POTENTIAL NEUROLOGICAL EFFECTS IN BANANA BATS FORAGING AT WASTEWATER TREATMENT WORKS

by

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ABSTRACT

Increasing rates of urbanisation cause ubiquitous infrastructures that remove anthropogenic contaminants – particularly Wastewater Treatment Works (WWTWs) – to become stressed, and hence pollute surrounding water systems. *Neoromicia nana* bats are suitable bioindicators because they exploit abundant pollutant-tolerant chironomid midges that breed at WWTWs, and consequently accumulate metals such as iron, copper and zinc in their livers and kidneys. If these metals persist in their circulatory systems, and cross the blood brain barrier (BBB) they can have adverse effects on critical functions such as flight and echolocation. The aim of this study was to investigate the potential neurological effects on *N. nana* foraging at WWTWs versus bats at reference sites in Durban, South Africa. My objectives were to 1) compare trace metal levels in brain and hair samples (as a proxy for circulating metals) between *N. nana* foraging at WWTWs and reference sites to determine if excess metals pass through the BBB via the circulatory system; and 2) compare biomarkers of neuron function (acetylcholinesterase activity), protection (antioxidant capacity), DNA integrity (DNA fragmentation), lipid integrity (lipid peroxidation) and cell viability (caspase-3 activity) between *N. nana* foraging at WWTWs and reference sites. I found a significantly higher concentration of arsenic in hair ($p<0.05$) and brain tissue ($p<0.1$) of WWTW bats compared to bats at reference sites. By contrast, acetylcholinesterase activity did not differ in bats among sites and there was no evidence of significant differences in lipid peroxidation, compromised DNA integrity or apoptosis in the brains between WWTW bats and reference site bats. However, total antioxidant capacity was significantly lower in brains of WWTW bats than bats at reference sites suggesting that antioxidant protection may be compromised. Long-term exposure to environmental pollutants at WWTWs may therefore affect cellular processes and protection mechanisms in brains of *N. nana* bats. It may also affect other mechanisms and functions in the brain such as mitochondrial efficiency and other neurotransmitters but that remains to be tested.

PREFACE

The experimental work described in this dissertation was carried out in the School of Life Sciences, University of KwaZulu-Natal, Westville campus, from January 2015 to September 2016, under the supervision of Dr Dalene Vosloo and Prof M. Corrie Schoeman.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

DECLARATION

I, ……. declare that

1. The research reported in this dissertation, except where otherwise indicated, is my original research.

2. This dissertation has not been submitted for any degree or examination at any other university.

3. This dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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LIST OF ABBREVIATIONS

MDGs	Millennium Development Goals
WWTWs	Waste Water Treatment Works
ROS	Reactive Oxygen Species
BBB	Blood Brain Barrier
CNS	Central Nervous System
CSF	Cerebral Spinal Fluid
AD	Alzheimer's disease
DNA	Deoxyribonucleic Acid
GABA	Glutamate and ɣ-Amino Butyric Acid
AChE	Acetylcholine Esterase
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
TAC	Total Antioxidant Capacity
WR	Working Reagent
FRAP	Ferric Reducing Antioxidant Power
TCEP	Tris (2-Carboxyethyl) Phosphine
ANOVA	One-Way Analysis Of Variance
BDL	Below Detection Limit
NMDS	Non-Metric multi-Dimensional Scaling
VE	Verulam
UM	Umbilo

BDF	BuffelsDrift Farm
UMD	Umdoni Forest
ATP	Adenosine-5’-Triphosphate
NADH	Nicotinamide Adenine Dinucleotide
PCR	Polymerase Chain Reaction

CHAPTER 1
INTRODUCTION

1.1. Urbanisation

Urbanisation is a major threat to many animal species as natural habitats are altered and destroyed to accommodate larger human populations, leaving minimal shelter or food resources (McKinney, 2006). This is echoed in the United Nations Millennium Development Goals (MDGs), which address the problems associated with the increase in the world's human population (United Nations, 2002). There are 8 MDGs in total which target a large range of worldwide issues pertaining to the growing population such as eradicating poverty and hunger, promoting gender equality and ensuring environmental sustainability. One of the MDGs focuses on the improvement of access to basic sanitation for under-privileged people. However, this goal puts enormous pressure on already overloaded infrastructures (United Nations, 2002). In South Africa, human population density (people per square km) increased from 36.2 in 2000 to 41.2 in 2010 and according to the South African 2030 National Development Plan,70% of the South African population will be living in urban areas (National Planning Commission, 2012). This National Development Plan was developed by the National Planning Commission and promotes eradicating poverty and reducing inequality in South Africa by improving the economy, education systems and transforming society. Immigration will further add a growth rate between 0.1 and 0.2 % a year to the increasing population. The population trajectories from 2010 to 2030 include an increase from 10.8 million to 14.1 million for Gauteng, increase from 5.3 million to 6.4 million in the Western Cape and an increase in KwaZulu-Natal from 10.4 million to 11.9 million. Johannesburg, eThekwini and Cape Town are the fastest-growing cities in the region therefore more permanent structures, particularly housing, are required to accommodate increases in urban population sizes. Increasing numbers of urban migrants leads to higher demands for basic services such as water and sanitation services. Wastewater Treatment Works (WWTWs) are tasked to provide some of these services in South Africa, but they struggle to cope with increasing demands of urban dwellers in cities (Muzenda *et al.*, 2012).

1.2. Wastewater Treatment Works and its effect on insectivorous bats

Balancing the supply and demand of sanitation to achieve both the UN's MDGs and South Africa's 2030 National Development Plan remains a challenge (CDE, 2010). In South Africa, >50% of

WWTWs have poor maintenance records (DWAF, 2011), hence poor quality wastewater is often released back into the environment.The release of industrial wastewater into surrounding water sources pollutes riparian environments, reduces water quality (Sacks and Buckley, 2004; Vaughan *et al.*, 1996), and causes nutrient and organic enrichment (Day and Dallas, 2011). Enrichment may reduce dissolved oxygen content and increase turbidity and bacterial contamination of water systems that, in turn, allows anaerobic organisms to thrive (Dallas and Day, 2004; Hynes, 1960). As a consequence of poor water quality, the structure and composition of resident aquatic biota, and the organisms that utilize them, change (Dallas and Day, 2004; Day and Dallas, 2011; Hynes, 1960; Pilosof *et al.*, 2014). Diversity of aquatic invertebrates often decreases in polluted water, yet a few groups may be highly abundant (Marques *et al.*, 1999). Invertebrate orders such as Ephemeroptera, Odonata and Lepidoptera are sensitive to aquatic pollutants and therefore less abundant (Dallas and Day, 2004; Dickens and Graham, 2002), whereas Hemiptera, Coleoptera and Diptera may be abundant in polluted environments (Dickens and Graham, 2002). For example, the dipteran family Chironomidae is able to inhabit and dominate environments polluted by WWTWs (Boonstra *et al.*, 2009; Marques *et al.*, 1999; Naidoo *et al.*, 2013). Organic sludge present in WWTW tanks creates an ideal breeding ground for chironomid midges (Boonstra *et al.*, 2009; Gagnon and Saulnier, 2003; Naidoo *et al.*, 2015). Chironomid midges accumulate toxicants from the surrounding water without adverse effects on their fitness (Krantzberg and Stokes, 1990), but this may make them harmful to their predators such as bats (Krantzberg and Stokes, 1990; Naidoo *et al.*, 2013; Vaughan *et al.*, 1996).

Insectivorous bats (Order Chiroptera) are small mammals that play an important role in riparian food webs and are the main nocturnal predator of aquatic insects in riparian environments (Kalcounis-Rueppell *et al.*, 2007). In fact, insectivorous bats often forage on aquatic insects from urban water sources (Kalcounis-Rueppell *et al.*, 2007; Vaughan *et al.*, 1996), including areas in the vicinity of WWTWs (Naidoo *et al.*, 2013; Park and Cristinacce, 2006). Moreover, certain species such as *Neoromicia nana* exploit industrial and urban environments thereby increasing the risk of contaminant exposure (Naidoo *et al.*, 2013, 2015; Zocche *et al.*, 2010; Zukal *et al.*, 2015). During foraging, insectivorous bats may consume insects up to 100% of their body weight (Kurta *et al.*, 1989) to sustain high ATP demands of flight and high metabolic rates (Clark and Shore, 2001; Encarnação and Dietz, 2006; Kunz and Fenton, 2006). This large food intake coupled with small body size and long lifespan make bats prone to accumulate contaminants (Zukal *et al.*, 2015). Indeed, there is evidence that bats accumulate metals and other toxicants by feeding

opportunistically on midges at WWTW-polluted sites (Naidoo *et al.*, 2013, 2016). Long-term exposure to metals via the food chain may negatively impact bat health (Flache *et al.*, 2015a, b; Naidoo *et al.*, 2013, 2015). Toxicant induced health effects in bats may be exacerbated by their high metabolic rates and long life span, and population level effects may manifest as a result of their low reproductive output.

