | |
| All Analytes | Select Analytes | All Analytes | Select Analytes |
| pH | pH | pH | pH |
| $NO_3^-$ | $Cl^-$ | $NO_3^-$ | $Cl^-$ |
| $Cl^-$ | $SO_4$ | $Cl^-$ | $SO_4$ |
| $SO_4$ | Ca | $SO_4$ | Ca |
| TDS | Fe | TDS | Fe |
| Hardness | Mn | Hardness | Mn |
| Ca | Na | Ca | Na |
| Cu | | Cu | |
| Fe | | Fe | |
| Mn | | Mn | |
| Na | | Na | |
| Zn | | Zn | |

**Table F.2.** List of low and high standards used in the calculation of Water Quality Index (WQI) values for each analyte included in the WQI calculations. Phosphorus and magnesium do not have a standard value and were not included in any of the WQI calculations nor statistical analysis.

| Analytes | Standard | |
|---|---|---|
| | Low | High |
| pH | 6.5 | 8.5 |
| Nitrate as Nitrogen (mg/L) | 10 | 10 |
| Nitrate as $NO_{3}^{-}$ (mg/L) | 45 | 50 |
| Hardness (mg/L) | 100 | 300 |
| Total Dissolved Solids (mg/L) | 500 | 600 |
| Chloride (mg/L) | 250 | 250 |
| Sulfate (mg/L) | 250 | 500 |
| Calcium (mg/L) | 100 | 300 |
| Phosphorus (mg/L) | 0.01 | 0.01 |
| Magnesium (mg/L) | — | — |
| Potassium (mg/L) | — | — |
| Sodium (mg/L) | 20 | 200 |
| Iron (mg/L) | 0.2 | 0.3 |
| Manganese (mg/L) | 0.05 | 0.4 |
| Zinc (mg/L) | 3.0 | 5.0 |
| Copper (mg/L) | 1.0 | 2.0 |
| Total Coliform | 0 | 0 |

**Table F.3.** Origin point Water Quality Index (WQI) values for all zoos (Non-Black Rhino and Black Rhino) and all four analytes. Ranked in order of highest to lowest WQI value for the Low: All Analytes formula.
(a) indicates a WQI value greater than or equal to 2.0, the highest 50th percentile value across all analyte formulas.
(b) indicates a WQI value greater than or equal to 13.2, the highest 90th percentile value across all analyte formulas.

| Zoo ID | WQI Calculations Origin Point | | | |
|---|---|---|---|---|
| | Low | | High | |
| | All Analytes | Select Analytes | All Analytes | Select Analytes |
| 1101 | 3358.2[b] | 3553.3[b] | 207.9[b] | 236.3[b] |
| 1012 | 195.8[b] | 207.0[b] | 38.9[b] | 44.1[b] |
| 1038 | 46.9[b] | 48.6[b] | 81.2[b] | 91.2[b] |
| 1026 | 7.5[a] | 7.9[a] | 13.1[a] | 14.8[b] |
| 1007 | 7.4[a] | 7.6[a] | 11.5[a] | 12.9[a] |
| 2004 | 7.3[a] | 7.7[a] | 11.6[a] | 13.2[b] |
| 1052 | 3.2[a] | 1.2 | 3.9[a] | 1.8 |
| 1136 | 2.9[a] | 0.9 | 3.6[a] | 1.7 |
| 1089 | 2.7[a] | 1.3 | 3.0[a] | 1.9 |
| 1004 | 2.4[a] | 1.1 | 2.8[a] | 1.7 |
| 1105 | 2.2[a] | 1.4 | 2.4[a] | 1.9 |
| 1109 | 2.2[a] | 0.7 | 2.9[a] | 1.6 |
| 1037 | 1.8 | 1.8 | 2.0[a] | 2.1[a] |
| 1096 | 1.8 | 1.7 | 2.0[a] | 1.8 |

**Table F.3.** (cont'd)

| Zoo ID | WQI Calculations Origin Point | | | |
|---|---|---|---|---|
| | Low | | High | |
| | All Analytes | Select Analytes | All Analytes | Select Analytes |
| 1017 | 1.6 | 1.5 | 2.0[a] | 1.9 |
| 1042 | 1.6 | 1.2 | 2.6[a] | 1.8 |
| 1029 | 1.5 | 1.4 | 2.0[a] | 1.8 |
| 1057 | 1.5 | 1.3 | 2.0[a] | 1.9 |
| 2016 | 1.5 | 1.4 | 2.4[a] | 2.2[a] |
| 2021 | 1.5 | 1.6 | 1.8 | 2.1[a] |
| 1128 | 1.4 | 1.3 | 1.9 | 1.8 |
| 1016 | 1.2 | 1.0 | 2.0[a] | 1.7 |
| 1084 | 1.2 | 1.1 | 1.7 | 1.8 |
| 1107 | 1.2 | 1.0 | 2.8[a] | 2.1[a] |
| 1126 | 1.2 | 0.8 | 2.7[a] | 1.7 |
| 1139 | 1.2 | 1.0 | 2.3[a] | 1.8 |
| 1021 | 1.1 | 1.1 | 1.8 | 2.1[a] |
| 1045 | 1.1 | 1.2 | 1.8 | 2.0[a] |
| 1068 | 1.1 | 1.0 | 2.0[a] | 2.0[a] |
| 1116 | 1.1 | 1.1 | 1.9 | 2.0[a] |
| 1130 | 1.1 | 0.9 | 1.8 | 1.7 |
| 1132 | 1.1 | 1.2 | 1.8 | 2.1[a] |

