Sex rather than wastewater associated stresses determines intestinal bacterial communities in the insectivorous bat, *Neoromicia nana*

by

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PREFACE

The research contained in this dissertation was completed by the candidate while based in the Discipline of Biological Sciences, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville, South Africa. The research was financially supported by the National Research Foundation through grant CSUR14080687212 to Dr Vosloo, Prof Schoeman, Prof Bezuidenhout and Prof Preiser; and a scarce skills bursary to Mr Mehl.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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DECLARATION 1: PLAGIARISM

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(ii) this dissertation has not been submitted in full or in part for any degree or examination to any other university;

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ABSTRACT

Wastewater treatment works (WWTWs) receive influent from domestic, agricultural and industrial sources, producing a cocktail of toxicants at these sites. WWTWs are unable to remove all the toxicants or bacterial and viral pathogens in the wastewater before it is released into surrounding ecosystems. Large amounts of nutrients in the wastewater supports abundant populations of chironomid midges (Diptera), that transfer these toxicants to their predators, such as *Neoromicia nana* (Vespertilionidae), resulting in numerous biochemical and metabolic effects. However, little is known if foraging at WWTWs affects the intestinal bacteria of bats. This study compared intestinal bacteria communities that play essential roles in nutrient absorption and immunity in their hosts between *N. nana* populations at WWTWs and reference sites. I hypothesised that bacterial communities of *N. nana* should differ between individuals foraging at WWTWs and reference sites. Next generation sequencing was used to identify intestinal bacteria of bats at two reference sites (Buffelsdrift and Inkunzi) and two WWTWs (Verulam and Umbilo). Differences in intestinal bacterial loads (at each taxonomic level) and host attributes (sex, body condition, locality) of individuals were quantified using the Gower distance measure. Hierarchical cluster analysis was used to identify the factors that determine the similarity between individuals. As predicted, bats at WWTW sites showed greater intestinal bacteria diversity than those at reference sites. This is likely due to exposure to the high diversity of bacteria within wastewater. Further, differences in certain bacterial taxa, such as the family Chitinophagaceae, may be due to differences in diet between WWTWs and reference site bats. Statistical analyses revealed that sex, and site to a lesser degree, were the best predictors of similarity in intestinal bacteria communities among *N. nana* bats. Because bacterial diversity did not correlate with body condition, sex-specific factors (such as sex hormones) may be the greatest drivers of these differences. Further, site specific factors such as toxicant and ectoparasite exposure likely had some influence on the difference observed between reference and WWTWs bats. Dysbiosis of intestinal bacterium communities, because of wastewater exposure, may have significant sex-biased impacts on host metabolism and immune functioning.

Keywords: Intestinal microbiota, wastewater, *Neoromicia nana*, sex

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ACRONYMS

WWTWs = wastewater treatment works

PCB = polychlorinated biphenyl

PAH = polycyclic aromatic hydrocarbon

GI = gastrointestinal

UV = ultraviolet

IBD = Inflammatory Bowel Disease

NMDS = non-metric multidimensional scaling

BCoAT = butyryl CoA transferase

FTHFS = formyltetrahydrofolate synthetase

PUFA = polyunsaturated fatty acid

Ig = immunoglobin

PCR = polymerase chain reaction

BCI = body condition index

EDTA = Ethylenediaminetetraacetic acid

rRNA = ribosomal ribonucleic acid

DNA = deoxyribonucleic acid

SARS = severe acute respiratory syndrome

OTU = operational taxonomic unit

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CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

The majority of the global human population now resides in urban areas (King, 2014). As urban populations rapidly increase (Angel et al., 2011), the urban microbiome grows increasingly more relevant (King, 2014). By 2050, the global human population size is predicted to reach between 8 and 10 billion people (Lutz and Samir, 2010), significantly increasing the strain on global ecosystems. The increase in pollution (Wei and Yang, 2010), infectious diseases (Alirol et al., 2010) and other stressors associated with urbanisation, puts urban inhabitants increasingly at risk of health complications. Further, urban expansion increasingly exposes animals in urban environments to anthropogenic stress factors (Vitousek et al., 1997), and humans to disease harbouring organisms (Plowright et al., 2011). Wastewater treatment works (WWTWs) are ubiquitous and one of the most prolific sources of pollution and environmental stress in urban landscapes (Cai et al., 2014).

WWTWs treat industrial and domestic wastewater. These sites are associated with high concentrations of metals (Naidoo et al., 2013), pharmaceuticals (Kolpin et al., 2002), microbial pathogens (Okoh et al., 2007), natural and synthetic hormones (Huang and Sedlak, 2001), antibiotics (Watkinson et al., 2009) and organic chemicals (Kolpin et al., 2002). Inorganic nitrogen and phosphorous released from these sites result in the eutrophication of downstream ecosystems (Abbott et al., 2009), thereby significantly increasing algal and moss growth while simultaneously decreasing the abundance and species richness of fish (Kalcounis-Rüppell et al., 2007), insects (Couceiro et al., 2006), clams (Powers et al., 2005), crabs and shrimp (Pihl et al., 1991). Further, the nutrient rich waters at and downstream from these sites allow large numbers of pollutant tolerant (Postma et al., 1995) insects to thrive (Abbott et al., 2009; Naidoo et al., 2013; Park et al., 2009). These insects accumulate toxicants such as metals, pesticides, polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) from the sediment and water during their larval stage and pass them on to predators such as bats and birds, which are attracted to these sites by the high concentrations of prey items (Reinhold et al., 1999, Naidoo et al., 2013). Recent studies have confirmed that bats foraging at WWTWs accumulate metals in their tissues (Naidoo et al.,

2013, 2015), leading to increased DNA damage, decreased antioxidant capacity (Naidoo et al., 2015) and lesion formation in the liver and kidneys (Naidoo et al., 2016).

The effects of toxic chemicals on fish and mammalian physiology is well-documented (Kannan et al., 2000; Sanderson et al., 2003; Tanabe, 2002), their general mode of action is mostly known (Álvarez, 2006; Narahashi, 1974), and some information exist about the physiological effects of pollutant mixtures (Schidt et al., 2005). In contrast, very little is known about the effects of pollutants on symbiotic intestinal bacteria (Liu et al., 2014) and almost nothing is known about the effect the pollutant cocktails associated with WWTWs on these symbionts. Intestinal bacteria play a key role in behaviour (Kraus et al., 2016), immune function (Collins and Bercik, 2009; Dillon et al., 2005; Fins et al., 2000), nutrient absorption (Collins and Bercik, 2009; Ley et al., 2005), the absorption and storage of fats (Bäckhed et al., 2004), and the detoxification of ingested metals and other pollutants (Breton et al., 2013a; Hooper et al., 2001). Because the hosts' health depends so heavily on their intestinal bacteria, the effects WWTWs have on this symbiosis is important.

Of significant importance to intestinal bacterial communities are the bacterial contaminants found in wastewater (Okoh et al., 2007). Bacteria found in wastewater influent reflect the human microbiome. These bacteria are largely unable to survive in the aerobic environment of the activated sludge at WWTWs (Cai et al., 2014). However, a diverse array of pathogenic bacteria can still be found in the effluent (Cai et al., 2014). Some bacterial pathogens - particularly those most resistant to chemical, filtration and UV treatments - may propagate and become more abundant in effluent (Cai et al., 2014). Because this effluent is released into nearby ecosystems, the chemical and biological contaminants therein are highly relevant to surrounding ecosystems and human communities. In fact, traces of human gut bacterial genes were found in river water tens of kilometres downstream from WWTWs (Meziti et al., 2016). Moreover, emerging Chironomid midges, exposed to *Vibrio cholerae* within wastewater from egg to adult stages, are able to transfer viable pathogenic bacteria from wastewater to clean waterbodies (Broza et al., 2005). Insectivorous bats such as *Neoromicia nana* feed on insects emerging from effluent (Abbott et al., 2009; Naidoo et al., 2013; Park et al., 2009), thereby exposing themselves to these bacterial pathogens. These pathogens may have significant implications for the health of these individuals (Pierlé et al., 2015). Although nothing in

known on the transmission of pathogens to humans via this species, bats in general are prolific reservoirs for viral and bacterial pathogens (Hayman et al., 2013).