Bats exhibit slow bodily development and reproduction rates. Most female bats produce a single offspring yearly (Barclay and Harder, 2005). Some species have the ability to manipulate the rate of foetal growth by either increasing or decreasing foetal growth time in response to changes in environmental conditions such as temperature and food supply (Jones *et al.*, 2009). However, if the female bat is exposed to pollution, it could prolong *in utero* exposure of the foetus. This prolonged exposure can potentially compromise foetal growth as well as the health of the female bat, which may lead to mortality. If exposure to pollution increases mortality rate, bat populations could markedly decline (Jones *et al.*, 2009). Vulnerability to bioaccumulation, high position on the trophic chain and long life span render bats ideal bioindicators of polluted environments and a suitable proxy for human health effects of pollutant accumulation and magnification through the food chain (Jones *et al.*, 2009; Zocche *et al.*, 2010; Zukal *et al.*, 2015).

In bats, long-term pollutant exposure results in the accumulation of both organic and inorganic contaminants in their tissues (Bayat *et al.*, 2014; Hernout *et al.*, 2016), with high concentrations in target tissues such as kidneys (Walker *et al.*, 2007), livers (Pikula *et al.*, 2010) and brains (Clark and Shore, 2001). After ingestion, metals are transported to the liver where they are deposited and sequestrated by metal-binding proteins such as metallothioneins (Boden *et al.*, 2008). Functions of metallothioneins include regulation of transcription factor activity, maintaining homeostasis of essential metal levels, detoxifying toxic metals and scavenging reactive oxygen species (ROS) (Lynes *et al.*, 2007; Vašák, 2005). Metal complexes are then excreted via the bile (Elder *et al.*, 2014) while unbound metals are either accumulated in the liver or re-enter circulation. From here metals accumulate in other tissue types such as the brain, and also into hair through the hair root (Vermeulen *et al.*, 2009). Metal load in feathers of birds (Dauwe *et al.*, 2002; Goede and de Bruin, 1984) and hair of mammals (Flache *et al.*, 2015a, b; McLean *et al.*, 2009; Vermeulen *et al.*, 2009) are often used to indicate metal levels circulating in the blood.

Hair offers a non-invasive method of assessing body metal load - especially non-essential metals ingested through food and drinking water (Hernout *et al.*, 2016; Kales and Christiani, 2005). On a

practical level, hair samples are readily attainable and may be stored, transported and analysed for metals at a later stage (Kales and Christiani, 2005). Metal ions bioaccumulated from food and the environment have previously been quantified from the hair of urban bats (Flache *et al.*, 2015a,b; Hernout *et al.*, 2016; Vermeulen *et al.*, 2009), and significant correlations were found between non-essential and essential metals in the fur and concentrations of these metals in the stomach, kidneys, liver and bones of bats (Hernout *et al.*, 2016). This suggests that bat hair could potentially be used as a biomarker of long-term metal exposure. Notably, non-essential metals in fur and tissues of bats were more significantly correlated than concentrations of essential metals, which may be attributed to regulation process to maintain homeostasis (D'Havé *et al.*, 2006). Exposure to metals, especially to non-essential metals, may interfere with physiological processes in bats therefore it needs to be explored further.

1.3. Metals function and potential neurotoxicity

Blood can carry metals into the central nervous system (CNS) through the blood-retina barrier (Lucchini *et al.*, 2014), the blood-cerebral spinal fluid (CSF) barrier and the BBB (Yokel, 2006). An additional route of exposure is through the olfactory neuron via the nasal cavity (Yokel, 2006).

The blood-CSF barrier comprises the choroid plexus that consists of differentiated epithelium cells with surrounding capillaries. Its function is to produce CSF and dispose of waste material and excess neurotransmitters (Laterra *et al.*, 1999). It is also an entry site for ions such as calcium that is crucial in maintaining calcium homeostasis in the brain (Laterra *et al.*, 1999; Lucchini *et al.*, 2014). The BBB plays many important roles to ensure the safety of the neuronal environment such as maintaining CNS homeostasis and using selective transportation to protect the brain from damaging substances (Zheng *et al.*, 2003). The BBB allows certain compounds to enter the brain via facilitated diffusion or transporter carriers. There are limitations to these transport mechanisms, for example molecules that diffuse across the BBB must be small and lipophilic (Yokel, 2006). Transporter proteins designed to transport metal ions do not discriminate between essential and non-essential metals. Non-essential metals (such as lead and mercury) with similar chemical properties and size as essential metals are able to pass through the BBB by mimicking the transporter's target molecule (Lucchini *et al.*, 2014). Environmental exposure to high metal concentrations may cause an influx of toxic levels of both essential and non-essential metals in the brain and result in neurotoxicity (Lucchini *et al.*, 2014).

Metal neurotoxicity depends on both concentration and type of metal implicated. Copper, iron and manganese are positively charged ions and can therefore partake in redox reactions, catalyze free radical reactions and act as pro-oxidants(Fraga, 2005). Furthermore, zinc ions are neutral and replace cations in free radical production and are therefore classified as anti-oxidants. Although these essential metals are required for certain functions in the brain, their levels need to be carefully regulated because high concentrations can compromise the CNS (Zheng *et al.*, 2003). Thus, each metal plays a unique role in the complex system of the brain.

1.3.1.Essential metals

Copper plays an important role in brain function because it forms a part of the ceruloplasmin protein-metal complex that is used to transport copper and aids in the formation of copper-activated enzymes (Fraga, 2005; Menkes, 1996). Both a surplus and deficiency of copper have been associated with neurodegenerative symptoms in Wilson's disease (Wright and Baccarelli, 2007). Additionally, iron is present in biological systems in varying oxidative states and plays important roles in energy processes. Iron can be oxidized via H_2O_2 to iron (III) and reduced back to its original form creating reactive hydroxyl radicals that can cause oxidative stress in mitochondria of cells and initiate lipid peroxidation. This may stimulate the formation of lipid hydroperoxides that can damage DNA, lipids and proteins, which, in turn, can induce apoptosis (Farina *et al.*, 2013). Moreover, manganese is important in multiple metabolism processes (Fraga, 2005). Although the role of manganese in neurotoxicity is poorly understood (Wright and Baccarelli, 2007), it may also be involved in creating free radicals and cause oxidative stress(Menkes, 1996).

Chromium is also required for physiological function (Farina *et al.*, 2013). It is used in many industrial processes and the anthropological enrichment in the environment is high, increasing the possibilities of exposure (Menkes, 1996). For example, chromium has been found in the kidneys, liver and muscle tissues of *Neoromicia nana* bats foraging at WWTWs (Naidoo *et al.*, 2013). Depending on its valency state, chromium has toxic effects on humans and animals (Bagchi *et al.*, 1997). Chromium (VI) generates higher oxidative stress levels than chromium (III) (Bagchi *et al.*, 1995; Bagchi *et al.*, 1997); this promotes the production of ROS that causes damage to proteins, DNA, and mitochondria (Pulido and Parrish, 2003) and may induce apoptosis (Bagchi *et al.*, 2000).

Nickel is also classified as an essential trace element and acts as a co-factor in transportation in the body, but has numerous toxic effects at high concentrations, such as nickel-induced lipid peroxidation and carcinogenesis (Cempel and Nikel, 2006). It can be transformed into alternative

forms in the body and can inhibit other metal-associated transport processes (Das *et al.*, 2008). Nickel can be high in wastewater (Poonkothai and Vijayavathi, 2012) and was found in the kidneys, liver and muscle tissues of *N. nana* foraging at WWTWs (Naidoo *et al.*, 2013)

Zinc plays an important role in synaptic activity and general neuronal signaling (Sensi *et al.*, 2011). Similar to copper and iron, excess zinc has been linked to neurodegenerative diseases and may play a role in free radical production and induction of apoptosis (Wright and Baccarelli, 2007). Exposure to excess zinc can increase cytosolic zinc levels, which could alter multiple downstream mechanisms and cause neuronal cell death and mitochondrial dysfunction via increased neuronal ROS. This can ultimately increase oxidative stress in the brain (Richetti *et al.*, 2011).

1.3.2.Non-essential metals

Non-essential metals are toxic at very low concentrations because they serve no biological purpose (Ewers and Schlipköter, 1991; Flache *et al.*, 2015b; Liu *et al.*, 2011; Pattee and Pain, 2002). Environmental exposure to non-essential metals over a long period of time can pose a threat to living organisms by accumulating in the brain and damaging brain tissue (Menkes, 1996). Lead, for example, can accumulate in brain cells and interfere with phosphorylation of cells and calcium regulatory processes. Lead can also hinder BBB development, allowing other toxic metals to enter the brain (Andrade *et al.*, 2015; Zheng *et al.*, 2003). Additionally, it interferes with cellular processes such as calcium metabolism (Zheng *et al.*, 2003). Mercury is present in aquatic food chains and can be methylated by bacteria in sediment. Mercury can be transported across the BBB via an L-type neutral amino acid, and cause neuronal loss (Farina *et al.*, 2013). Methyl mercury can also be transported through the BBB and interact with multiple sulfhydryl-containing proteins such as neurotransmitter transporters causing neuronal loss and damage in brain tissue (Farina *et al.*, 2013). Arsenic is a metalloid that has many oxidative states and is highly toxic (Flora, 2016; Rodrıguez *et al.*, 2003). Arsenic can induce oxidative stress that can cause neuronal death. It has also been linked to Alzheimer's disease (AD) because arsenic encourages the hyperphosphorylation of the tau protein and can interfere with transcription of amyloid precursor protein thereby potentially increasing the formation of amyloid plaques and neurofibrillary tangles (Gong and O'Bryant, 2010).