**Table F.3.** (cont'd)

| Zoo ID | WQI Calculations Origin Point | | | |
|---|---|---|---|---|
| | Low | | High | |
| | All Analytes | Select Analytes | All Analytes | Select Analytes |
| 1143 | 1.1 | 1.0 | 2.0[a] | 1.6 |
| 2003 | 1.1 | 1.0 | 2.0[a] | 2.1[a] |
| 1019 | 1.0 | 1.0 | 1.6 | 1.7 |
| 1034 | 1.0 | 0.8 | 1.6 | 1.6 |
| 1066 | 1.0 | 0.9 | 1.8 | 1.8 |
| 1074 | 1.0 | 0.9 | 1.7 | 1.7 |
| 2008 | 1.0 | 1.0 | 1.9 | 2.0[a] |
| 1036 | 0.9 | 0.9 | 1.8 | 2.0[a] |
| 1091 | 0.9 | 0.9 | 1.6 | 1.7 |
| 1131 | 0.9 | 0.9 | 1.7 | 1.7 |
| 2019 | 0.9 | 0.7 | 1.6 | 1.6 |
| 1035 | 0.8 | 0.8 | 1.6 | 1.8 |
| 1127 | 0.8 | 0.8 | 1.6 | 1.7 |
| 2011 | 0.8 | 0.8 | 1.5 | 1.7 |
| 2012 | 0.8 | 0.8 | 1.7 | 1.8 |
| 2020 | 0.8 | 0.8 | 1.6 | 1.8 |

**Table F.3.** (cont'd)

| Zoo ID | WQI Calculations Origin Point | | | |
|---|---|---|---|---|
| | Low | | High | |
| | All Analytes | Select Analytes | All Analytes | Select Analytes |
| 1069 | 0.7 | 0.8 | 1.5 | 1.7 |
| 2006 | 0.7 | 0.8 | 1.5 | 1.7 |

**Table F.4.** Exhibit Water Quality Index (WQI) values for all Black Rhino zoos and all four analytes. Ranked in order of highest to lowest WQI value for the Low: All Analytes formula.
(a) indicates a WQI value greater than or equal to 2.0, the highest 50th percentile value across all analyte formulas.
(b) indicates a WQI value greater than or equal to 10.2, the highest 90th percentile value across all analyte formulas.

| Zoo ID | WQI Calculations Black Rhino Exhibit | | | |
|---|---|---|---|---|
| | Low | | High | |
| | All Analytes | Select Analytes | All Analytes | Select Analytes |
| 2020 | 5.5[a] | 5.8[a] | 9.7[a] | 11.1[b] |
| 2004 | 1.7 | 1.8 | 1.8 | 2.0[a] |
| 2016 | 1.5 | 1.4 | 2.4[a] | 2.3[a] |
| 2021 | 1.4 | 1.5 | 1.9 | 2.0[a] |
| 2011 | 1.1 | 0.8 | 1.8 | 1.7 |
| 2003 | 1.0 | 1.0 | 2.0[a] | 2.1[a] |

**Table F.4.** (cont'd)

| Zoo ID | WQI Calculations Black Rhino Exhibit | | | |
|---|---|---|---|---|
| | Low | | High | |
| | All Analytes | Select Analytes | All Analytes | Select Analytes |
| 2008 | 1.0 | 1.0 | 1.9 | 1.9 |
| 2012 | 0.8 | 0.8 | 1.6 | 1.8 |
| 2006 | 0.7 | 0.7 | 1.5 | 1.7 |
| 2019 | 0.7 | 0.7 | 1.5 | 1.7 |

**Table F.5.** Difference in drinking water quality between the origin and Black Rhino exhibit sampling points, as shown by a change in calculated Water Quality Index (WQI) value between the two points across all four analyte formulas. The Difference was calculated by subtracting the Black Rhino exhibit WQI value from the origin WQI value (Origin-Black Rhino Exhibit=Difference).
(a) Indicates a decrease in drinking water quality from the origin to the Black Rhino exhibit sample points. Negative zero (-0.0) being possible due to the rounding of minor changes between water quality at the two sampling points (e.g., -0.0067 rounding down to -0.0).

| Zoo ID | WQI Values | | |
|---|---|---|---|
| | Low: All Analytes | | |
| | Origin | Exhibit | Difference |
| 2003 | 1.1 | 1.0 | 0.0 |
| 2004 | 7.3 | 1.7 | 5.6 |
| 2006 | 0.7 | 0.7 | 0.0 |
| 2008 | 1.0 | 1.0 | 0.0 |
| 2011 | 0.8 | 1.1 | -0.3 [a] |
| 2012 | 0.8 | 0.8 | 0.0 |
| 2016 | 1.5 | 1.5 | -0.0 [a] |
| 2019 | 0.9 | 0.7 | 0.1 |
| 2020 | 0.8 | 5.5 | -4.7 [a] |
| 2021 | 1.5 | 1.4 | 0.1 |
| | Low: Select Analytes | | |
| | Origin | Exhibit | Difference |
| 2003 | 1.0 | 1.0 | 0.0 |
| 2004 | 7.7 | 1.8 | 5.9 |
| 2006 | 0.8 | 0.7 | 0.0 |
| 2008 | 1.0 | 1.0 | 0.0 |
| 2011 | 0.8 | 0.8 | 0.0 |
| 2012 | 0.8 | 0.8 | -0.0 [a] |

**Table F.5.** (cont'd)

| | Low: Select Analytes (cont'd) | | |
|---|---|---|---|
| | Origin | Exhibit | Difference |
| 2016 | 1.4 | 1.4 | -0.0 [a] |
| 2019 | 0.7 | 0.7 | -0.0 [a] |
| 2020 | 0.8 | 5.8 | -4.9 [a] |
| 2021 | 1.6 | 1.5 | 0.1 |
| | High: All Analytes | | |
| | Origin | Exhibit | Difference |
| 2003 | 2.0 | 2.0 | 0.0 |
| 2004 | 11.6 | 1.8 | 9.8 |
| 2006 | 1.5 | 1.5 | 0.0 |
| 2008 | 1.9 | 1.9 | 0.0 |
| 2011 | 1.5 | 1.8 | -0.3 [a] |
| 2012 | 1.7 | 1.6 | 0.1 |
| 2016 | 2.4 | 2.4 | -0.0 [a] |
| 2019 | 1.6 | 1.5 | 0.1 |
| 2020 | 1.6 | 9.7 | -8.2 [a] |
| 2021 | 1.8 | 1.9 | -0.0 [a] |
| | High: Select Analytes | | |
| | Origin | Exhibit | Difference |
| 2003 | 2.1 | 2.1 | 0.0 |
| 2004 | 13.2 | 2.0 | 11.2 |
| 2006 | 1.7 | 1.7 | 0.0 |