1.2 Factors influencing the intestinal microbiota

The intestinal microbiota comprises all organisms (bacteria, fungi, viruses, etc.) in the gastrointestinal (GI) tract of organisms (Virgin, 2014). In mammals, an infant's microbiome composition is initially determined during birth when the infant is inoculated with the mother's vaginal and faecal microbiota (Koenig et al., 2011). The mother may also influence the infant microbiota before birth, by transferring bacterial or viral infections to the foetus (Loughran and Tuomanen, 2016), and after birth by transferring probiotic lactic acid bacteria during breastfeeding (Martín et al., 2003). This early inoculation of the foetal or infant microbiota is in part essential for development of their immune system, hormonal control, mood, and behaviour (Jenkins et al., 2016). Later in life the microbiome is influenced by many factors including the host's environment, physiology and social interactions (Archie and Theis, 2011). Diurnal changes in some intestinal bacterial loads may occur naturally, driven by the host's natural circadian rhythm (Leone et al., 2015). For example, the phylum Cyanobacteria (Ley et al., 2005) have their molecular clock controlled by a transcriptional feedback loop similar to that in humans, that controls their circadian rhythm (Voight et al., 2016). Further, exercise can significantly alter the intestinal microbiome composition through the increased (such as the phylum Firmicutes, specifically the order Lactobacillales) or decreased (such as the phyla Tenericutes and Bacteroidetes) abundance of certain bacteria (Choi et al., 2013). Factors such as sex and diet may also significantly impact Fusobacteria (Bolnick et al., 2014). For example, high-fat diets increase *Lactobacillus* and *Clostridium* abundances in males, but have the opposite effect in females (Bolnick et al., 2014). The interaction of intestinal microbiota with sex-hormones may have sex-biased effects on immunity and disease progression, such that females are typically more susceptible to certain autoimmune diseases than males (Dominianni et al., 2015; Gomez et al., 2015). Differences in intestinal microbiota between sexes, where females generally have a greater diversity than males (Cong et al., 2016; Phillips et al., 2012), are reversed by male castration, thereby confirming the role of sex-hormones in these differences (Yurkovetskiy et al., 2013). Host sex-specific differences in intestinal microbiota also modulate the hippocampal serotonergic system (Clarke et al., 2013) and gut microbial dysbiosis in males can cause a decrease in

serum serotonin levels and an increase in anxiety-like or depressive behaviour (Clarke et al., 2013). Further, host body condition (as a ratio of body mass and height or limb length) shows a significant correlation with intestinal microbiota in females but not males (Dominianni et al., 2015). The gut microbiome may differ between populations with different diets and across geographical distances (Yatsunenko et al., 2012).

A high diversity of intestinal microbiota is essential as it increases the community's resilience to environmental changes (Elmqvist et al., 2003). Further, the disruption of a healthy microbial community can directly affect an organism's health by causing diseases such as obesity, diabetes, osteoporosis (Hernandez et al., 2016), and inflammatory bowel disease (IBD) (Lozupone et al., 2012); and affect behaviour through poor memory, reduced cognition (Jenkins et al., 2016) and reduced aggression (Sylvia et al., 2017). Describing a species' typical microbiome composition is a difficult task due to the high degree of interindividual and temporal variation (Yatsunenko et al., 2012), and many endogenous and exogenous factors that influence their community composition (Lozupone et al., 2012). However, altering the intestinal microbiome may significantly reduce the intestinal barrier, thereby exposing the host to bacterial infection (Compare et al., 2012). Because wastewater contains a diverse array of bacterial pathogens (Okoh et al., 2007), which can potentially be passed to insectivorous bats such as *N. nana* feeding on emerging insects (Broza et al., 2005), this urban ecosystem provides a unique example of human-animal pathogen transfer.

1.3 The impact of wastewater exposure on intestinal bacteria

Organisms at WWTWs may be exposed to environmental pollutants and pathogens through contact with or the ingestion of contaminated water (Sadhukhan et al., 1997) or food (Broza et al., 2005; Naidoo et al., 2013). Further, inhaled particulate air pollution at these sites, often containing metals (Costa and Dreher, 1997) and PAHs (Sarkar and Khillare, 2013), is transported from the lungs to the gut via mucociliary clearance (Beamish et al., 2011) where it may cause inflammation of the intestine, altered immune gene expression, decreased immune response and increased gut permeability (Kish et al., 2013). The interaction of metals and intestinal bacteria is antagonistic where bacteria may detoxify ingested metals (Upreti et al., 2004) or metals may impair bacterial growth (Liu et al., 2014).

The ability of hosts to cope with prolonged metal exposure is dependent on intestinal bacteria both directly through their metabolism of metals, and indirectly through their ability to alter host metallothionein gene expression (Breton et al., 2013a). However, both acute and chronic metal exposure may result in reduced intestinal bacterial diversity and altered microbial community structure (Lapanje et al., 2007). For example, chronic mercury exposure decreases intestinal bacterial species richness in the terrestrial isopod *Porcellio scaber*, favouring species with a higher mercury tolerance (Lapanje et al., 2010; Sadhukhan et al., 1997). The loss of metal intolerant bacteria provides vacant niches which are subsequently filled by more tolerant bacterial species (Coolon et al., 2010). In mice, prolonged oral exposure to lead and cadmium results in an altered commensal bacterial community with a decrease in the family *Lachnospiraceae* and increases in the families *Lactobacillaceae* and *Erysipelotrichaceacae* (specifically *Turicibacter spp*) (Breton et al., 2013b). However, many factors influence intestinal microbiota and their reaction to toxicants. Thus, metal pollution does not always have similar effects on these microbial communities in different host species. For instance, prolonged exposure to cadmium resulted in significantly reduced *Bacteroidetes* growth in mice (Liu et al., 2014), whereas metal (including cadmium) pollution, favoured *Bacteroidetes* in the toad, *Bufo raddei* (Zhang et al., 2016). Because metals such as cadmium impair gut barrier and decreased mucus layer thickness (Liu et al., 2014), animals exposed to these chemicals may be increasingly susceptible to gastrointestinal infection (Compare et al., 2012).

Untreated or inadequately treated wastewater, released into surrounding ecosystems, may expose organisms to bacterial, viral, protozoal, fungal and helminth infections (Okoh et al., 2007). One of the most commonly used wastewater treatment systems, the activated sludge system, uses large densities of bacteria (as well as fungi, protozoa, rotifers and nematode worms) to break down organic waste (Okoh et al., 2007). Viruses and bacteria, shed from humans and other hosts, make their way into WWTWs (Okoh et al., 2010) where they are removed via several methods including filtration, chemical and UV treatment (Bohrerova and Linden, 2006). However, even chlorine and UV irradiation disinfectant techniques are unable to completely remove bacterial and viral pathogens (Okoh et al., 2010, 2007) and helminth eggs (Mahvi and Kia, 2006). Contact with or the consumption of water polluted with bacterial pathogens (such as *Escherichia coli*, *Staphylococcus aureus* and *Salmonella spp*) may result in outbreaks of gastrointestinal diseases throughout the food web (Okoh et al., 2007).