1.4. Physiological effects of metal exposure

Previous studies have described the accumulation of pollutants and resultant adverse health effects in bats foraging at WWTWs (Kalcounis-Rueppell *et al.*, 2007; Naidoo *et al.*, 2013, 2015, 2016;

Pilosof *et al.*, 2014). Toxic metals (cadmium, chromium and nickel) accumulated in the liver, kidneys and pectoral muscles of bats foraging at WWTWs(Naidoo *et al.*, 2013), and WWTW bats also exhibited haematological and genotoxic effects such as higher DNA damage in blood and a lower antioxidant response in pectoral muscles than bats at unpolluted reference sites (Naidoo *et al.*, 2015). Similarly, higher DNA damage and metal content were present in the liver and blood of bats exposed to toxic metals in mining areas (Zocche *et al.*, 2010). This suggests that the capacity of metal binding proteins in the liver to render metals unreactive was exceeded and high metal levels were present in circulation.

Metals in the blood stream, whether essential or not, can therefore reach other tissues in the body and potentially cross the blood brain barrier (BBB) causing harmful effects to the brain (Zheng *et al.*, 2003). These effects include interference with neurotransmitter function (Frasco *et al.*, 2005; Fulton and Key, 2001), lipid peroxidation (Bagchi *et al*., 1997; Travacio *et al*., 2000; Bashir *et* al., 2006), DNA damage and activation of apoptosis through oxidative stress (Pulido and Parrish, 2003).

1.4.1.Interference with neurotransmitter function

Metal-induced effects may interfere with neurotransmitters in the various regions of the brain and may be cell-type specific. For example, cultured astrocytes are more vulnerable to highmanganese concentration than neurons (Aschner *et al*., 1992). Astrocytes control the glutamine-glutamate cycle where glutamine is hydrolyzed and converted to the neurotransmitter glutamate in the neuron via phosphate-activated glutaminase (Daikhin and Yudkoff, 2000; Sidoryk-Węgrzynowicz *et al*., 2009). Exposure to excess amounts of manganese may alter transport efficiency of glutamine and possible impairment has the ability to affect glutamate and ɣ-amino butyric acid (GABA) neurotransmitter functions (Sidoryk-Węgrzynowicz *et al*., 2009; Farina *et al*., 2013). Similarly, exposure to methylmercury leads to interference with glutamate because methylmercury has the ability to inhibit glutamate uptake in astrocytes and stimulate the release of glutamate from neurons thereby increasing extracellular glutamate levels and potentially causing excitotoxic injury (Aschner *et al*., 2007; Farina *et al*., 2013).Release of the excitatory neurotransmitter glutamate is also inhibited by cadmium exposure (Minami *et al*., 2001; Sadiq *et al*., 2012). Another neurotransmitter affected by metals is acetylcholine. Acetylcholine is transported through the synaptic cleft and combines with receptors causing the receiving ganglion cell membrane to depolarize. An enzyme known as acetylcholinesterase (AChE) dismantles acetylcholine to restore the membrane to a polarized state (Koelle, 1962). Arsenic may inhibit AChE activity in certain regions of the brain as shown in rats

exposed to arsenic (Nagaraja and Desiraju, 1994). Rodrıguez *et al.* (2003) reviews the numerous studies pertaining to arsenic-induced neurotoxicity that show evidence of a decreased AChE activity due to arsenic exposure.

1.4.2.Lipid peroxidation

Pollutant insult and/or the inefficiency of mitochondrial respiration can cause the formation of free radicals (Machlin and Bendich, 1987). Metals, such as iron and copper, can further generate damaging hydroxyl radicals through the Haber-Weiss reaction, and these radicals are highly damaging to biomolecules such as lipids and DNA if antioxidant defenses are insufficient (Ercal *et al.*, 2001; Farina *et al.*, 2013). When free radicals, such as ROS, cause oxidation of polyunsaturated fatty acids it generates lipid hydroperoxides (Gutteridge, 1995).

The brain is especially susceptible to an increase in free radical production because it consumes ~20% of the body's available oxygen. Furthermore, it has a high polyunsaturated fatty acid content therefore making it more vulnerable to ROS attack (Travacio *et al*., 2000). Exposure to chromium (VI) and cadmium (II) may increase lipid peroxidation in the brain because of an increase in ROS production (Bagchi *et al*., 1997; Travacio *et al*., 2000). Arsenic-induced oxidative stress increases free radical production and may cause cellular damage (Ercal *et al.*, 2001) as evident in the enhanced lipid peroxidation in the brains of rats directly exposed to arsenic (Bashir *et al*., 2006).

1.4.3.DNA damage

Metal-induced ROS may also directly or indirectly damage DNA and interfere with DNA repair mechanisms. For example, iron can form hydroxyl radicals through the Haber-Weiss reaction as discussed in 1.7.3 (Farina *et al*., 2013) thereby causing DNA damage and arsenic is known to inhibit DNA repair mechanisms (Beyersmann and Hartwig, 2008). Further, low non-cytotoxic concentrations of arsenic as well as nickel, cobalt and cadmium inhibit DNA repair processes thereby allowing DNA damage to persist in cells (Hartwig *et al*., 2002).Previous studies have demonstrated that exposure to toxic metals also increase DNA damage in bats (Naidoo *et al*., 2015; Zocche *et al*., 2010).

1.4.4.Activation of caspase-3 and apoptosis

Apoptosis, alternatively known as programmed cell death, is vital for normal brain function and development (Porter and Janicke, 1999). Stress-induced apoptosis can be induced via different pathways. Firstly, through intrinsicapoptosis pathway metals and metal-induced ROS can directly damage mitochondrial membrane permiability and cause multiple apoptosis promoting factors to be

released. Cytochrome c is freed from the mitochondria, binds to an apoptosis protease-activating factor-1 protein and activates caspase-9 that in turn initiates caspase-3 to induce apoptosis (Chen *et al.*, 2001; Pulido and Parrish, 2003). Alternatively, the intrinsic pathway can be initiated through activation of the p53 transcription factor. DNA damage also activates both p53 and p53 independent pathways (Benchimol, 2001). Secondly, metals can potentially activate the extrinsic apoptosis pathway through mitogen-activated protein kinases that in turn activate caspase-3 driven apoptosis via activation of caspase-8 (Chen *et al.*, 2001). In this way, damaged cells can be removed from tissue to prevent autoimmunity, tumorigenesis, and maintain function (Rana, 2008).

1.5. Brain function in bats

The brain plays a vital role in communicating signals from nerve cells to muscle cells during movement of the body (Jewett and Rayner, 1984). In insectivorous bats, the brain is used for a variety of functions, which include the regulation of hibernation (Chen *et al.*, 2008), and flight and echolocation to avoid obstructions and capture prey (Schwartz and Smotherman, 2011; Wenstrup and Portfors, 2011). Alterations in mechanisms of the brain due to accumulation of contaminants may negatively impact the fitness of bats. For example, inhibition of neurotransmitter acetylcholinesterase will result in involuntary muscle spasms (Frasco *et al.*, 2005; Fulton and Key, 2001). Oxidative stress increases ROS production and excess ROS may target glial cells and neurons in the brain causing neurodegeneration leading to loss of function in coordination or sensory dysfunction (Gilgun-Sherki *et al.*, 2001; Uttara *et al.*, 2009). It is therefore essential to determine whether foraging at WWTW affects neurological functioning of resident insectivorous bats.

1.6. Rationale for this study

Neoromicia nana (family Vespertilionidae) is a small insectivorous bat (3-4g) that roosts in rolled-up banana tree leaves (LaVal and LaVal, 1977). They are often found in riparian habitats throughout sub-Saharan Africa, particularly in forest and savannah biomes (Monadjem *et al.*, 2010a; Monadjem and Reside, 2008). *N. nana* bats have short, broad wings and frequency-modulated low duty-cycle echolocation calls (Monadjem *et al.*, 2010b). *N. nana* opportunistically feed on pollution-tolerant chironomid midges at WWTW (Naidoo *et al.*, 2013), and consequently accumulate metals in their kidney, liver and muscle tissue (Naidoo *et al.*, 2013, 2015). This may cause DNA damage (Naidoo *et al.*, 2015). Furthermore, exposure to pollutants, such as metals, at WWTWs was associated with histopathological lesions in the liver and kidneys of WWTW bats (Naidoo *et al.*, 2016). Because metal levels at the tank sites of Verulam and Umbilo WWTWs

exceed the chronic exposure value of the SA target water quality guidelines for ecosystem health (Naidoo et al, 2013; DWAF, 1996), the liver and kidneys of WWTW bats may be unable to detoxify these high metal levels, and metals may enter circulation. Excess metals in the blood stream may then accumulate in other tissues in the body and potentially cross the BBB (Zheng *et al.*, 2003) and adversely affect brain function. However, there are few studies that investigate neurological responses to toxic metals in bats and therefore it is important to explore this further.

1.7. Aims and objectives of this study

I hypothesized that *N. nana* bats foraging at the Verulam and Umbilo WWTWs are at risk from trace metals entering circulation and crossing the BBB, thereby decreasing neuron functioning and increasing antioxidant responses. If antioxidant capacities of *N. nana* are exceeded, I predicted damage to DNA integrity and lipids in the brain and high levels of apoptosis in the brain.