**Table F.5.** (cont'd)

| | High: Select Analytes (cont'd) | | |
|---|---|---|---|
| | Origin | Exhibit | Difference |
| 2008 | 2.0 | 1.9 | 0.0 |
| 2011 | 1.7 | 1.7 | 0.0 |
| 2012 | 1.8 | 1.8 | -0.0[a] |
| 2016 | 2.2 | 2.3 | -0.0 [a] |
| 2019 | 1.6 | 1.7 | -0.0 [a] |
| 2020 | 1.8 | 11.1 | -9.3 [a] |
| 2021 | 2.1 | 2.0 | 0.1 |

**Table F.6.** Measures of Central Tendency for Non-Black Rhino zoo Water Quality Index (WQI) values at the origin sampling point calculated using the four different analyte formulas.

| | WQI: Origin | | | |
|---|---|---|---|---|
| | Low | | High | |
| | All Analytes | Select Analytes | All Analytes | Select Analytes |
| *N* | 40 | 40 | 40 | 40 |
| Mean | 91.6 | 96.6 | 10.7 | 11.6 |
| Median | 1.2 | 1.1 | 2.0 | 1.8 |
| Mode | 1.1 | 0.9 | 2.0 | 1.7 |
| Std. Deviation | 530.7 | 561.6 | 34.8 | 39.6 |
| Min | 0.7 | 0.7 | 1.5 | 1.6 |
| Max | 3358.2 | 3553.3 | 207.9 | 236.3 |

**Table F.7.** Percentiles for Water Quality Index (WQI) values at the origin point for Non-Black Rhino zoos, calculated using the four different analyte formulas.

| | WQI: Origin | | | |
|---|---|---|---|---|
| | Low | | High | |
| | All Analytes | Select Analytes | All Analytes | Select Analytes |
| *N* | 40 | 40 | 40 | 40 |
| Percentiles | | | | |
| 10th | 0.9 | 0.8 | 1.6 | 1.7 |
| 50th | 1.2 | 1.1 | 2.0 | 1.8 |
| 90th | 7.5 | 7.9 | 12.9 | 14.6 |

**Table F.8.** Measures of Central Tendency for Black Rhino zoo Water Quality Index (WQI) values at the origin sampling point calculated using the four different analyte formulas.
(a) Multiple modes exist for the data; smallest value shown.

| | WQI: Origin | | | |
|---|---|---|---|---|
| | Low | | High | |
| | All Analytes | Select Analytes | All Analytes | Select Analytes |
| *N* | 10 | 10 | 10 | 10 |
| Mean | 1.6 | 1.7 | 2.8 | 3.0 |
| Median | 1.0 | 0.9 | 1.8 | 1.9 |
| Mode | 0.8 | 0.8 | 1.5[a] | 1.7[a] |
| Std. Deviation | 2.0 | 2.1 | 3.1 | 3.6 |
| Min | 0.7 | 0.7 | 1.5 | 1.6 |
| Max | 7.3 | 7.7 | 11.6 | 13.2 |

**Table F.9.** Measures of Central Tendency for Black Rhino zoo Water Quality Index (WQI) values within the Black Rhino exhibit calculated using the four different analyte formulas.
(a) Multiple modes exist for the data; smallest value shown.

| | WQI: Black Rhino Exhibit | | | |
|---|---|---|---|---|
| | Low | | High | |
| | All Analytes | Select Analytes | All Analytes | Select Analytes |
| *N* | 10 | 10 | 10 | 10 |
| Mean | 1.5 | 1.6 | 2.6 | 2.8 |
| Median | 1.1 | 1.0 | 1.9 | 2.0 |
| Mode | 0.7[a] | 0.7[a] | 1.5[a] | 1.7 |
| Std. Deviation | 1.4 | 1.5 | 2.5 | 2.9 |
| Min | 0.7 | 0.7 | 1.5 | 1.7 |
| Max | 5.5 | 5.8 | 9.7 | 11.1 |

**Table F.10.** Percentiles for Black Rhino zoo Water Quality Index (WQI) values at the origin sampling point calculated using the four different analyte formulas.

| | WQI: Origin | | | |
|---|---|---|---|---|
| | Low | | High | |
| | All Analytes | Select Analytes | All Analytes | Select Analytes |
| *N* | 10 | 10 | 10 | 10 |
| Percentiles | | | | |
| 10th | 0.7 | 0.7 | 1.5 | 1.7 |
| 50th | 1.0 | 0.9 | 1.8 | 2.0 |
| 90th | 6.7 | 7.1 | 10.7 | 12.1 |

**Table F.11.** Percentiles for Black Rhino zoo Water Quality Index (WQI) values within the Black Rhino exhibit calculated using the four different analyte formulas.

| | WQI: Black Rhino Exhibit | | | |
|---|---|---|---|---|
| | Low | | High | |
| | All Analytes | Select Analytes | All Analytes | Select Analytes |
| *N* | 10 | 10 | 10 | 10 |
| Percentiles | | | | |
| 10th | 0.7 | 0.7 | 1.5 | 1.7 |
| 50th | 1.1 | 1.0 | 1.9 | 2.0 |
| 90th | 5.1 | 5.4 | 9.0 | 10.2 |