Another wastewater contaminant important to intestinal bacteria is the large quantity of antibiotics administered to humans and livestock which make their way into rivers and WWTWs (Kümmerer et al., 2000; Watkinson et al., 2009). Filtration techniques used by most WWTWs are unable to adequately remove many of these antibiotics from the water (Batt et al., 2006; Watkinson et al., 2007). These antibiotics, when released into the environment, may reduce beneficial bacteria while promoting drug resistant bacterial strains (Batt et al., 2006; Renew and Huang, 2004; Watkinson et al., 2007). For example, ingestion of ciprofloxacin, one of the antibiotics most commonly found in wastewater (Watkinson et al., 2007), results in the rapid loss of gut bacterial diversity and a significant shift in intestinal bacteria community structure in humans, which is unlikely to return to the initial state (Dethlefsen and Relman, 2011).

Faced with the overwhelming number of toxicants and pathogens at WWTWs, the indirect effects of association with these sites on an organism's health are often overlooked. The chironomid, and resultant polyunsaturated fatty acid (PUFA)-rich, diet of bats at WWTWs results in increased leptin production (Mehl et al., 2016), thereby possibly influencing their ability to arouse from torpor. With gut microbiota playing a role in functions such as protein absorption and lipid and carbohydrate storage, dysbiosis of this community may result in changes in metabolism and body mass (Wong et al., 2014). Moreover, Liu et al. (2014) found that the genes involved in metabolising carbohydrates to short chain fatty acids (*BcoAT* and *FTHFS*) are downregulated when mice are exposed to cadmium. This has further implications for substrate utilisation, fat storage and arousal capacity. An altered fatty acid intake may further enhance the growth of certain harmful bacteria, increase gut permeability and potentially cause infection (Shen et al., 2014). Because the liver, the body's first defence against intestine-derived pathogens, receives 70% of its blood from the intestine, dysbiosis of intestinal microbiota and increased gut permeability may cause translocation of gut microbes into the hepatic portal system (Compare et al., 2012), and exacerbate metal-induced liver damage. Intestinal bacteria also play an important role in the abundance and diversity of viruses within the GI tract of animals because the majority of these viruses are bacteriophages (Reyes et al., 2011). Because bacteriophages have varying degrees of host specificity (Koskella and Meaden, 2013), changes in virus diversity are often closely linked to changes in intestinal microbiota (Minot et al., 2011).

Some of the viruses receiving much attention today (such as SARS, Hendra, Ebola, Marburg and Nipah viruses) can be found in bats (Donaldson et al., 2010; Wang et al., 2011). Moreover, bats also harbour bacterial pathogens from the phyla Chlamydiae (Pierlé et al., 2015), Actinobacteria (Alirol et al., 2010) and Proteobacteria (Banskar et al., 2016). As humans encroach on natural ecosystems and impose increasing stress on natural resources, humans may be increasingly exposed to infection-carrying animals (Calisher et al., 2008). At the same time, natural ecosystems are increasingly exposed to human influences. WWTWs are a unique ecosystem where animals are exposed to a cocktail of pollutants and pathogens derived from human influence.

In this study, the intestinal microbiota of *Neoromicia nana* at two WWTWs (Verulam and Umbilo) and two reference sites (Buffelsdrift and Inkunzi) in KwaZulu-Natal, South Africa were compared. The aims of this study were twofold: (1) to determine how the intestinal bacterial communities differ between bats at WWTWs and reference sites, and (2) to determine the most significant determining factors of intestinal bacteria communities (i.e. sex, site or body condition) in these bats. Intestinal bacterial communities, determined using next-generation sequencing, and host attributes are compared using network analyses of the Gower distances between individuals. A higher diversity of intestinal bacteria was expected in bats at WWTWs due to their exposure to wastewater. Further, host sex and site were predicted to have the greatest influence on intestinal bacterium communities.

CHAPTER 2: MATERIALS AND METHODS

2.1 Sampling

Neoromicia nana were collected at four sites during April 2015: Umbilo (29.845283°S 30.890776°E) and Verulam (29.646241°S 31.063543°E) WWTWs and Inkunzi Lodge (28.565201°S 31.241312°E) and Buffelsdrift (29.756730°S 30.678980°E) reference sites. Bats were captured using mist nets at WWTWs and by hand from roosts at reference sites. Individuals were identified to species using a taxonomic key (Monadjem et al., 2010). Non-target animals were released at the capture site. Captured *N. nana* bats were sexed and, by examining the ossification of the finger bones (Kunz and Anthony, 1982), aged (adult or sub-adult). Forearm length (to the nearest 0.1 mm) and mass (to the nearest 0.5 g) were measured using calipers and a Pesola scale, respectively. Body condition index (BCI) was calculated as body mass/forearm length (Speakman and Racey, 1986). Scat samples were collected and placed in RNAlater for virus analysis. Bats were kept individually in cotton bags overnight. The following morning, bats were humanely euthanised (University of KwaZulu-Natal Animal Ethics Committee, Reference: 014/15/Animal) and dissected. Tissues were weighed and frozen in dry ice or liquid nitrogen before being stored at -80°C until further analysis.

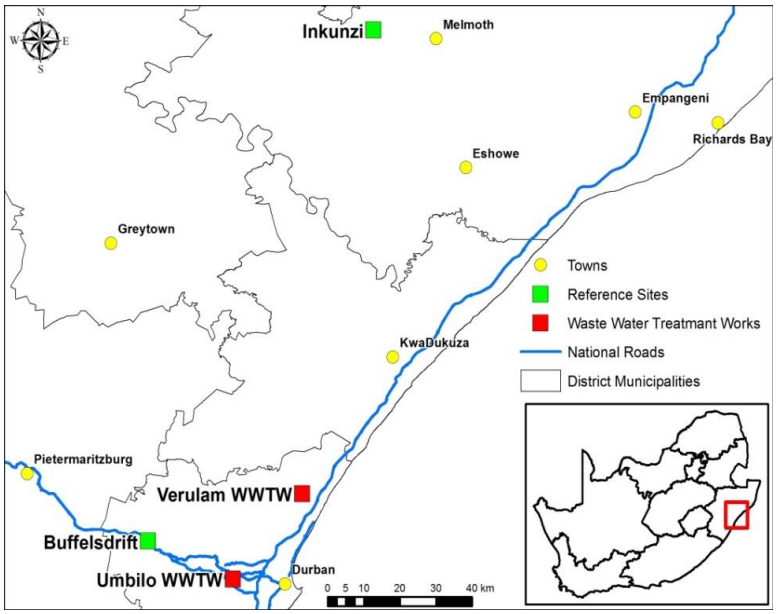

Figure 1: Locality map of wastewater treatment works (WWTWs) and reference sites, KwaZulu-Natal, South Africa. Red squares represent WWTWs and green squares represent reference sites. Figure taken from Mehl et al. (2016).

2.2 DNA extraction and quantification from intestinal scrapings

Using sterilized equipment, the bat intestines were dissected from the stomach, cut longitudinally and the interior was scraped to remove gut microbes. Genomic DNA was extracted from these scrapings using a NucleoSpin® Tissue kit (Macherey-Nagel, Germany). DNA concentrations were measured using a NanoDrop (Thermo Scientific).