The first aim of this study was to determine if more metals pass through the BBB via the circulatory system in bats collected from WWTWs than in bats collected from reference sites. The second aim was to determine if biomarkers of neuron function, protection, DNA integrity, lipid integrity and cell viability differ between *N. nana* foraging at WWTW and reference sites.

My objectives were to:

1. Measure and compare metal levels in brain and hair samples of *N. nana* at two WWTWs and two reference sites in KwaZulu-Natal;
2. Measure and compare the following biomarkers in brain samples of N. nana collected from two WWTWs and two reference sites in KwaZulu-Natal:
    a. Acetylcholinesterase activity levels as a biomarker of neuron function;
    b. Antioxidant capacity as a biomarker of protection;
    c. DNA fragmentation levels as a biomarker of DNA integrity;
    d. Lipid hydroperoxidation levels as a biomarker of lipid integrity; and
    e. Caspase-3 activity levels as a biomarker of cell viability.

CHAPTER 2

METHODS

2.1. Study sites

This study was conducted in eThekwini Municipality, KwaZulu-Natal province, South Africa (Durban; 29º58' S; 30º57' E). eThekwini Municipality is home to a population of ca. 3, 5 million people (Stats SA, 2011) spanning over an area of approximately 2297 km^2. Naidoo *et al.* (2013, 2015, 2016) and Hill *et al.* (2016) sampled bats at two polluted sites: Verulam WWTW (29°38'43.1" S; 31°03'52.8" E) and Umbilo WWTW (29°50'46.1" S; 30°53'26.8" E) because these

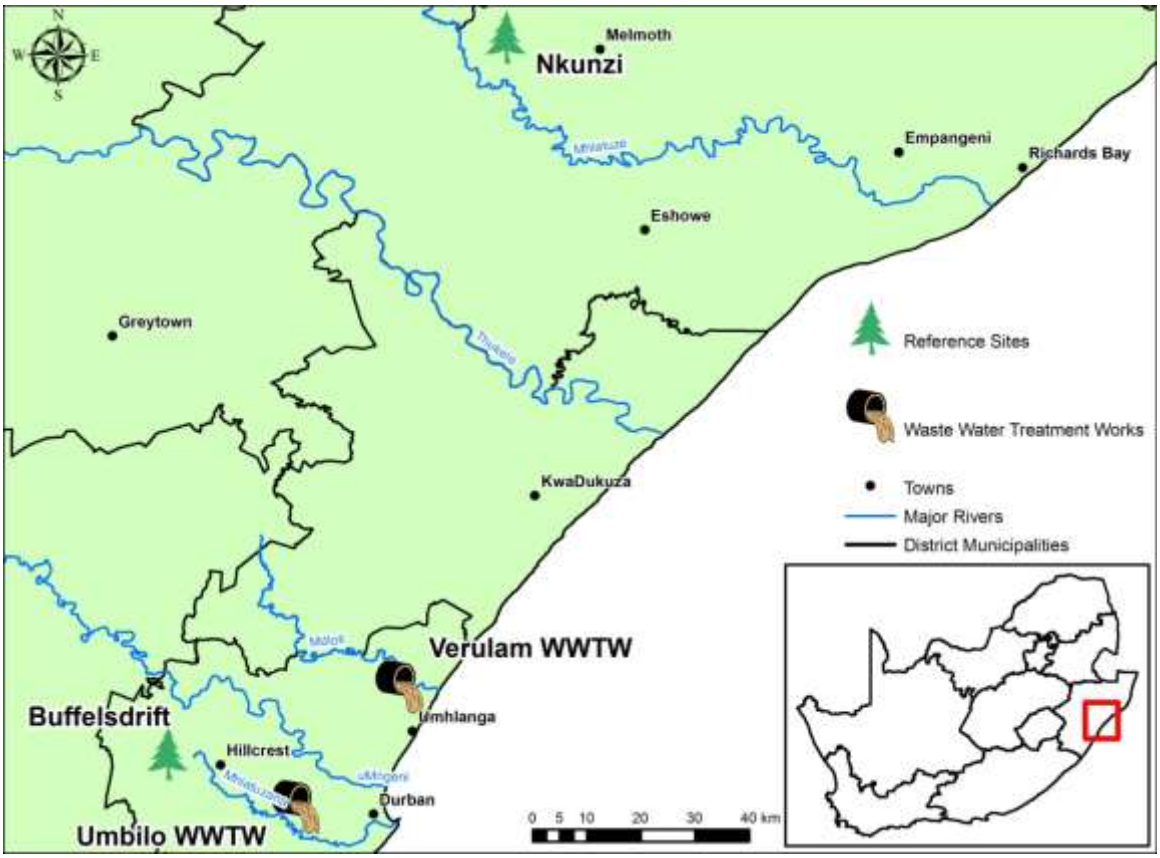

Fig.1. Map indicating the study sites in Durban, South Africa showing Verulam WWTW, Umbilo WWTW, Buffelsdrift farm and Nkunzi Lodge.

two WWTWs, compared to 32 in and around Durban, had poor quality compliance scores in the most recent Green Drop Report (DWAF, 2011). Verulam WWTW is situated on the Mdloti river ca.40 km outside the center of Durban, whereas Umbilo WWTW is situated on the Umbilo river

about 22 km outside Durban (Fig. 1). Buffelsdrift farm (29°45'14.1" S; 30°40’38.6” E) and Nkunzi Lodge (28º33’42.3” S; 31º14’25.3” E) were selected as reference sites. Buffelsdrift farm is a small organic small holding situated about 40km west of Durban (Hill *et al.*, 2016) and about 11 km from the nearest WWTW. Nkunzi Lodge is situated in a relatively secluded forest area about 148 km from Durban and 78 km from the nearest WWTW.

2.2 Bat sampling

Bats were captured during a single sampling in the summer of 2015 using mist nets, except at Buffelsdrift where bats were retrieved by hand from their roosts inside rolled-up banana tree leaves. Bats were transferred individually into cotton bags. *N. nana* bats were identified using a taxonomic key (Monadjem *et al.*, 2010b) and sexed. All other bat species were released at the site of capture. Only adults were used in this study - developmental stage was determined by the presence/absence of cartilaginous epiphyseal plates in the finger joints (Anthony, 1988). The total number of bats collected from each site were as follows: 13 (5 male; 8 female) from Umbilo WWTW, 12 (10 male; 2 female) from Verulam WWTW, 12 (5 male; 7 female) from Buffelsdrift Farm, and 4 (1 male; 3 female) from Nkunzi Lodge. Forearm length was measured using calipers (to nearest 0.1 mm) and body mass was measured to nearest 0.5 g using a Pesola scale (Baar, Switzerland).Bats were euthanized humanely (University of KwaZulu-Natal Animal Ethics Committee reference: 014/15/Animal) and dissected to collect brain tissue which were transferred into 2 ml microcentrifuge tubes and stored at -80 °C.

2.3 Brain and hair metal analysis

The hair and brain of bat samples collected in the previous year (summer of 2014; see Hill *et al*., 2016) were used to determine metal concentrations because there was not a sufficient quantity of brain sample left from the 2015 samples for accurate analyses. Brain tissue from Verulam WWTW bats and reference site bats were used. Hair samples were cleaned to remove external metal contamination (Hickey *et al.*, 2001): samples were washed three times using Acetone (Merck Group, Darmstadt, Hesse, Germany), once with 10% Triton-X® (Sigma-Aldrich, St Louis, Missouri, USA) and rinsed three times with ultrapure water (Merck Millipore, Massachusetts, USA). Reference samples were prepared in the same way, using certified reference material (Mussel tissue ERM-CE278k, European Reference Material®, IMMR, Geel, Belgium). All samples were weighed and transferred into pre-weighed 15 mL tubes and placed into an oven at 60 ºC for at least 48 hours. Once a constant weight was reached, 0.5 mL Nitric acid (Sigma-Aldrich, St Louis,

Missouri, USA) was added and samples were allowed to digest overnight. Reference samples contained 1.5 mL nitric acid and were allowed to digest overnight. Hair and brain samples were diluted with 9.5 mL ultrapure water (Merck Millipore, Massachusetts, USA) whilst reference samples were diluted with 13.5 mL ultrapure water. Samples and reference material were sent to the Central Analytical Facilities at Stellenbosch University for inductively coupled plasma mass spectrometry (ICP-MS) analysis. Where metal levels were below the detection limit of the ICP-MS, the detection limit value was used in analyses. Tissue metal concentrations were expressed in $mgkg^{-1}$and the extraction efficiency (%) of certified reference material metal levels was expressed relative to certified reference values.

2.4 AChE activity in brain tissue

An amount of ~10 mg of brain tissue was placed in a pre-weighed tube. A minimum of 10 samples were used per site. The brain tissue was homogenized with a stainless steel bead and 50 µl 0.1 $mol.L^{-1}$ potassium phosphate (pH 7.2) in a Tissue Lyser (Qiagen®, Hilden, Germany) for 3 minutes. A magnet was used to remove the stainless steel bead before samples were centrifuged for 5 minutes at 16873 x *g*. Aliquots of the protein-containing supernatant were transferred into 1.5 mL microcentrifuge tubes for analysis of total antioxidant capacity (TAC) and caspase-3.