**Table F.12.:** Sign Test summary table for the water quality difference between the origin and Black Rhino exhibit sampling points for Black Rhino zoos calculated using the four different analyte formulas. The Difference was calculated by subtracting the Black Rhino exhibit Water Quality Index (WQI) value from the origin WQI value (Origin-Black Rhino Exhibit=Difference).

| WQI Difference Between the Origin and Black Rhino Exhibit | | | | |
|---|---|---|---|---|
| | Low | | High | |
| | All Analytes | Select Analytes | All Analytes | Select Analytes |
| *P* | 0.688 | 0.625 | 1.000 | 1.000 |
| *Effect Size* | 0.13 | 0.16 | 0.00 | 0.00 |
| Origin Median | 0.95 | 0.90 | 1.75 | 1.90 |
| Exhibit Median | 1.05 | 1.00 | 1.85 | 1.95 |
| Difference Median | 0.00 | 0.00 | 0.00 | 0.00 |

**Table F.13.:** Sign Test summary table for the sign change of the drinking water quality between the origin and Black Rhino exhibit sampling points for Black Rhino zoos calculated using the four different analyte formulas. The change in sign indicates whether the water quality is worse at the origin point (+), worse within the Black Rhino exhibit (-), or whether there was no change in water quality between the two sampling points.

| Water Quality Index (WQI) Change Between the Origin and Black Rhino Exhibit | | | | |
|---|---|---|---|---|
| | Low | | High | |
| | All Analytes | Select Analytes | All Analytes | Select Analytes |
| Positive (+) | 4 | 3 | 3 | 3 |
| Negative (-) | 2 | 1 | 3 | 3 |
| No Change | 4 | 6 | 4 | 4 |

**Table F.14.:** Kruskal-Wallis H Test summary table for the effect zoo age has on the water quality difference between the origin point and Black Rhino exhibit sampling points for Black Rhino zoos calculated using the four different analyte formulas. The change in drinking water quality between the origin point and Black Rhino exhibit decreases as the mean rank value increases; meaning the quality of the drinking water is lower as the mean rank increases.

| Zoo Age Effect on the Water Quality Index (WQI) Difference Between the Origin and Black Rhino Exhibit | | | | |
|---|---|---|---|---|
| | Low | | High | |
| | All Analytes | Select Analytes | All Analytes | Select Analytes |
| *P* | 0.314 | 0.248 | 0.088 | 0.248 |
| $\chi 2$ | 2.318 | 2.789 | 4.860 | 2.789 |
| *Effect Size* | 0.13 | 0.10 | 0.10 | 0.10 |
| ≤ 50 years Mean Rank | 6.00 | 6.00 | 7.50 | 6.00 |
| 51-100 years Mean Rank | 3.75 | 2.50 | 2.75 | 2.50 |
| ≥ 100 years Mean Rank | 4.13 | 4.75 | 3.88 | 4.75 |

**Table F.15.** Summary table showing the p-value, Mann-Whitney U statistic, z-value, effect size, and mean ranks for the difference in Water Quality Index (WQI) values sampled at the origin point for zoos that did and did not use municipal water as their primary drinking water source. The WQI values for all four analyte formulas are shown in the table. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping.

| Difference in WQI Origin Between Zoos That Did and Did not Use a Municipal Water Source | | | | |
|---|---|---|---|---|
| | Low | | High | |
| | All Analytes | Select Analytes | All Analytes | Select Analytes |
| *P* | 0.027 | 0.004 | 0.126 | 0.009 |
| *U* | 13.50 | 5.00 | 24.00 | 8.00 |
| *z* | -2.144 | -2.599 | -1.591 | -2.460 |
| Effect Size | -0.34 | -0.42 | -0.25 | -0.39 |
| Mean Rank for zoos that do use Municipal water | 18.88 | 18.64 | 19.17 | 18.72 |
| Mean Rank for zoos that do not use Municipal water | 33.50 | 36.33 | 30.00 | 35.33 |

**Table F.16.** Summary table showing the p-value, Mann-Whitney U statistic, z-value, effect size, and mean ranks for the difference in WQI values sampled at the origin point for zoos that did and did not use well (bore) water as their primary drinking water source. The Water Quality Index (WQI) values for all four analyte formulas are shown in the table. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping.

| Difference in WQI Origin Between Zoos That Did and Did not Use a Well(bore) Water Source | | | | |
|---|---|---|---|---|
| | Low | | High | |
| | All Analytes | Select Analytes | All Analytes | Select Analytes |
| *P* | 0.526 | 0.314 | 0.823 | 0.192 |
| *U* | 100.5 | 110.0 | 90.50 | 117.0 |
| *z* | 0.654 | 1.057 | 0.232 | 1.364 |
| Effect Size | 0.10 | 0.17 | 0.04 | 0.22 |
| Mean Rank for zoos that do use Well(bore) water | 23.10 | 25.00 | 21.10 | 26.40 |
| Mean Rank for zoos that do not use Well(bore) water | 19.54 | 19.26 | 19.84 | 19.06 |

**Table F.17.** Summary table showing the p-value, Mann-Whitney U statistic, z-value, effect size, and mean ranks for the difference in Water Quality Index (WQI) values sampled at the origin point for zoos that did and did not use river water as their primary drinking water source. The WQI values for all four analyte formulas are shown in the table. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping.

| Difference in WQI Origin Between Zoos That Did and Did not Use a River Water Source | | | | |
|---|---|---|---|---|
| | Low | | High | |
| | All Analytes | Select Analytes | All Analytes | Select Analytes |
| *P* | 0.564 | 0.821 | 0.356 | 0.410 |
| *U* | 10.00 | 15.50 | 4.50 | 7.50 |
| *z* | -0.803 | 0.00 | -1.296 | -1.037 |
| Effect Size | -0.13 | -0.05 | -0.21 | -0.17 |
| Mean Rank for zoos that do use River water | 11.00 | 16.50 | 5.50 | 8.50 |
| Mean Rank for zoos that do not use River water | 20.24 | 20.09 | 20.38 | 20.30 |