2.3 Bacterial 16S rRNA gene sequence amplification

A partial gene sequence for bacterial 16S rRNA was amplified using universal 16S rRNA 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACTT-3') primers (Inqaba Biotec, Pretoria, RSA) and a Prime Elite thermocycler (Techne, United Kingdom). The PCR mix (final volume of 25 µL) contained 1 µL (100-200 ng) DNA, 10.5 µL nuclease-free water, 1 µL primers (1 µmol L^{-1}) and 12.5 µL 2x DreamTaq PCR Master Mix (ThermoFisher Scientific, Massachusetts, United States). The PCR cycle consisted of a 5 min initial denaturation at 95°C followed by 35 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 53 °C and 1 min elongation at 72°C, and a final elongation at 72°C for 10 min. This produced a 1465bp amplicon (Lane, 1991). The PCR products and a molecular marker (GeneRuler 1kb DNA Ladder, ThermoFisher Scientific, Massachusetts, United States) were then resolved by gel electrophoresis in a 1% agarose gel (w/v) in 1x TAE buffer (40 mmol L^{-1} Tris, 20 mmol L^{-1} acetic acid, 1 mmol L^{-1} EDTA) at 80 V for 45 min. GelRed™ Nucleic Acid Gel Stain (Biotium, USA), incorporated with the loading dye, was used to visualise bands under UV light using a Gene Genius Bio-Imaging System (Syngene, Synoptics, UK).

2.4 Optimising DNA amplification

The initial bacterial 16S rRNA gene sequence amplification (above) resulted in a smear of amplicons and could not be used directly for sequencing purposes. These amplicons were therefore reamplified with universal 16S rRNA primers 27F and 1492R. This PCR was carried out using 1 µL (100-200 ng) DNA, 2x KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Massachusetts, United States) and 0.5 µM of each of the 27F and 1492R primers in a final volume of 10 µl. The PCR cycle consisted of an initial 2 min denaturation at 98°C followed by 25 cycles of denaturation at 98°C for 15 sec, annealing at 55°C for 30 sec and

elongation at 72°C for 20 sec, with a final elongation step at 72°C for 5 min. The product and a molecular marker (GeneRuler 1kb DNA Ladder, ThermoFisher Scientific, Massachusetts, United States) were resolved on a 1% agarose gel in 1x TAE buffer (40 mmol L^{-1} Tris, 20 mmol L^{-1} acetic acid, 1 mmol L^{-1} EDTA) at 80 V for 30 min. The gel was stained with ethidium bromide and visualised under UV light, but only a smear of PCR products were visible.

A nested PCR was carried out with 0.5 µL of a 1:50 dilution of the PCR product from the previous step. This was done using primers recommended by Illumina for the preparation of the amplicon library following the Illumina 16S metagenomics sequencing library preparation guide (Illumina), with the exception of the PCR reaction conditions. The hypervariable V3-V4 region (≈460 bp) of the bacterial 16S rRNA gene was targeted using locus-specific primers 341F and 805R (Klindworth et al., 2013) attached to Illumina forward and reverse overhang adapters (Illumina) (16S forward primer 5'–TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG –3': 16S reverse primer 5'– GTCTCGTGGGCTCGGAGATGTGTATAAG AGACAGGACTACHVGGGTATCTAATCC –3'). All PCRs were performed in a PCRmax thermal cycler (PCRmax, Staffordshire, United Kingdom). The region of interest was amplified using the larger fragment as template using 2x KAPA HiFi HotStart ReadyMix and 0.4 µmol L^{-1} of the Illumina primers (above) in a final volume of 25 µL, using the same cycling protocol from the previous amplification. The product and a molecular marker (GeneRuler 1kb DNA Ladder, ThermoFisher Scientific, Massachusetts, United States) were resolved on a 1% agarose gel in 1x TAE buffer (40 mmol L^{-1} Tris, 20 mmol L^{-1} acetic acid, 1 mmol L^{-1} EDTA) at 80 V for 30 min. The gel was stained with ethidium bromide and visualised under UV light.

2.5 PCR clean-up, quantification and next generation sequencing

Agencourt AMPure XP beads (Beckman Coulter Genomics, California, USA) were used to clean-up the amplicons obtained in 4.4. Thereafter, a PCR reaction attaching dual indexes (Nextera XT Index Kit) was performed using 5 µL of the PCR amplification product, 5 µL of Illumina Nextera XT Index Primer 1 (N7xx), 5 µL of Nextera XT Index Primer 2 (S5xx), 25 µL of 2x KAPA HiFi HotStart Ready Mix, and 10 µL of PCR-grade water. This was performed with an initial denaturation step at 95°C for 3 minutes followed by 8 cycles of

denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, elongation at 72°C for 30 sec and a final elongation step at 72°C for 5 minutes. This was followed by a second PCR clean-up step of the index PCR products using Agencourt AMPure XP beads (Beckman Coulter Genomics, California, USA).

A Qubit fluorometer (Qubit 3.0, Life Technologies, Malaysia) was used to quantify the partial 16S rRNA libraries which were normalised, pooled to a final concentration of 20 pmol L^{-1}, and denatured in 0.2 mol L^{-1} NaOH. Prior to loading samples on the MiSeq V3 reagent cartridge (Illumina, San Diego, CA, USA), the pooled library was diluted to a final concentration of 4 pmol L^{-1}, spiked with 5% PhiX control and heat denatured for 2 min. A 2 x 300 bp paired-end reads sequencing run on the Illumina MiSeq was then performed. This was followed by de-multiplexing and secondary analyses of the reads using the MiSeq reporter software (Illumina, San Diego, CA, USA) as per manufacturers protocol.

2.6 Sequence Analysis

Prior to the merging of forward and reverse reads, primer and adapter sequences as well as low quality (score less than 15) and short reads (fewer than 25 bp) were removed using Trimmomatic (v0.36) (Bolger et al., 2014). Quantitative Insights Into Microbial Ecology (QIIME™, Caporaso et al., 2011) was used to analyse trimmed sequences. PandaSeq was used to merge forward and reverse reads (Masella et al., 2012). Only sequences of length 200-675bp were used with an overlap of 50-200bp and a threshold of 0.8. Singletons were removed and open reference OTUs were selected from the Silva 128 database (Quast et al., 2013) using usearch61 (Edgar, 2010). A single rarefaction (to the number of reads from the sample with the least number of reads) to 19688 reads was performed to reduce bias between samples of unequal numbers of reads and a summarised taxa table was constructed.

2.7 Statistical analysis

R software v. 3.2.2 (R Development Core Team 2015) was used to perform statistical analyses. Shapiro-Wilk tests and Levene's tests were used to determine normality and homogeneity of variance, respectively. Spearman-rank order correlation matrices were used to determine the relationship between BCI and bacterial diversity at each taxonomic level (n=39

for all correlation matrices). Assumptions were violated for all data, and transformation was unsuccessful in normalising data. Therefore, non-parametric tests were used: Kruskall-Wallis rank sums test to compare taxa loads, forearm length, body mass and BCI between sites, and Wilcoxon signed ranks test to compare taxa loads, forearm length, body mass and BCI between grouped sites and sexes. Dunn's test was used as the post hoc test for the Kruskall-Wallis rank sums test using the dunn.test package (Dinno, 2017) in R. Non-metric multidimensional scaling (NMDS) was used to plot differences between sites in terms of bacterial species occurrence using the vegan package (Oksanen et al., 2017).

Differences between individuals in terms of their gut bacteria does not consider differences between hosts that may influence this microbiome composition. Therefore, differences between individuals in terms of their bacterial diversity and factors that may influence this diversity, including forearm length, body mass, sex and site were quantified with the Gower distance using the cluster package (Maechler et al., 2017) in R. The Gower distance can handle categorical values (such as sex and site) and standardises data. It calculates the final dissimilarity between individuals from the combined distances that were calculated for each variable. This distance measure was used to quantify the differences between all individuals based on these factors. Hierarchical cluster analysis was used to identify clusters in this distance matrix using the pvclust package (Suzuki and Shimodaira, 2015). The distance matrix was used to produce a network with the qgraph package (Epskamp et al., 2012). The network was converted to an igraph object in qgraph (Epskamp et al., 2012) and clusters calculated by the hierarchical cluster analysis were plotted as subgraphs of this network in igraph (Csardi and Nepusz, 2006).