For AChE analysis, a kit (Sigma-Aldrich, St Louis, Missouri, USA) was used based on the Ellman's method (Ellman *et al.*, 1961). Working Reagent (WR) was prepared as per the protocol and then two replicates of 200 µl of assay blank and 200 µl of calibrator were transferred into separate wells in a 96-well microcentrifuge plate. 1 µl of the whole cell extract isolated as in 1.6 were added into separate wells in replicates of two together with 190 µl WR. Samples were incubated at room temperature for 2 minutes and initial absorbance was measured at 412 nm (Powerwave XS microplate spectrophotometer). AChE activity was calculated as follows as per protocol (Sigma-Aldrich, St Louis, Missouri, USA):

$$\text{AChE activity (units.L}^{-1}) = \frac{\text{A}_{412\text{ final}} - \text{A}_{412\text{ initial}}}{\text{A}_{412\text{ calibrator}} - \text{A}_{412\text{ blank}}} \times n \times 200$$

Where: 200 = equivalent activity ($units.L^{-1}$) of calibrator when assayed is read at 2 minutes and 10 minutes.

n = dilution factor

A412 final = Absorbance value of sample at 10 minutes

A412 initial =Absorbance value of sample at 2 minutes

A412 calibrator = Absorbance value of calibrator at 10 minutes

A412 blank =Absorbance value of blank at 10 minutes

AChE activity was then expressed as µmol $min^{-1}g^{-1}$ tissue.

2.5 Total antioxidant capacity (TAC) in brain tissue

TAC of brain tissue was determined using a protocol from Griffin and Bhagooli (2004) using ferric reducing antioxidant power (FRAP) assay. A standard series of $FeSO_2.7H_2O$ (0, 25, 50, 75, 100, 150, 200, 1000 µmol.L^{-1}) was used to determine TAC. Whole cell isolates (as extracted in 1.6) were diluted to a concentration of 2 µg.µL^{-1} using ultrapure water and placed in a 96-well microplate. FRAP reagent was prepared using a 10:1:1 ratio of acetate buffer: TPTZ: $FeCl_3$. $6H_2O$ and placed in all occupied wells. The microplate was incubated at 37 ºC for 20 minutes and absorbance was determined at a wavelength of 600 nm (Powerwave XS microplate spectrophotometer). A standard curve was constructed and results were extrapolated using the standard curve. Results were expressed in pmoles of Fe^{2+}.μg^{-1} tissue.

2.6 Brain lipid peroxidation levels

Brain lipids were extracted using an adaptation of the Bligh and Dyer method (White *et al.*, 1979). Samples of ~30 mg brain tissue were placed in 2 mL microcentrifuge tubes with mixed stock solution of methanol, chloroform and 50 mmol.L^{-1} phosphate buffer (pH 7.4) at a volume ratio of 2.0:1.0:0.8 with a stainless steel bead (Qiagen®, Hilden, Germany). Tissue was then disrupted for 3 minutes using a Tissue Lyser (Qiagen®, Hilden, Germany). Samples were left to stand in room temperature for 2 hours to allow phase separation. Equal amounts of chloroform and ultrapure water were added and the final solution to contain a volume ratio of 1:1:0.9 for chloroform: methanol: water. Samples were centrifuged for 15 minutes at 344 x *g* and the lower organic phase, containing the lipids, was transferred into a clean 2 mL microcentrifuge tube.

Lipid peroxidation levels in brain tissue of *N. nana* were determined using a PeroxiDetect™ kit (Sigma-Aldrich, St Louis, Missouri, USA) that measures the amount of lipid hydroperoxides in the sample tissue. The colour change produced by the oxidation of Fe^{2+} to Fe^{3+} ions by hydrogen peroxides allows for quantification of lipid hydroperoxides and the addition of tris (2-carboxyethyl) phosphine (TCEP) (Nourooz-Zadeh *et al.*, 1995) allows the total lipid hydroperoxides to be

measured. A sample aliquot (18 µl) was transferred into four 2 mL microcentrifuge tubes; two of the tubes contained 2 µl 90% methanol (control) and two tubes contained 2 µl TCEP (10 mol.L^{-1}). Samples were vortexed (vortex-genie 2; Scientific Industries™) and incubated at 25 °C for 30 minutes. Standards of 200 µM *tert*-Butyl hydroperoxide (t-BuOOH) (0, 1, 2, 4, 8, 12, 16 nmoles/reaction volume) were diluted whilst samples were being incubated. After incubation, 200 µl of WR (100 parts of organic peroxide colour reagent to 1 part of ferrous ammonium sulfate reagent) was placed into each standard and sample microcentrifuge tube, vortexed and incubated at 25 °C for 30 minutes. Standards and samples were transferred to a 90-well microcentrifuge plate and the absorbance was measured at 560 nm using KC4 software version 3.2 (Powerwave XS microplate spectrophotometer, BIO-TEK®, Winooski, Vermont, USA). Blank values were subtracted from relevant absorbance values and lipid peroxidation levels were extrapolated from the standard curve. Lipid peroxidation values were converted into nmoles.g^{-1} of tissue.

2.7 DNA integrity in brain tissue

N. nana brain samples were thawed and ~10 mg sample was placed in microcentrifuge tubes that contained 40 µl lysis buffer (1 mol.L^{-1}TRIS-HCL pH 8.5; 0.5 molL^{-1} EDTA; 10% SDS; 5 mol.L^{-1}NaCl) and 1.2 µl Proteinase K (10 mg.mL^{-1}). Tissue samples were incubated for 4 hours at 56 °C and placed on an orbital shaker (IKA® Works, Inc., Wilmington, USA) at 320 rpm for an hour. Samples were then centrifuged (Eppendorf®) for 10 minutes at 8609 x *g* and the supernatant was transferred to a new 2 mL microcentrifuge tube containing ~80 µl of ice-cold 99% ethanol alcohol and 8 µl sodium acetate (3 mol.L^{-1}). Samples were agitated until DNA precipitated and then centrifuged at 8609 x *g* for 10 minutes. The DNA pellet was washed in 40 µl of 70% ethanol, centrifuged for 5 minutes at 8609 x *g* and left to dry at room temperature. The DNA pellet was suspended in 20 µl 1x TE buffer, left in -4 °C for two days and then stored into -80 °C.

DNA was quantified using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington). 1 ng DNA was resolved on a 2% agarose gel stained with ethidium bromide at 85 V for 2 hours in TBE buffer against a DirectLoad™ wide range DNA marker (Sigma-Aldrich, St Louis, Missouri, USA) containing 16 bands from 50 bp to 10 000 bp. A photograph was taken under UV light (DOC XRS^{+}, Bio-Rad, Hercules, California, USA). DNA integrity was analysed as follows with Image Lab™ software (version 2.0.1, Bio-Rad®, Hercules, California, USA): The density of all intact DNA per lane was divided by the density measured for the entire lane (intact + fragmented DNA) and was expressed relative to tissue mass to obtain the level of intact DNA per unit tissue mass.

2.8 Caspase-3 activity in brain tissue

A Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to quantify protein samples extracted from the brain for caspase-3 and TAC analyses. Samples were diluted 10 fold with ultrapure water and added together with the standard series (0, 25, 125, 250, 500, 750, 1000, 1500, and 2000) into a 96-well microwell plate. WR was prepared according to the protocol, using a 50:1 ratio of Reagent A and Reagent B respectively, and 200 µl was added to each well. The microwell plate was incubated for 30 minutes at 37 °C and the absorbance was measured at 562 nm (Powerwave XS microplate spectrophotometer).

A colorimetric assay kit (BioVision Incorporated, Milpitas, CA, USA) was used to measure caspase-3 activity. Protein samples were diluted to 50 µg protein to 50 µl with ultrapure water and pipetted into a 96-well microwell plate. An amount of 50 µl reaction buffer, containing 10 mmol.L^{-1} DTT, and 5 µl of 4 mmol.L^{-1} DEVD-pNA substrate were added to each sample. Samples were incubated at 37 °C for 1 hour and absorbance was measured at 405 nm (Powerwave XS microplate spectrophotometer). Absorbance values for blank wells were subtracted from average absorbance of each sample and reference sites values were combined and averaged. Each sample from each WWTW site was divided by reference site average to calculate fold-change to compare the WWTW relative to the reference sites.

2.9 Statistical analyses

Data collected from Nkunzi Lodge bats were combined with those from Buffelsdrift farm. Assumptions for normality and equality of variances were tested using a Kolmogorov-Smirnov test and a Levene's test, respectively. All statistical analyses were performed with R (version 3.2.5, R Core Team, 2014). Graphs were constructed using R (version 3.2.5, R Core Team, 2014) and GraphPad Prism software (version 7, www.graphpad.com). To compare the mean AChE activity, apoptosis, DNA damage and TAC among sites, one-way analysis of variance (ANOVA) followed by Tukey post-hoc tests were used. For data that did not satisfy the assumptions, non-parametric Kruskal Wallis tests were conducted followed by pairwise comparisons using the Tukey and Kramer (Nemenyi) test in the PMCMR package in R (version 3.2.5, R Core Team, 2014).

Metal concentrations of hair and brain samples between bats from WWTWs and reference sites were compared using one-way ANOVAs for each individual metal and non-metric multidimensional scaling (NMDS) analyses based on Bray-Curtis dissimilarities using the vegan package in R (version 3.2.5, R Core Team, 2014).For data that did not satisfy the assumptions of

normality and/or equal variances, non-parametric Kruskal Wallis tests were conducted followed by Dunn's post hoc test.