**Table F.18.** Summary table showing the P-value, Kruskal-Wallis H statistic reported as the $X_2$, effect size, and mean ranks for the different zoo size categories reported in numbers of species. The p-value reported is asymptotic and not exact. The Water Quality Index (WQI) values for all four analyte formulas are shown in the table. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping.

| Zoo Size Effect on the WQI at the Origin Point | | | | |
|---|---|---|---|---|
| | Low | | High | |
| | All Analytes | Select Analytes | All Analytes | Select Analytes |
| *P* | 0.265 | 0.189 | 0.365 | 0.316 |
| $X_2$ | 2.655 | 3.336 | 2.014 | 2.302 |
| Effect Size | 0.10 | 0.10 | 0.10 | 0.10 |
| ≤ 100 Species Mean Rank | 18.73 | 16.32 | 18.64 | 16.14 |
| 101-200 Species Mean Rank | 24.12 | 24.46 | 23.62 | 23.12 |
| > 200 Species Mean Rank | 17.37 | 18.83 | 17.87 | 20.13 |

**Table F.19.** Summary table showing the p-value, Mann-Whitney U statistic, z-value, and effect size for the difference in Water Quality Index (WQI) values between the origin point and the Black Rhino exhibit for zoos that have replaced their drinking water pipes within the last 5 years versus zoos that have not replace their drinking water pipes with the last 5 years. The WQI values for all four analyte formulas are shown in the table. Only Black Rhino zoos are included in this WQI grouping.

| WQI Difference Between Zoos That Had and Had Not Replaced Drinking Water Pipes | | | | |
|---|---|---|---|---|
| | Low | | High | |
| | All Analytes | Select Analytes | All Analytes | Select Analytes |
| *P* | 0.114 | 0.200 | 0.114 | 0.200 |
| *U* | 2.0 | 3.0 | 2.0 | 3.0 |
| *z* | -1.845 | -1.654 | -1.845 | -1.654 |
| Effect Size | -0.65 | -0.58 | -0.65 | -0.85 |

**Table F.20.** Summary table showing the p-value, Mann-Whitney U statistic, z-value, and effect size for the difference in Water Quality Index (WQI) values sampled within the Black Rhino exhibit for zoos that cleaned the drinking water receptacles provided to the black rhino daily or less than daily. The WQI values for all four analyte formulas are shown in the table. Only Black Rhino zoos are included in this WQI grouping.

| Drinking Water Receptacle Cleaning Frequency Effect on WQI in Black Rhino Zoos | | | | |
|---|---|---|---|---|
| | Low | | High | |
| | All Analytes | Select Analytes | All Analytes | Select Analytes |
| *P* | 0.786 | 1.00 | 0.393 | 0.786 |
| *U* | 8.5 | 7.0 | 10.5 | 9.0 |
| *z* | 0.300 | -0.151 | 0.905 | 0.45 |
| Effect Size | 0.11 | -0.05 | 0.32 | 0.16 |

**Table F.21.** Summary table showing the p-value, Mann-Whitney U statistic, z-value, and effect size for the difference in Water Quality Index (WQI) values sampled at the origin point for zoos that did and did not routinely analyze the drinking water provided to their animal collections. The WQI values for all four analyte formulas are shown in the table. Both Black Rhino and Non-Black Rhino zoos are included in this WQI grouping.

| Difference in WQI Origin Between Zoos That Did and Did not Routinely Analyze the Drinking Water | | | | |
|---|---|---|---|---|
| | Low | | High | |
| | All Analytes | Select Analytes | All Analytes | Select Analytes |
| *P* | 0.629 | 1.00 | 0.530 | 0.378 |
| *U* | 98.0 | 113.0 | 94.0 | 136.5 |
| *z* | -0.515 | 0.037 | -0.663 | 0.910 |
| Effect Size | -0.08 | 0.01 | -0.11 | 0.15 |

## LITERATURE CITED

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Addiscott, T. M., & Benjamin, N. (2004). Nitrate and human health. *Soil Use and Management, 20*(2), 98–104. https://doi.org/10.1079/Sum2004256

Akter, T., Jhohura, F. T., Akter, F., Chowdhury, T. R., Mistry, S. K., Dey, D., Milan, K.B., Islam, M.A., & Rahman, M. (2016). Water Quality Index for measuring drinking water quality in rural Bangladesh: A cross-sectional study. *Journal of Health, Population and Nutrition, 35*(4), 1–12. https://doi.org/10.1186/s41043-016-0041-5

Alberts, B., Bray, D., Hopkin, K., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2013). *Essential Cell Biology* (4th ed.). New York, NY: Garland Science.

Batal, A., Fairchild, B., Ritz, C., & Vendrell, P. (2005). The effect of water manganese on broiler performance. *Poultry Science, 84*(Supplement 1), 13–14.

Beede, D. K. (1991). Mineral and water nutrition. *Veterinary Clinics of North America-Food Animal Practice, 7*(2), 373–390.

Beede, D. K. (2005). The most essential essential nutrient: water. In *Western Dairy Management Conference* (p. 22). Reno, Nevada.

Beede, D. K. (2009). Solving bad water problems for thirsty cows. In *Western Dairy Management Conference* (p. 9). Reno, Nevada.

Beutler, E., West, C., Speir, J. A., Wilson, I. A., & Worley, M. (2001). The HFE gene of browsing and grazing rhinoceroses: a possible site of adaptation to a low-iron diet. *Blood Cells, Molecules, and Diseases, 27*(1), 342–350. https://doi.org/10.1006/bcmd.2001.0386

Bjørklund, G., Aaseth, J., Skalny, A. V., Suliburska, J., Skalnaya, M. G., Nikonorov, A. A., & Tinkov, A. A. (2017). Interactions of iron with manganese, zinc, chromium, and selenium as related to prophylaxis and treatment of iron deficiency. *Journal of Trace Elements in Medicine and Biology, 41*(8610), 41–53. https://doi.org/10.1016/j.jtemb.2017.02.005

Boyd, C. E. (2015). *Water Quality: An Introduction* (2nd ed.). Switzerland: Springer. Retrieved from http://dx.doi.org/10.1007/978-3-319-17446-4

British Columbia Ministry of Environment. (2017). *Source Drinking Water Quality Guidelines: Guideline Summary*. Victoria, B.C.