CHAPTER 3: RESULTS

3.1 General differences

Sample size and sex ratio varied between sites (Verulam: 10 males and 2 females, Umbilo: 4 males and 8 females, Buffelsdrift: 5 males and 6 females, Inkunzi: 1 male and 3 females). There were no significant differences in forearm length (χ^2=2.07, df=3, P=0.6), body mass (χ^2=4.2, df=3, P=0.24) or BCI (χ^2=7.29, df=3, P=0.63) between sites. However, females had significantly greater forearm length ($W_{(38)}$=304.5, P=0.0009), body mass ($W_{(38)}$=307.5, P=0.0003) and BCI ($W_{(38)}$=293, P=0.003) than males. There was no significant correlation between BCI and bacterial phylum diversity in males or females (r^2=0.003, P=0.32 and r^2=-0.01, P=0.39, respectively). This was observed at class, order and family levels (class: r^2=-0.033, P=0.54 and r^2=0.07, P=0.14 order: r^2=-0.05, P=0.76 and r^2=0.08, P=0.13, family: r^2=-0.04, P=0.59 and r^2=0.14, P=0.06, respectively. Although no correlation was seen between BCI and bacterial genus diversity in males (r^2=-0.06, P=0.92), a positive relationship was seen in females (r^2=0.18, P=0.04).

3.2 Distribution of intestinal bacterium taxa in bats at different sampling sites

The total number of intestinal bacterium phyla found at Verulam WWTWs was greater than at other sites (Figure 2A). This trend was true at all taxonomic levels measured, with the greatest difference in the number of genera (Figures 2B-2E). Further, bats at WWTWs, specifically Verulam WWTW, had greater numbers of taxa unique to those sites. Bats caught at Buffelsdrift had more taxa in common with bats caught at WWTWs than those caught at Inkunzi (Figures 2A-2E). When grouped, WWTWs sites and reference sites had no significant differences in the number of intestinal bacterium taxa at any taxonomic level (P>0.05). However, when examined separately, bats caught at Verulam WWTWs had, on average, significantly more intestinal bacterium phyla (χ^2=8.94, df=3, P=0.03, Dunn's Test z=-2.99, P=0.001), classes (χ^2=3.70, df=3, P=0.3, Dunn's Test z=-1.73, P=0.042), orders (χ^2=9.68, df=3, P=0.02, Dunn's Test z=-2.95, P=0.002) and families (χ^2=3.77, df=3, P=0.29, Dunn's Test z=-1.70, P=0.045) than bats caught at Umbilo WWTWs (Figure 3). Further, bats caught at Verulam WWTWs had, on average, significantly more intestinal bacterium orders (χ^2=9.68, df=3, P=0.02, Dunn's Test z=-1.92, P=0.027) than bats caught at Inkunzi, and significantly

more genera (χ^2=4.24, df=3, P=0.24, Dunn's Test z=-1.95, P=0.026) than bats caught at Buffelsdrift (Figure 3). The loads of gut bacterial phyla differed greatly between sites (Figure 4). These differences are further explained below.

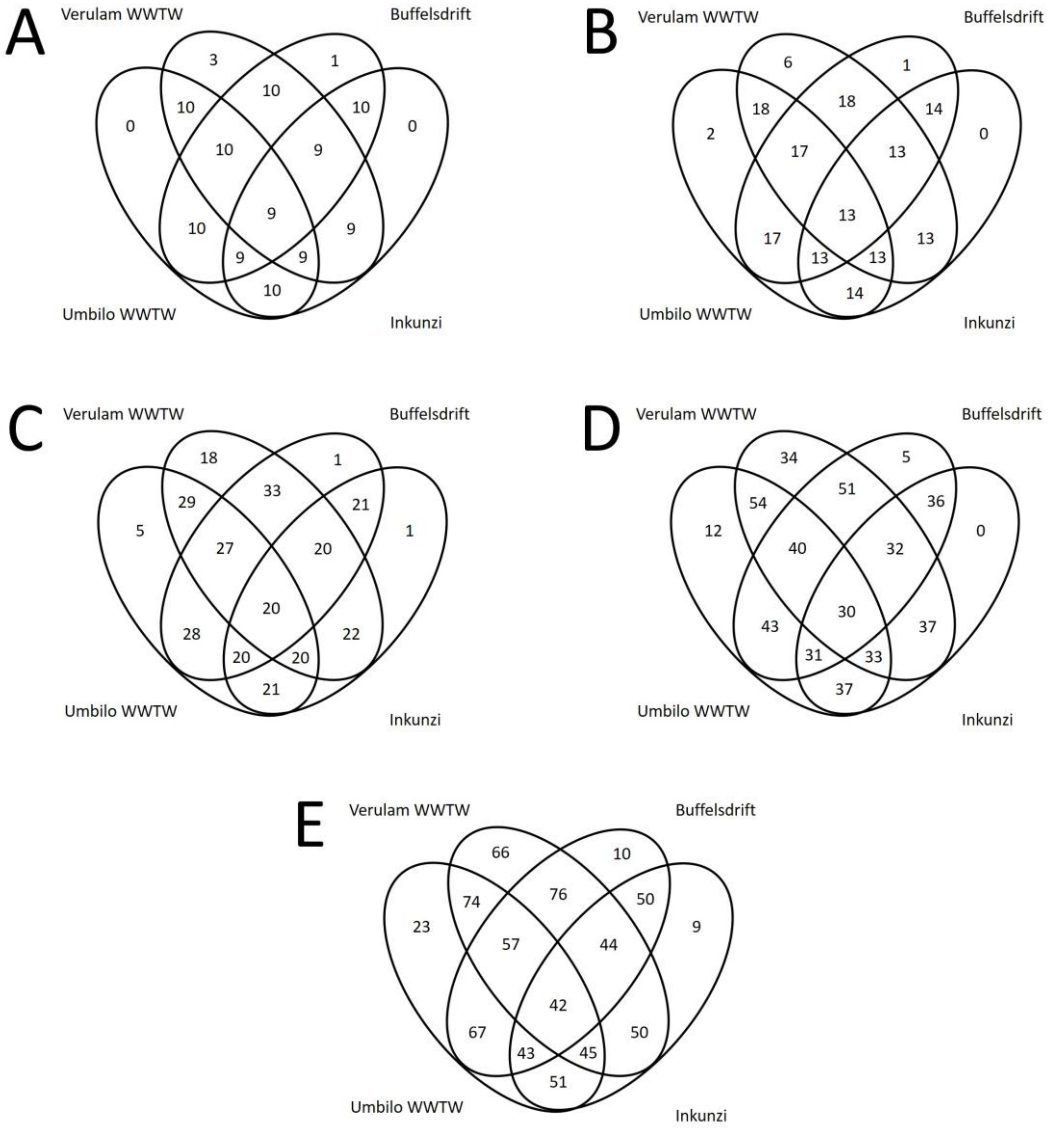

Figure 2: The total number of intestinal bacterial phyla (A), classes (B), orders (C), families (D) and genera (E) in *Neoromicia nana* caught at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa.