CHAPTER 3

RESULTS

3.1 Metal concentrations in hair and brain tissue

Among the metals tested, only mean concentration of arsenic in hair samples was significantly higher in Verulam WWTW bats (1.514µg.g^{-1} dry tissue mass) than at other sites (Kruskal Wallis: $\chi^2 = 9.964$, d.f. = 2, $p<0.05$; Dunn's Test: $p<0.05$, Table 1). Similarly, mean concentration of arsenic in brain samples was significantly higher in Verulam WWTW bats (0.220 µg.g^{-1} dry tissue mass) than bats at reference sites (Kruskal Wallis:$\chi^2 = 3.267$, d.f. = 1, $p<0.1$; Dunn's test: $p<0.05$, Table 1). Essential trace element concentrations in hair and brain tissue were not different between WWTW bats and bats from reference sites (Table 2).

Table.1. Mean (± s.d.) and range of non-essential trace element concentrations (µg.g^{-1} dry weight)with the number of samples below the detection limit (BDL) for *N. nana* hair and brain tissue from Verulam WWTW, Umbilo WWTW and reference sites. Number of samples is indicated in brackets. Significant differences between sites are indicated by an asterisk(* = p<0.05; =p<0.01; *** = p< 0.001).**

		Verulam WWTW		Umbilo WWTW	Reference sites	
		Brain (n=8)	Hair (n=9)	Hair (n=10)	Brain (n=6)	Hair (n=5)
Arsenic	Mean±s.d.	0.413±0.553	1.514±0.657*	0.785±0.492*	0.128±0.080	0.418±0.469*
	Range	0.092-1.768	0.588-2.563	0.392-1.974	0.039-0.238	0.049-1.177
	BDL	0	0	0	0	0
Cadmium	Mean± s.d.	0.029±0.033	0.074±0.094	0.119±0.236	0.010±0.013	0.056±0.058
	Range	0.001-0.103	0.014-0.285	0.014-0.788	0.002-0.033	0.018-0.159
	BDL	1	0	1	0	0
Lead	Mean± s.d.	1.169±3.091	1.859±5.373	1.576±2.603	0.723±1.648	5.943±11.621
	Range	0.050-8.818	0.050-16.187	0.050-6.951	0.050-4.087	0.050-26.609
	BDL	6	6	5	5	3

Table.2. Mean (± s.d.) and range of essential trace element concentrations (µg.g^{-1} dry weight)with the number of samples below the detection limit (BDL) for *N. nana* hair and brain tissue from Verulam WWTW, Umbilo WWTW and reference sites. Number of samples is indicated in brackets. None of the average concentrations were different between sites.

		Verulam WWTW		Umbilo WWTW	Reference sites	
		Brain (n= 8)	Hair (n=9)	Hair (n=10)	Brain (n=6)	Hair (n=5)
Chromium	Mean±s.d.	0.076±0.075	0.566±1.087	0.225±0.322	0.05±0.000	0.247±0.270
	Range	0.050-0.261	0.050-3.365	0.050-1.071	0.050-0.050	0.050-0.545
	BDL	7	3	5	6	3
Copper	Mean±s.d.	1.363±3.715	5.236±15.558	5.937±11.156	4.004±9.685	37.699±84.186
	Range	0.050-10.557	0.050-46.724	0.050-34.555	0.050-23.774	0.050-188.295
	BDL	7	8	6	5	4
Iron	Mean± s.d.	119.730±42.625	59.478±38.291	84.565±37.917	105.326±8.537	61.27 ±40.416
	Range	78.518-182.359	22.501-134.097	23.798-170.520	93.737-115.762	24.621-125.323
	BDL	0	0	0	0	0
Manganese	Mean± s.d.	2.083±0.717	9.637±7.377	11.998±8.485	2.342±1.138	30.681±21.758
	Range	1.255-3.154	3.125-26.822	3.142-31.328	1.469-4.502	9.681-67.218
	BDL	0	0	0	0	0
Zinc	Mean± s.d.	101.515±43.435	298.726±48.511	308.605±126.830	96.671±34.315	255.798±70.094
	Range	57.214-193.837	247.241-391.349	206.608-554.400	71.208-158.197	161.907-357.951
	BDL	0	0	0	0	0

NMDS of metals in hair samples (linear fit of ordination distance and observed dissimilarity R^2=0.947) did not clearly separate bats from WWTW and reference sites – polygons linking bats from polluted vs. unpolluted sites overlapped (Fig.2A). This may partially be due to the small sample size of the reference sites. NMDS of metals in brain samples (linear fit of ordination distance and observed dissimilarity R^2=0.974) separated into WWTW and reference sites – polygons linking bats from polluted vs. unpolluted sites exhibited a relatively small area of overlap (Fig.2B). Arsenic and cadmium are more associated with WWTW sites than reference sites.

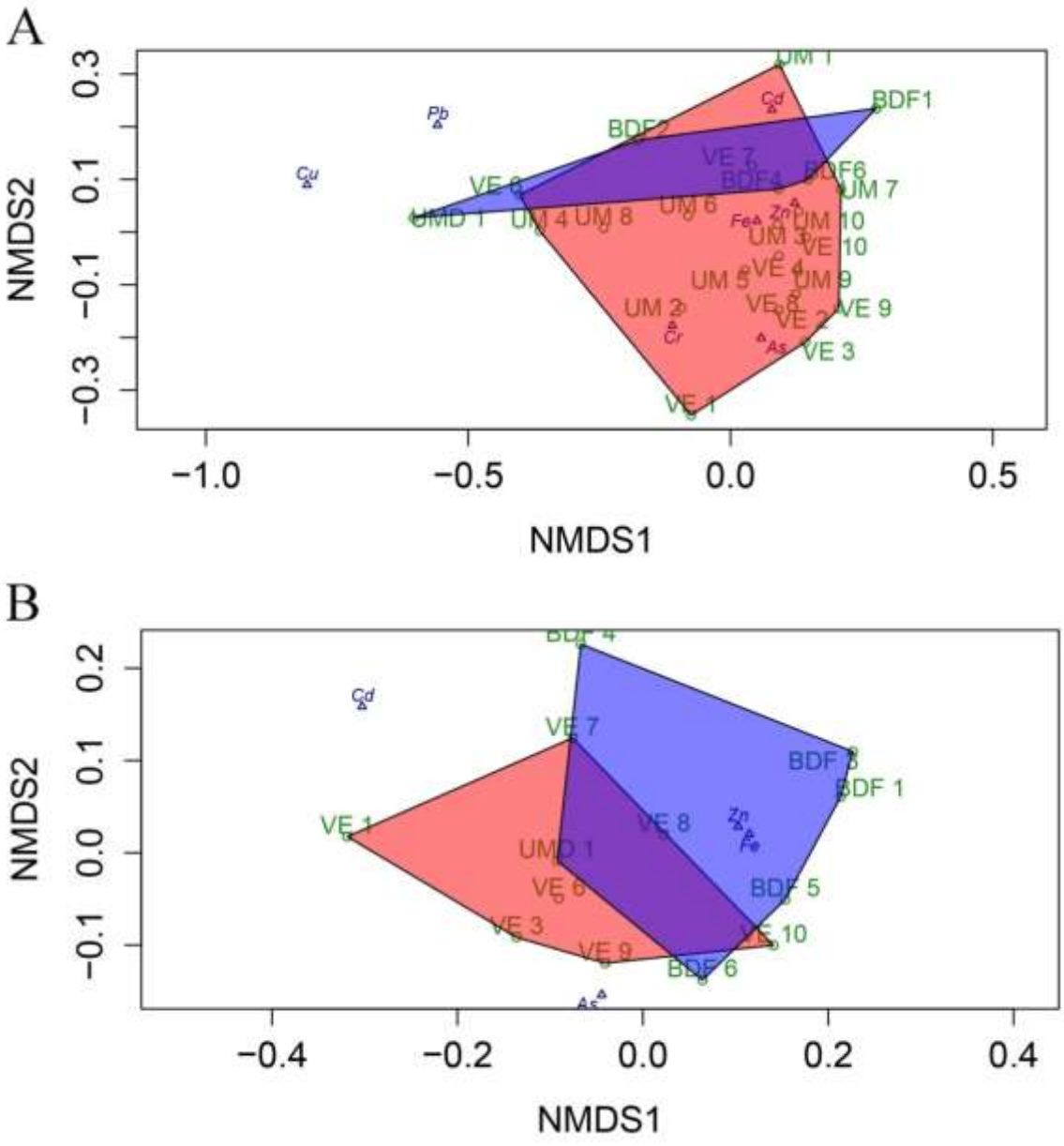

Fig.2. Non-metric multidimensional scaling of metals concentrations in bat hair (A) and brain (B). The red polygon indicates WWTW bats [VE (Verulam WWTWs) and UM (Umbilo WWTWs)] and the blue polygon indicates reference site bats [BDF (Buffelsdrift) and UMD (Umdoni)]. Cu=Copper, Pb=Lead, Fe=Iron, Zn=Zinc, Cr=Chromium, As=Arsenic, Cd=Cadmium.

A Pearson product-moment correlation shows a significant positive correlation between arsenic concentrations in hair and brain of bats at Verulam WWTW and reference sites (r=0.749, n=11, P =0.008, Fig.3).