Brown, R. M., McClelland, N. I., Deininger, R. A., & O'Connor, M. F. (1973). A water quality index – crashing the psychological barrier. In S. H. Jenkins (Ed.), *Advances in Water Pollution Research* (pp. 787–797). Oxford, U.K.: Pergamon Press Limited. https://doi.org/10.1016/b978-0-08-017005-3.50067-0

Brown, R. M., McClelland, N. I., Deininger, R. A., & Tozer, R. G. (1970). A water quality index-do we dare? *Water Sewage Works, 117*(10), 339–343.

Church, D. C. (1993). *The Ruminant Animal: Digestive Physiology and Nutrition*. (D. C. Church, Ed.) (2nd ed.). Waveland Press.

Clauss, M., Dierenfeld, E., Goff, J., Klasing, K., Koutsos, L., Lavin, S., Livingston, S., Nielsen, B., Schlegel, M., Sullivan, K., Valdes, E., & Ward, A. (2012). IOD in rhinos—nutrition group report: report from the nutrition working group of the international workshop on iron overload disorder in browsing rhinoceros (February 2011). *Journal of Zoo and Wildlife Medicine*, *43*(3), S108–S113. Retrieved from https://www-jstor-org.proxy1.cl.msu.edu/stable/pdf/41681918.pdf?refreqid=excelsior%3A7fd311d700b5b10da56d3acffaab6f98

Clauss, M., & Paglia, D. E. (2012). Iron storage disorders in captive wild mammals: the comparative evidence. *Journal of Zoo and Wildlife Medicine*, *43*(3), S6–S18. Retrieved from https://www.jstor.org/stable/41681907

CNN. (2018). Flint water crisis fast facts. Retrieved March 19, 2019, from https://www.cnn.com/2016/03/04/us/flint-water-crisis-fast-facts/index.html

Costagliola, A., Roperto, F., Benedetto, D., Anastasio, A., Marrone, R., Perillo, A., Russo, V., Papparella, S., & Paciello, O. (2014). Outbreak of fatal nitrate toxicosis associated with consumption of fennels (*Foeniculum vulgare*) in cattle farmed in Campania region (southern Italy). *Environmental Science and Pollution Research*, *21*(9), 6252–6257. https://doi.org/10.1007/s11356-014-2520-9

Cumberland Valley Analytical Services. (2019). Water Evaluation Services. Retrieved March 18, 2019, from https://www.foragelab.com/Lab-Services/Water

Dadone, L. I., Schilz, A., Friedman, S. G., Bredahl, J., Foxworth, S., & Chastain, B. (2016). Training giraffe (*Giraffa camelopardalis reticulata*) for front foot radiographs and hoof care. *Zoo Biology*, *35*(3), 228–236. https://doi.org/10.1002/zoo.21279

Department of Health and Environmental Control. (2013). Drinking water: common water quality problems and their treatment. Columbia, SC: South Carolina Department of Health and Environmental Control. Retrieved from https://scdhec.gov/sites/default/files/Library/CR-006453.pdf

Dierenfeld, E. S. (1996). Rhino SSP Husbandry Manual.

Dinneen, L. C., & Blakesley, B. C. (1973). Algorithm AS 62: A generator for the sampling distribution of the Mann- Whitney U statistic. Applied Statistics, 22(2), 269–273. Retrieved from https://www.jstor.org/stable/2346934

Dorst, J., & Dandelot, P. (1969). *A Field Guide to the Larger Mammals of Africa*. Boston:

Houghton Mifflin Company.

Doster, E., Chislock, M. F., Roberts, J. F., Kottwitz, J. J., & Wilson, A. E. (2014). Recognition of an important water quality issue at zoos: prevalence and potential threat of toxic cyanobacteria. *Journal of Zoo and Wildlife Medicine*, *45*(1), 165–168. Retrieved from https://doi.org/10.1638/2013-0040R1.1

Dryden, G. (2008). *Animal Science Nutrition* (1st ed.). Wallingford, UK: CABI.

Genther, O. N., & Beede, D. K. (2013). Preference and drinking behavior of lactating dairy cows offered water with different concentrations, valences, and sources of iron. *Journal of Dairy Science*, *96*(2), 1164–1176. https://doi.org/10.3168/jds.2012-5877

Gibson, K. M., Nguyen, B. N., Neumann, L. M., Miller, M., Buss, P., Daniels, S., Ahn, M.J., Crandall, K.A., & Pukazhenthi, B. (2019). Gut microbiome differences between wild and captive black rhinoceros - implications for rhino health. *Scientific Reports*, *9*(1), 7570. https://doi.org/10.1038/s41598-019-43875-3

Gray, N. F. (2008). *Drinking Water Quality: Problems and Solutions* (2nd ed.). Cambridge University Press.

Helary, S. F., Shaw, J. A., Brown, D., Clauss, M., & Owen-Smith, N. (2012). Black rhinoceros (*Diceros bicornis*) natural diets: comparing iron levels across season and geographical locations. *Journal of Zoo and Wildlife Medicine*, *43*(3), S48–S54. Retrieved from https://www.jstor.org/stable/41681911

Horton, R. K. (1965). An index-number system for rating water quality. In *J. Water Pollu. Cont. Fed.* (Vol. 37, pp. 300–305). Retrieved from https://www.jstor.org/stable/25035251

Huuskonen, A., Tuomisto, L., & Kauppinen, R. (2011). Effect of drinking water temperature on water intake and performance of dairy calves. *Journal of Dairy Science*, *94*(5), 2475–2480. https://doi.org/10.3168/jds.2010-3723

IBM Corp. (2017). IBM SPSS statistics for mac. Armonk, NY: IBM Corp.