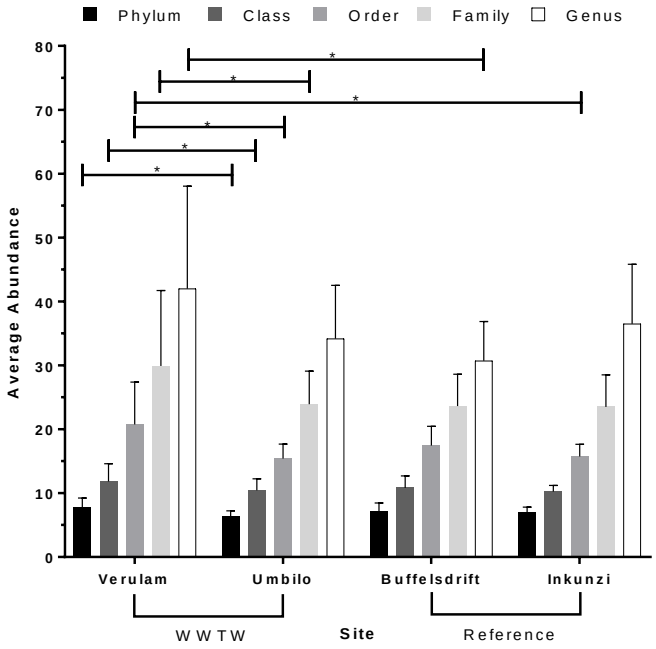

Figure 3: Average intestinal bacterium taxa loads in *Neoromicia nana* caught at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa. Asterisks indicate a significant difference between sites ($p<0.05$). Error bars = Standard deviation.

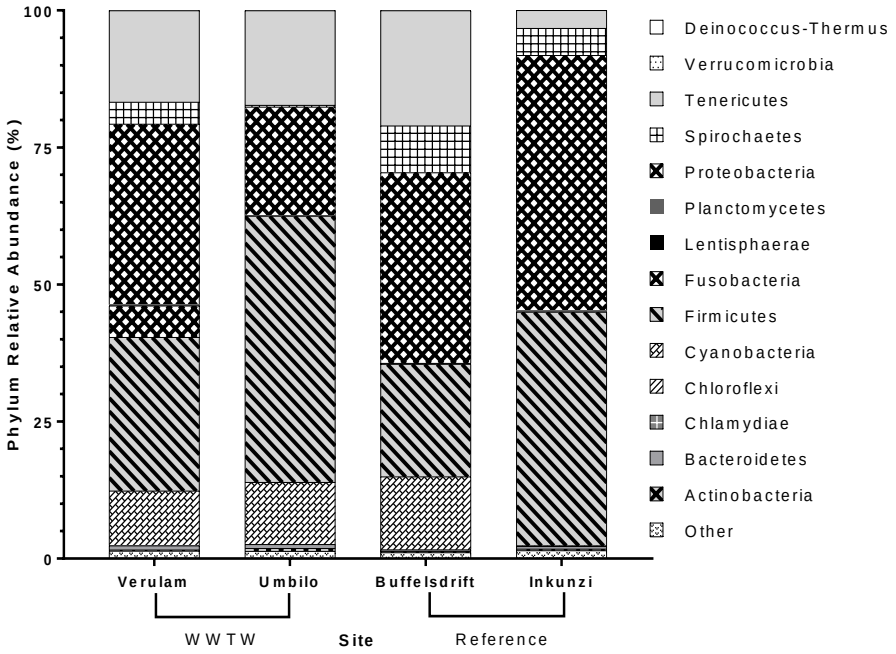

Figure 4: Average relative loads of intestinal bacterium phyla in *Neoromicia nana* caught at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa.

3.3 Phyla Chlamydiae, Chloroflexi and Tenericutes

Bats caught at Inkunzi had significantly higher load of Chlamydiae than bats at Verulam WWTWs and Buffelsdrift (χ^2=4.79, df=3, P=0.19, Dunn's Test z=-1.99, P=0.024 and z=-1.97, P=0.025, respectively; Figure 5). This difference was at all taxonomic levels within this phylum (Table 1). Bats caught at Verulam WWTWs had a significantly higher load of Chloroflexi than those caught at Buffelsdrift and Umbilo WWTWs (χ^2=4.62, df=3, P=0.2, Dunn's Test z=1.79, P=0.037 and z=1.83, P=0.034, respectively; Figure 4). This phylum contained just two classes, namely C0119 and S085 (Table 1). Within the phylum Tenericutes, the genus *Ureaplasma* had a significantly greater load at Buffelsdrift than Verulam WWTWs (χ^2=3.11, df=3, P=0.38, Dunn's Test z=1.66, P=0.049; Figure 5).

Table 1: Taxonomic classification of the intestinal bacteria within the phyla Chlamydiae, Chloroflexi, and Tenericutes in *Neoromicia nana* caught at wastewater treatment works and reference sites in KwaZulu-Natal, South Africa.

Phylum	Chlamydiae	Chloroflexi		Tenericutes		
Class	Chlamydiia	C0119	S085	Mollicutes		
Order	Chlamydiales	C0119 sp	S085 sp	Mycoplasmatales		
Family	Chlamydiaceae			Mycoplasmataceae		
Genus	*Chlamydia*			Other	*Mycoplasma*	*Ureaplasma*

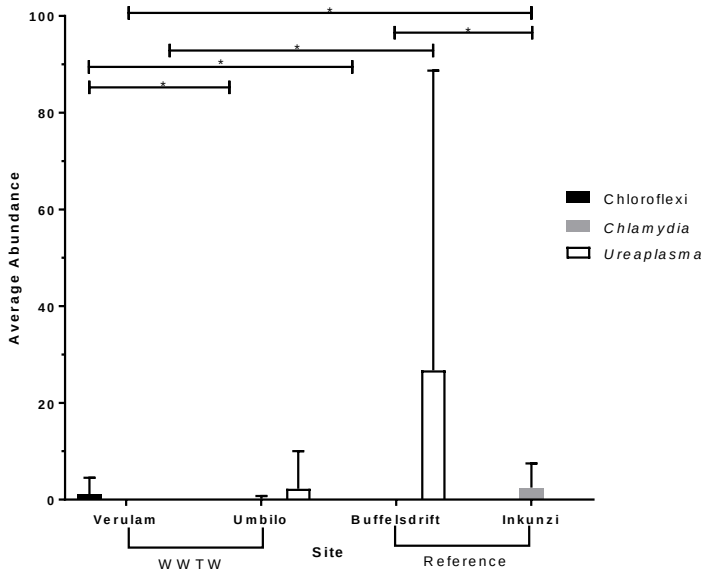

Figure 5: Significant differences in the average intestinal phyla Chlamydiae, Chloroflexi and Tenericutes load in *Neoromicia nana* caught at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa. Asterisks indicate a significant difference between sites ($p<0.05$). Error bars = Standard deviation.

3.4 Phylum Spirochaetes and Fusobacteria

When grouped together, bats caught at reference sites had significantly greater load of Spirochaetes than those caught at WWTWs sites ($W_{(38)}$=246, P=0.047; Table 2; Figure 6A). Further analyses showed that bats caught at Verulam WWTWs, Buffelsdrift and Inkunzi had significantly greater number of Spirochaetes than those caught at Umbilo WWTWs (χ^2=11.71, df=3, P=0.01, Dunn's Test z=2.77, P=0.003, z=-2.96, P=0.002 and z=-2.08, P=0.019, respectively; Figure 6B). These differences were present at all taxonomic levels within this phylum (Table 2). Fusobacteria were significantly more abundant in bats caught at Verulam WWTWs than those caught at all other sites (χ^2=13.89, df=3, P=0.0004, Dunn's Test Umbilo: z=3.36, P=0.0004, Buffelsdrift: z=2.40, P=0.008 and Inkunzi: z=2.60, P=0.005; Figure 6B). This difference was mainly attributed to differences in the genus *Fusobacterium*, which showed the same trend as the phylum to which it belongs (Table 2; Figure 6A). Further, male bats had significantly greater load of Fusobacteria than females ($W_{(38)}$=115, P=0.01; Figure 6C). This difference was also mainly attributed to differences in the genus *Fusobacterium* (Figure 6C).