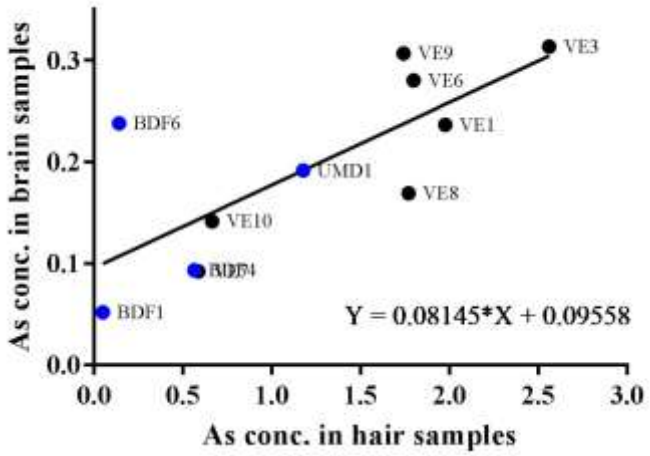

Fig.3. Significant positive correlation between arsenic (As) concentration in hair and brain samples of bats at Verulam WWTW and reference sites.

3.2 Physiological responses

The mean AChE activity in bats did not significantly differ among those foraging at Verulam and Umbilo WWTWs and reference sites (Kruskal-Wallis: $\chi^2 = 0.222$, d.f. = 2, $p>0.05$; Fig.4A). Mean TAC in *N. nana* brain tissue at the reference site significantly differed from those foraging at Verulam and Umbilo WWTW (Kruskal-Wallis: $\chi^2 = 18.34$, d.f. = 2, $p<0.1$; Fig.4B). The Tukey and Kramer (Nemenyi) test revealed Verulam and Umbilo WWTWs had lower TAC values than bats from the reference sites (p-values = 0.004 and 0.0003, respectively). I found no detectable amount of lipid hydroperoxides in the brains of bats. The PeroxiDetect™ kit has a detection limit range for lipid hydroperoxides in organic solvents of 1-16 nmoles per reaction volume. The mean DNA integrity was higher in bats at reference sites than bats at Verulam and Umbilo WWTW but these differences were not statistically significant (Kruskal Wallis: $\chi^2 = 3.286$, d.f. = 2, $p>0.05$; Fig.4C). Caspase-3 activity in the brains did not differ between bats foraging at WWTW and reference sites (Kruskal Wallis:$\chi^2=0.772$,d.f. =2, $p>0.05$; Fig.4D).

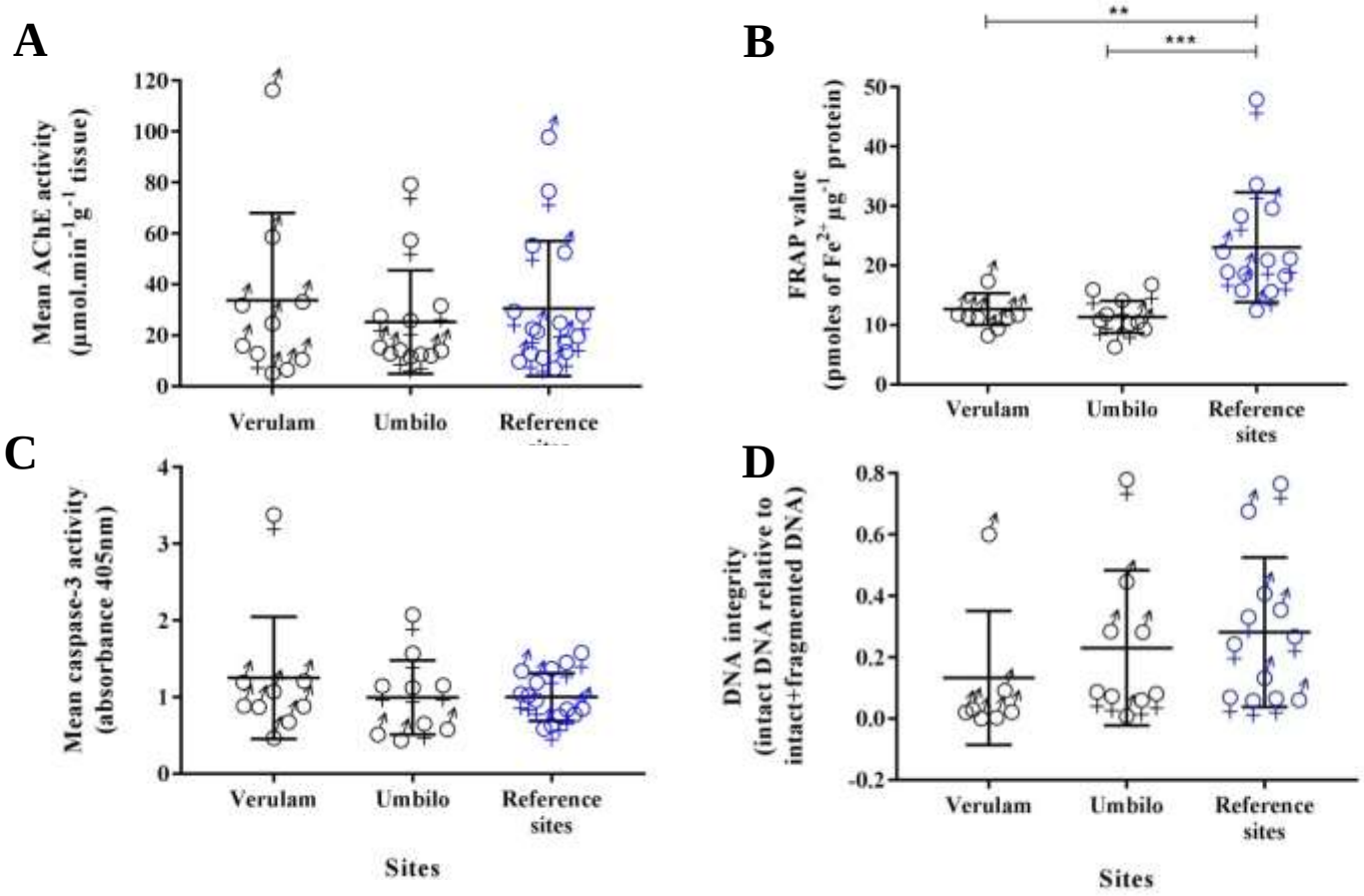

Fig.4. (A) Acetylcholinesterase activity in brains did not differ between*N. nana* foraging at WWTW and reference sites. (B) The TAC in *N. nana* brain tissue was significantly higher at the reference sites than WWTW sites. (C) DNA integrity (intact DNA relative to total intact + fragmented DNA) in brains did not differ among banana bats foraging at reference sites and Verulam and Umbilo WWTW. (D)The level of apoptosis (as represented by caspase-3 activity) in brains did not differ among bats foraging at Verulam and Umbilo WWTW and reference sites. Data are presented as mean±s.d and males and females are indicated by ♂ and ♀ respectively. Significance codes: * - p< 0.05; ** - p< 0.01; *** - p< 0.001

CHAPTER 4
DISCUSSION

4.1. High arsenic levels in WWTW bats

In support of my prediction that certain metal pollutants may enter circulation in bats, arsenic levels were significantly high in hair of bats foraging at WWTW. Metal concentrations in hair samples reflect metal concentrations in the bloodstream because the bloodstream is in contact with the hair root where metal ions can be absorbed into the hair as it grows (Hernout *et al.*, 2016; Vermeulen *et al.*, 2009). Arsenic can be transported through blood to other parts of the body by binding to globin and thus also reflect tissue metal levels (Flache *et al.*, 2015a,b; Flora, 2016). Furthermore, elevated levels of arsenic in brain tissue of WWTW bats compared to that of reference site bats support previous observations that arsenic can cross the BBB (Andrade *et al.*, 2015). Arsenic can cause symptoms of neurodegeneration similar to that of AD (Andrade *et al.*, 2015). These symptoms include a reduction in AChE activity in certain regions of the brain (Nagaraja and Desiraju, 1994; Tyler and Allan, 2014; Yadav *et al.*, 2011), overproduction of amyloid-ß, and induction of apoptosis via ROS-induced damage to DNA, lipids and proteins (Gong and O'Bryant, 2010). Furthermore, arsenic exposure may induce hyper-phosphorylation of many amino acid residues in tau and oxidative stress *in vivo*, which may disrupt tau protein function and cause neurodegeneration(Giasson *et al.*, 2002). Collectively these studies provide support for the hypothesis that arsenic exposure may be involved in the pathogenesis of AD.Regardless, arsenic concentrations in brain tissue of bats from both WWTWs and reference sites were lower than that in hair, which supports evidence that lower concentrations of arsenic accumulate in the brain than liver, kidneys and other tissues(Flora, 2016).

4.2. AChE activity is not impaired in bats foraging at WWTW

Although short-term exposure to elevated levels of arsenic reduced AChE activity in rats (Rodrıguez *et al.*, 2003), arsenic accumulation in brain tissue of bats at WWTW was not associated with a decrease in AChE activity. Moreover, raw sewage may contain other AChE inhibitors such as carbamates and organophosphates, and treatment of sewage may fail to eliminate these AChE inhibitors (Hendricks and Pool, 2012). I did not quantify levels of these inhibitors in bat tissues, but their presence can potentiate the effects of iron and arsenic.

Arsenic-induced oxidative stress can increase free radical production (Ercal *et al.*, 2001) and induce an antioxidant response, which may have prevented the inhibition of AChE activity, and one would therefore expect increased TAC of brain tissue in WWTW bats that exhibit high arsenic levels.