Johnson, D. L., Ambrose, S. H., Bassett, T. J., Bowen, M. L., Crummey, D. E., Isaacson, J. S., Johnson, D.N., Lamb, P., Saul, M., & Winter-Nelson, A. E. (1997). Meanings of environmental terms. *Journal of Environment Quality*, *26*(3), 581–589. https://doi.org/10.2134/jeq1997.00472425002600030002x

Johnson, S. P., Venn-Watson, S. K., Cassle, S. E., Smith, C. R., Jensen, E. D., & Ridgway, S. H. (2009). Use of phlebotomy treatment in Atlantic bottlenose dolphins with iron overload. *Journal of the American Veterinary Medicinal Association*, *235*(2), 194–200.

Kaneko, J. J., Harvey, J. W., & Bruss, M. L. (2008). *Clinical Biochemistry of Domestic Animals*.

(Jiro Jerry Kaneko, J. W. Harvey, & M. L. Bruss, Eds.) (6th ed.). Amsterdam: Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-370491-7.X0001-3

Kurnit, J. (2009). *Diceros bicornis*. Retrieved March 26, 2019, from https://animaldiversity.org/accounts/Diceros_bicornis/

Laerd Statistics (2015). Statistical tutorials and software guides. Retrieved from https://statistics.laerd.com/

Lardner, H. A., Braul, L., Schwartzkopf-Genswein, K., Schwean-Lardner, K., Damiran, D., & Darambazar, E. (2013). Consumption and drinking behavior of beef cattle offered a choice of several water types. *Livestock Science*, *157*(2–3), 577–585. https://doi.org/10.1016/j.livsci.2013.08.016

Lavin, S. R. (2012). Plant phenolics and their potential role in mitigating iron overload disorder in wild animals. *Journal of Zoo and Wildlife Medicine*, *43*(3), S74–S82. Retrieved from https://www.jstor.org/stable/41681915

Linebach, J. A., Tesch, B. P., & Kovacsiss, L. M. (2014). Nonparametric statistics for applied research. Nonparametric Statistics for Applied Research. https://doi.org/10.1007/978-1-4614-9041-8

Malyszko, J. (2009). Hemojuvelin: the hepcidin story continues. *Kidney Blood Pressure Research*, *32*(2), 71–76. https://doi.org/10.1159/000208988

McKenzie, V. J., Song, S. J., Delsuc, F., Prest, T. L., Oliverio, A. M., Korpita, T. M., Alexiev, A., Amato, K. R., Metcalf, J. L., Kowalewski, M., Avenant, N. L., Link, A., Di Fiore, A., Seguin-Orlando, A., Feh, C., Orlando, L. Mendelson, J. R., Sanders, J., & Knight, R. (2017). The effects of captivity on the mammalian gut microbiome. *Integrative and Comparative Biology*, *57*(4), 690–704. https://doi.org/10.1093/icb/icx090

Muya, S. M., & Oguge, N. O. (2000). Effects of browse availability and quality on black rhino (*Diceros bicornis michaeli* Groves 1967) diet in Nairobi National Park, Kenya. *African Journal of Ecology*, *38*(1), 62–71. https://doi.org/10.1046/j.1365-2028.2000.00213.x

Mylniczenko, N. D., Sullivan, K. E., Corcoran, M. E., Fleming, G. J., & Valdes, E. V. (2012). Management strategies of iron accumulation in a captive population of black rhinoceroses (*Diceros bicornis minor*). *Journal of Zoo and Wildlife Medicine*, *43*(3), S83–S91. Retrieved from https://www.jstor.org/stable/41681916

National Research Council (U.S.). (1974). *Nutrients and toxic substances in water for livestock and poultry: a report*. (T. J. Cunha, J. P. Bowland, C. W. Deyoe, W. H. Hale, J. E. Halver, E. C. Naber, … W. H. Pfander, Eds.). Washington, D.C.: National Academy of Sciences. Retrieved from http://hdl.handle.net/2027/uc1.$b504402

National Research Council (U.S.). (2005). *Mineral tolerance of animals* (2nd rev.). Washington,

D.C.: National Academies Press.

National Research Council (U.S.). (2007). *Nutrient requirements of horses* (6th rev.). Washington, D.C.: National Academies Press.

Nelson, D. L., & Cox, M. M. (2013). *Lehninger principles of biochemistry* (6th ed.). New York, NY: W.H. Freeman and Company.

Paglia, D. E., & Dennis, P. (1999). Role of chronic iron overload in multiple disorders of captive black rhinoceroses (*Diceros bicornis*). In *Proceedings of the American Association of Zoo Veterinarians Annual Meeting* (pp. 163–171).

Pond, W. G., Church, D. C., Pond, K. R., & Schoknecht, P. A. (2005). *Basic Animal Feeding and Nutrition* (5th ed.). New York, NY: Wiley.

Ross, A. C., Caballero, B., Cousins, R. J., Tucker, K. L., & Ziegler, T. R. (2014). *Modern nutrition in health and disease*. (A. C. Ross, B. Caballero, R. J. Cousins, K. L. Tucker, & T. R. Ziegler, Eds.) (11th ed.). Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins.