Table 2: Taxonomic classification of the intestinal bacteria within the phyla Spirochaetes and Fusobacteria in *Neoromicia nana* collected at wastewater treatment works and reference sites in KwaZulu-Natal, South Africa.

Phylum	Spirochaetes	Fusobacteria	
Class	Spirochaetes	Fusobacteriia	
Order	Borreliales	Fusobacteriales	
Family	Borreliaceae	Fusobacteriaceae	Leptotrichiaceae
Genus	*Spironema*	*Fusobacterium*	*Leptotrichia*

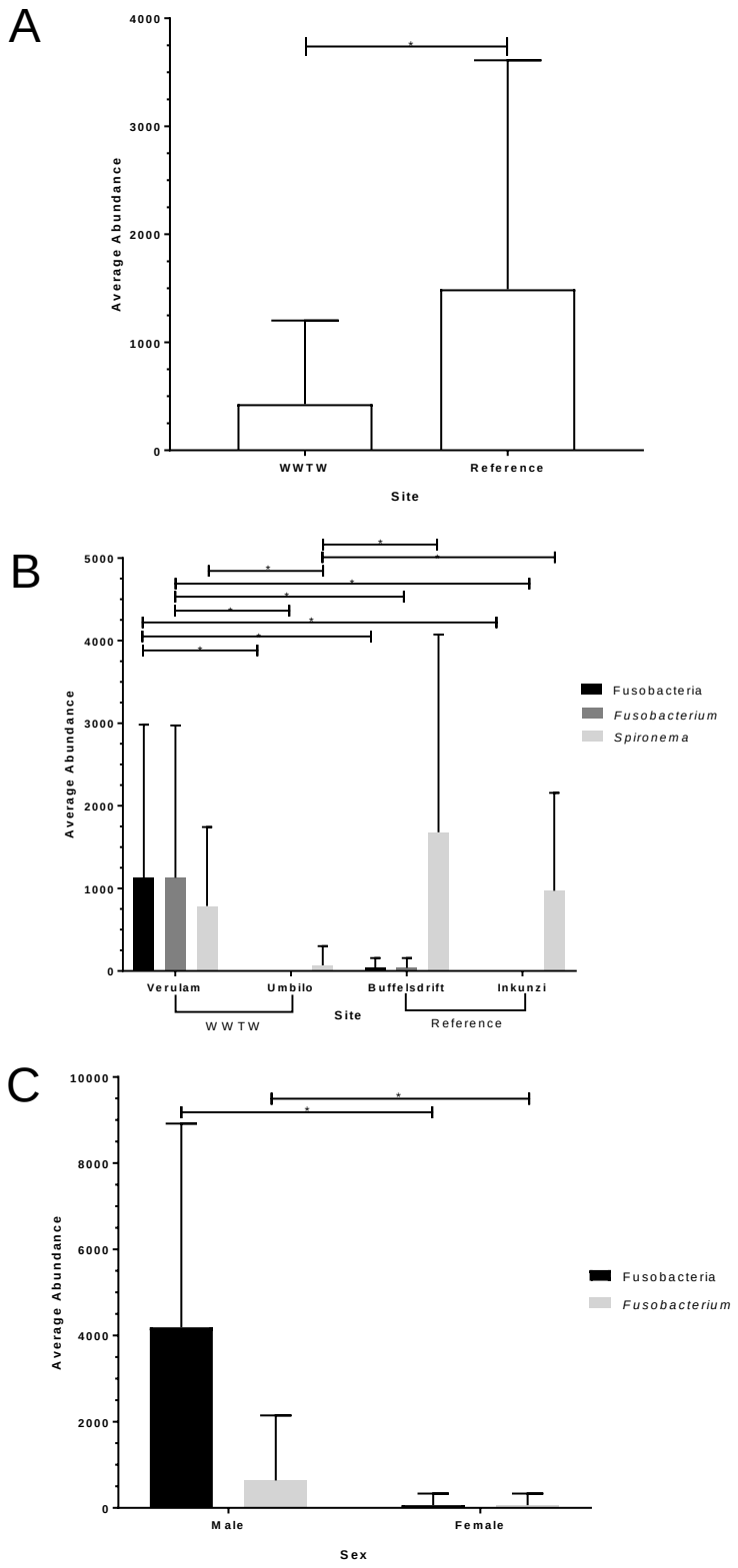

Figure 6: (A) Grouped average intestinal Spironema (phylum: Spirochaetes) load, (B) significant differences in the average intestinal Fusobacteria load and (C) significant differences between sexes in the average intestinal Fusobacteria load in *Neoromicia nana* caught at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa. Asterisks indicate a significant difference between sites ($p<0.05$). Error bars = Standard deviation.

3.5 Phylum Planctomycetes

Within the phylum Planctomycetes, bats at Verulam WWTWs had a significantly greater load of the family Pirellulaceae than those at Umbilo WWTWs (χ^2=4.17, df=3, P=0.24, Dunn's Test z=-1.84, P=0.033; Figure 7). The genus *Gemmata* was significantly more abundant in bats at Verulam WWTWs than those at Buffelsdrift and Inkunzi (χ^2=4.62, df=3, P=0.2, Dunn's Test z=-1.79, P=0.037 and z=-1.83, P=0.034, respectively; Table 3; Figure 7).

Table 3: Taxonomic classification of the intestinal bacteria found within the phylum Planctomycetes in *Neoromicia nana* caught at wastewater treatment works and reference sites in KwaZulu-Natal, South Africa.

Phylum	Class	Order	Family	Genus
Planctomycetes	Phycisphaerae	Phycisphaerales		
	Planctomycetia	B97		
		Gemmatales	Gemmataceae	Other
				Gemmata
			Isosphaeraceae	
		Pirellulales	Pirellulaceae	*Pirellula*
				Other
		Planctomycetales	Planctomycetaceae	*Planctomyces*

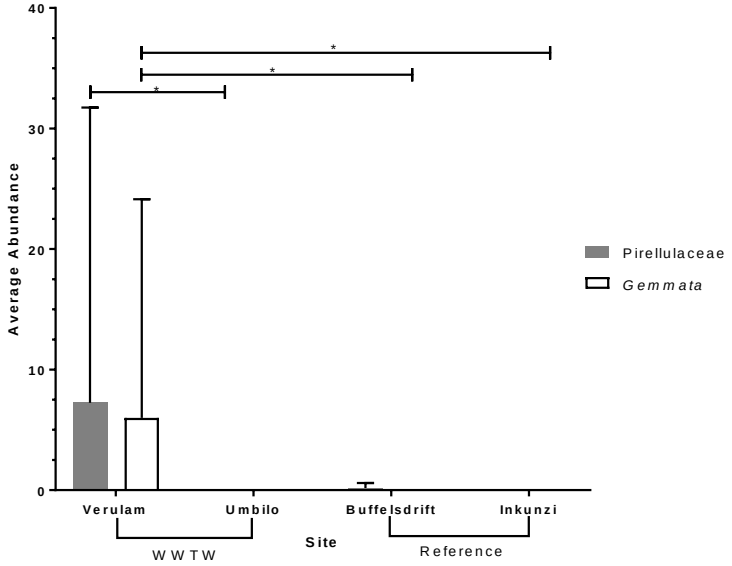

Figure 7: Significant differences found in the average intestinal Planctomycetes load in *Neoromicia nana* caught at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa. Asterisks indicate a significant difference between sites ($p<0.05$). Error bars = Standard deviation.