4.3. Total antioxidant capacity in brain tissue of WWTW bats is compromised

I found significant lower TAC in brains of bats foraging at WWTW than bats at reference sites, supporting evidence significantly low TAC in muscle tissue of WWTW bats (Naidoo *et al*., 2015). Measurements of TAC do not fully quantify all antioxidants present (Young, 2001). Regardless, the low TAC levels in the brain warrants a full analysis of redox state in the brain tissue of WWTW bats (Naidoo *et al*., 2015). Arsenic exposure has previously been implicated in decreasing antioxidant capacity (Wu *et al.*, 2001), and potentially contributing to AD (Gong and O'Bryant, 2010), specifically when the decreased antioxidant capacity co-incides with lipid peroxdation or damage to DNA and proteins.

4.4. Lipids are not damaged by foraging at WWTWs

Despite high lipid content, there were no detectable amounts of lipid peroxidation present in the brains of WWTW bats. The high polyunsaturated fatty acid content of the brain and its high demand for oxygen make it a target for lipid peroxidation (Sultana *et al.*, 2006), especially if antioxidant capacity is compromised. My results suggest that antioxidant responses, although compromised, are still adequate in protecting the brain against lipid damage induced by metals or metal-induced oxidative stress. Arsenic-induced AD (Gong and O'Bryant, 2010) is therefore unlikely as long as lipids are still intact, unless other biomolecules such as DNA and protein are sufficiently damaged to result in neurodegeneration.

4.5. DNA integrity was not compromised nor was apoptosis induced in WWTW bats

Contrary to my prediction, mean DNA integrity for bats foraging at Verulam and Umbilo WWTWs was not significantly lower than bats collected from the reference sites, nor was the caspase-3 activity (indicating the onset of apoptosis) elevated in the WWTW bats. An increase in metal-induced ROS production causes DNA damage and activates apoptosis if DNA damage is beyond repair (Zocche *et al.*, 2010; Chandra *et al*., 2000; Chen *et al*., 2001). In addition, metals such as arsenic may induce oxidative stress resulting in oxidative DNA damage (Piao *et al*., 2005) and may inhibit DNA repair mechanisms by disrupting damage-specific DNA-proteins that identify damaged DNA and other processes associated with nucleotide excision repair involved in DNA repair(Hartwig, 1998; Beyersmann and Hartwig, 2008). Conversely, Naidoo *et al.* (2015) found

significantly higher DNA damage in blood of bats at Verulam and Umbilo WWTWs. Because I found significantly high amounts of arsenic in the hair of bats at these WWTWs, it could be possible that circulating metals are causing DNA damage via increased metal-induced ROS production (Zocche *et al.*, 2010), whereas metal levels in the brain might be too low to cause significant DNA damage. Because the BBB provides a barrier for compounds into the brain, one would expect brain metal levels to be lower than that in circulation. In addition, DNA repair mechanisms may be efficient in protecting the brain from DNA damage – by repairing and removing direct or indirect metal-induced DNA lesions (Lodish *et al.*, 2000). However, DNA repair mechanisms in the brain may not necessarily be more efficient than in other tissues given that DNA glycosylase levels (involved in excision base repair) do not differ in brain, heart, muscle or kidney tissues (Karahalil *et al*., 2002). Metal-induced ROS may also damage DNA via an intrinsic pathway, activating p53 proteins, which in turn activate caspase-3 thereby inducing apoptosis (Chen *et al*., 2001; Pulido and Parrish, 2003).

Contrary to my predictions, I found no significant differences in caspase-3 levels in brain tissue among sites. This may indicate that the concentration of metals is not high enough to induce apoptosis in cellular tissue or DNA repair mechanisms may be effective in repairing low levels of damage in brain tissue. For example, the prion protein can efficiently stimulate base excision repair in mammals while also maintaining cell survival in neurons where the DNA was damaged bygenotoxicants (Bravard *et al*., 2015). Conversely, environmental metals may disable and suppress apoptosis thereby causing an accumulation of abnormal cells, which may be involved in the pathogenesis of autoimmunity (Rana, 2008). One would then however expect an accumulation of DNA damage (Chukhlovin *et al.*, 2001), which was not found in the current study.

Although more sensitive methods may reveal subtle differences in DNA integrity between bats at WWTWs and non-polluted reference sites, the absence of AChE inhibition, lipid peroxidation and apoptosis suggests that DNA repair mechanisms in brains of bats from WWTWs are either highly efficient or metal levels are too low to cause significant damage.

4.6. Future work

I used rather crude biomarkers of damage in this exploratory study. To better understand the subtle effects of bioaccumulation of toxic metals through the food chain on bats, my work can be refined and supplemented by other physiological biomarkers of exposure, such as molecular lesions and disease markers (Kakkar and Jaffery, 2005). A more thorough analysis should now be done on the

specific mechanisms of arsenic toxicity and the implications it may have for neurodegeneration. The reduced antioxidant capacity measured in my study can result in arsenic-induced oxidative stress, and cause neuronal death and aid in the formation of brain amyloid plaques and neurofibrillary tangles (Gong and O'Bryant, 2010; Prakash *et al.*, 2016). Quantifying these signs of neurodegeneration through the use of cell cultures or magnetic resonance imaging could give more insight into other neurological effects of pollutant exposure on bat brains that may still affect their ability to effectively feed, fly and echolocate.

Given that Naidoo *et al.* (2015) found DNA damage in the blood of WWTW bats and arsenic may play a role in the inhibition of DNA repair (Beyersmann and Hartwig, 2008), I suggest supplementing my existing data with analyses to quantify DNA adducts and DNA-protein cross links, two forms of DNA damage that might not necessarily result in DNA fragmentation (Bau *et al*., 2002). In addition, mitochondrial efficiency analysis (Prakash *et al.*, 2016) may shed light on the decrease in antioxidant capacity because arsenic exposure can down regulate mitochondrial antioxidant enzymes such as superoxide dismutase and catalase in different regions of rat brains (Ram Kumar *et al*., 2013). Furthermore, arsenic may impair mitochondrial function by decreasing the production of adenosine-5'-triphosphate (ATP) and inhibiting the reduction of NADH to $NAD^+ + H^+$ during oxidative phosphorylation (Hughes, 2002; Prakash *et al.*, 2016).

Pollutants present in wastewater as a consequence of inadequate treatment processes and/or raw sewage, such as organophosphates from pharmaceutical waste and carbamates (Hendricks and Pool, 2012), should be quantified carefully because these chemicals may inhibit synapses and other neurotransmitters. To further explore the effects of these contaminants on the brain, neurotoxicity PCR Array (Qiagen®, Hilden, Germany) may be a promising approach. It quantifies gene expression changes before cellular/physiological changes occur. Although it is a human-specific array, neurotoxicity PCR Array has an array of potential toxicity biomarkers to detect many neurotoxic responses including motor neuron axonopathy and developmental neurotoxicity, and therefore may give insight into neurotoxicity in bats.

4.7. Conclusion

This study shows that WWTW bats may be vulnerable to bioaccumulation of metals, especially the non-essential metal arsenic, in their brain tissue. The high concentrations of arsenic in the hair suggest that although the brain has a protective barrier, a fraction of the circulating arsenic can cross the BBB (Prakash *et al.*, 2016). This may cause adverse effects such as neurodegeneration (Gong

and O'Bryant, 2010) in the long-term. The biomarkers of neuron function, lipid integrity, DNA integrity and cell viability measured in this study did not differ significantly between *N. nana* foraging at WWTWs and reference sites, but antioxidant response was compromised. Long-term exposure to anthropogenic pollutants at WWTWs may therefore affect cellular processes and protection mechanisms in brains of *N. nana* bats and may in extreme circumstances result in mortality, and ultimately, declines of populations (Jones *et al.*, 2009). Although not quantified in this study, these effects may be exacerbated with age (Kurta *et al.*, 1989) because long lifespans make bats more susceptible to toxic metals through increased exposure time.This increasing effect of age may explain some of the variation in the data.

Furthermore, exposure to pollutants at WWTWs is not limited to *N. nana* bats but may also affect other bat species such as *Myotisbocagii*, *Chaerephonpumilus* and *Pipistrellushesperidus*, and other animals that utilize these habitats. Regardless, bats may be particularly good models for gauging the possible physiological changes in mammals exposed to high levels of anthropogenic pollution (Zukal *et al.*, 2015).

In South Africa, many of the WWTWs are not adequately maintained, resulting in effluent of questionable quality (DWAF, 2011; Mema, 2010; Sacks and Buckley, 2004). In addition, the effects of rapid urbanisation such as increased industrial effluent, mining activities, and natural weathering of rocks may result in water hotspots with high metal concentrations (Dallas and Day, 2004). Long-lived mammals, including humans, utilizing these environments may be particularly vulnerable to exposure. Communities living in proximity to polluted rivers rely on these areas as their primary source of scarce resources such as water and fish, and exposure to pollutants is therefore inevitable (Mema, 2010; Obi *et al*., 2002). These links in the food chain can cascade into other ecosystems. Long-term accumulation of metals such as arsenic and iron may potentially result in neurodegenerative diseases. Currently, we can only speculate on the ramifications and effects of pollutants on brain function in all species utilizing WWTWs and other polluted water sources.

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