Shapiro, M. E., Shapiro, H. G., & Ehmke, E. E. (2018). Behavioral responses of three lemur species to different food enrichment devices. *Zoo Biology*, *37*(3), 146–155. https://doi.org/10.1002/zoo.21414

Shim, H., & Dierenfeld, E. S. (2017). Added dietary vegetables and fruits improved coat quality of capybara in Seoul Zoo, Republic of Korea: A case study. *Zoo Biology*, *36*(1), 50–55. https://doi.org/10.1002/zoo.21334

Skinner, J. D., & Chimimba, C. T. (2005). *The Mammals of the Southern African Subregion* (3rd Edition). Cape Town, South Africa: Cambridge University Press. https://doi.org/10.1192/bjp.111.479.1009-a

St. Clair, K. (2019). Nitrite rule for transient water systems. Retrieved March 23, 2019, from https://www.epa.gov/sites/production/files/2018-05/documents/nitrate_and_nitrite_rule_training_presentation.pdf

Stein, L. Y., & Klotz, M. G. (2016). The nitrogen cycle. *Current Biology*, *26*(3), R94–R98. https://doi.org/https://doi.org/10.1016/j.cub.2015.12.021

Taste and Smell. (2012). Retrieved March 27, 2019, from http://www.brainfacts.org/Thinking-Sensing-and-Behaving/Taste/2012/Taste-and-Smell

Tomczak, M., & Tomczak, E. (2014). The need to report effect size estimates revisited. An overview of some recommended measures of effect size. TRENDS in Sport Sciences (Vol. 1).

Tricker, A. R., & Preussmann, R. (1991). Carcinogenic N-nitrosamines in the diet: occurrence, formation, mechanisms and carcinogenic potential. *Mutation Research*, *259*(3–4), 277–289. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/2017213

Tyagi, S., Singh, P., Sharma, B., & Singh, R. (2014). Assessment of water quality for drinking purpose in district Pauri of Uttarakhand, India. *Applied Ecology and Environmental Sciences*, *2*(4), 94–99. https://doi.org/10.12691/aees-2-4-2

U.S. Centers for Disease Control and Prevention. (2014). Safe water system: chlorination. Retrieved March 27, 2019, from https://www.cdc.gov/safewater/chlorine-residual-testing.html

U.S. Environmental Protection Agency. (2004). *Drinking Water Health Advisory for Manganese*. Washington, D.C. https://doi.org/EPA-822-R-04-003

U.S. Environmental Protection Agency. (2015). *Drinking Water Health Advisory for the Cyanobacterial Toxin Cylindrospermopsin*. Washington, D.C. https://doi.org/doi:10.2514/6.1979-763

U.S. Environmental Protection Agency. (2017). Secondary drinking water standards: guidance for nuisance chemicals. Retrieved March 27, 2019, from https://www.epa.gov/dwstandardsregulations/secondary-drinking-water-standards-guidance-nuisance-chemicals

U.S. Environmental Protection Agency. (2018a). *2018 Edition of the Drinking Water Standards and Health Advisories Tables*. Washington, D.C. https://doi.org/EPA 822-S-12-001

U.S. Environmental Protection Agency. (2018b). National Primary Drinking Water Regulations.

U.S. Environmental Protection Agency. (2019a). EPA Drinking Water Health Advisories for Cyanotoxins.

U.S. Environmental Protection Agency. (2019b). Learn about Cyanobacteria and Cyanotoxins.

U.S. Geological Survey. (2016a). Water Hardness and Alkalinity. Retrieved March 27, 2019, from https://water.usgs.gov/owq/hardness-alkalinity.html

U.S. Geological Survey. (2016b). Water Properties: Turbidity. Retrieved March 27, 2019, from https://www.usgs.gov/special-topic/water-science-school/science/turbidity-and-water?qt-science_center_objects=0#qt-science_center_objects

Van Sant, S. (2018). Critically endangered black rhinos die after transportation to Kenya National Park. Retrieved March 23, 2019, from https://www.npr.org/2018/07/13/628862085/critically-endangered-black-rhinos-die-after-transportation-to-kenya-national-pa

Vigil, K. M. (2003). *Clean Water: An Introduction to Water Quality and Water Pollution Control* (2nd ed.). Corvallis, Oregon: Oregon State University Press.

Villar, D., Carson, T. L., Janke, B. H., Pallares, F. J., Frenandez, G., & Kinker, J. A. (2002). Retrospective study of chronic copper poisoning in sheep. *An. Vet. (Murcia)*, *18*, 53–60.

Washington State Department of Health. (2019). Blue-Green Algae. Retrieved March 27, 2019, from https://www.doh.wa.gov/CommunityandEnvironment/Contaminants/BlueGreenAlgae

Wilks, D. L., Coppock, C. E., Lanham, J. K., Brooks, K. N., Baker, C. C., Bryson, W. L., Elmore, R.G., & Stermer, R. A. (1990). Responses of lactating Holstein cows to chilled drinking-water in high ambient-temperatures. *Journal of Dairy Science*, *73*(4), 1091–1099. Retrieved from https://doi.org/10.3168/jds.S0022-0302(90)78768-1

World Health Organization. (2003). Algae and cyanobacteria in fresh water. In *Guidelines for Safe Recreational Water Environments, Volume 1: Coastal and Fresh Waters* (pp. 136–158). Geneva, Switzerland: World Health Organization. Retrieved from https://ebookcentral-proquest-com.proxy1.cl.msu.edu/lib/michstate-ebooks/detail.action?docID=284656

World Health Organization. (2011a). *Manganese in Drinking-water*. Geneva, Switzerland.

World Health Organization. (2011b). *Molybdenum in Drinking-water*. Geneva, Switzerland.

World Health Organization. (2017). *Guidelines for Drinking-water Quality* (4th ed.). Geneva, Switzerland: World Health Organization. Retrieved from https://apps.who.int/iris/bitstream/handle/10665/254637/9789241549950-eng.pdf?sequence=1

World Wildlife Fund. (2019). Fact sheet: black rhino. Retrieved March 27, 2019, from https://www.worldwildlife.org/species/black-rhino

Yisa, J., & Jimoh, T. (2010). Analytical studies on water quality index of River Landzu J. Yisa and T. Jimoh. *American Journal of Applied Sciences*, *7*(4), 453–458.