3.6 Phylum Actinobacteria

The family Dermacoccaceae, and the only genus (*Dermacoccus*) therein, was significantly more abundant in bats at Buffelsdrift than those at Verulam WWTWs and Umbilo WWTWs (χ^2=5.22, df=3, P=0.16, Dunn's Test z=-1.95, P=0.026 and z=-1.95, P=0.026, respectively; Table 4; Figure 8). The family Nocardioidaceae was found in significantly greater numbers in bats at Inkunzi than those at Buffelsdrift and Verulam WWTWs (χ^2=4.45, df=3, P=0.22, Dunn's Test z=-1.87, P=0.031 and z=1.89, P=0.030 respectively; Table 4; Figure 8). Further, within this family bats at Inkunzi had significantly greater loads of bacteria not assigned to a genus than bats at all other sites (χ^2=8.75, df=3, P=0.03, Dunn's Test Verulam: z=2.70, P=0.003, Umbilo: z=2.70, P=0.003 and Buffelsdrift: z=-2.67, P=0.004). Bats at Inkunzi also had a significantly greater load of the family Micrococcaceae (χ^2=4.61, df=3, P=0.2, Dunn's Test z=1.89, P=0.0295 and z=1.89, P=0.03, respectively; Table 4) and the genus *Mycobacterium* (χ^2=4.94, df=3, P=0.18, Dunn's Test z=1.99, P=0.024 and z=1.99, P=0.024, respectively; Figure 8) than those at Umbilo WWTWs and Verulam WWTWs. Within the family Micrococcaceae, bats at Inkunzi had a significantly greater load of the genus

Microbispora (χ^2=4.61, df=3, P=0.2, Dunn's Test z=1.89, P=0.03 and z=1.89, P=0.03, respectively; Figure 8), and bacteria not assigned to a genus (χ^2=4.94, df=3, P=0.18, Dunn's Test z=1.99, P=0.03 and z=1.99, P=0.024, respectively) than those at Umbilo and Verulam WWTWs. The genus *Microbacterium* was significantly more abundant in bats at Inkunzi than those at Buffelsdrift and Verulam WWTWs (χ^2=4.45, df=3, P=0.22, Dunn's Test z=-1.87, P=0.03 and z=1.89, P=0.03, respectively; Figure 8).

Table 4: Taxonomic classification of the intestinal bacteria found within the phylum Actinobacteria in *Neoromicia nana* caught at wastewater treatment works and reference sites in KwaZulu-Natal, South Africa.

Phylum	Class	Order	Family
Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae
			Corynebacteriaceae
			Dermacoccaceae
			Gordoniaceae
			Intrasporangiaceae
			Microbacteriaceae
			Micrococcaceae
			Mycobacteriaceae
			Nocardiaceae
			Nocardioidaceae
			Promicromonosporaceae
			Propionibacteriaceae
			Streptomycetaceae
			Other

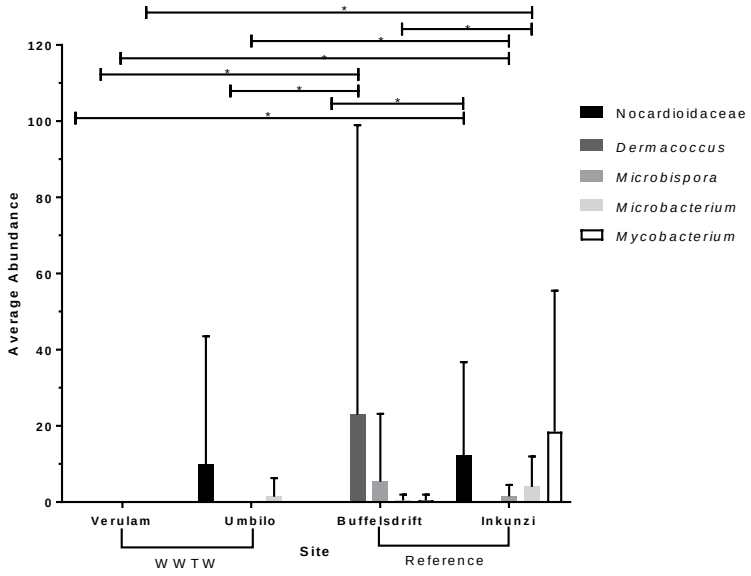

Figure 8: Significant differences found in the average intestinal Actinobacteria load in *Neoromicia nana* caught at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa. Asterisks indicate a significant difference between sites ($p<0.05$). Error bars = Standard deviation.

3.7 Phylum Firmicutes

Firmicutes and Proteobacteria were the two most abundant phyla found in bats at all sites. Firmicutes accounted for ~48.6%, 42.6%, 28.0% and 20.5% of all intestinal bacterial diversity in bats caught at Umbilo WWTWs, Inkunzi, Verulam WWTWs and Buffelsdrift, respectively (Figure 4). Firmicutes were significantly more abundant in bats at Umbilo WWTWs than those caught at Verulam WWTWs and Buffelsdrift (χ^2=9.55, df=3, P=0.02, Dunn's Test z=-1.99, P=0.02 and z=2.80, P=0.003, respectively; Figure 9). Further, bats caught at Inkunzi had a significantly higher number of Firmicutes than bats caught at Buffelsdrift (χ^2=9.55, df=3, P=0.02, Dunn's Test z=-1.90, P=0.03; Figure 9). The class Bacilli accounted for ~42.9%, 42.6%, 27.6% and 14.2% of all intestinal bacterial diversity in bats caught at Umbilo WWTWs, Inkunzi, Verulam WWTWs and Buffelsdrift, respectively. Bats caught at Umbilo WWTWs had a significantly greater number of Firmicutes not assigned to one of these three classes than bats caught at Buffelsdrift and Verulam WWTWs (χ^2=7.12, df=3, P=0.07, Dunn's Test z=-2.22, P=0.01 and z=2.27, P=0.012, respectively).

Table 5: Taxonomic classification of the intestinal bacteria found within the phylum Firmicutes in *Neoromicia nana* caught at wastewater treatment works and reference sites in KwaZulu-Natal, South Africa.

Phylum	Class	Order
Firmicutes	Bacilli	Bacillales
		Lactobacillales
		Turicibacterales
		Other
	Clostridia	Clostridiales
		OPB54
	Erysipelotrichi	Erysipelotrichales
	Other	

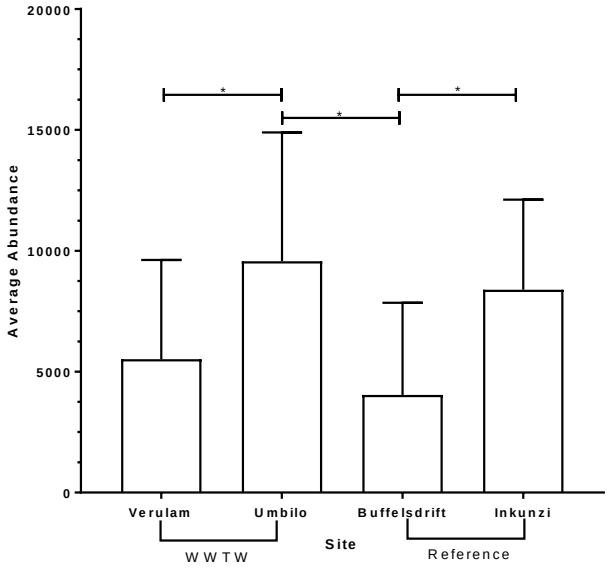

Figure 9: Average intestinal Firmicutes load in *Neoromicia nana* caught at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa. Asterisks indicate a significant difference between sites ($p<0.05$). Error bars = Standard deviation.

3.7.1 Class Clostridia

Bats caught at Inkunzi had a significantly lower load of the class Clostridia than those caught at Buffelsdrift and Umbilo WWTWs (χ^2=4.97, df=3, P=0.17, Dunn's Test z=1.94, P=0.02 and z=-1.93, P=0.03, respectively; Figure 10). The order OPB54 occurred in a significantly greater load in bats at Verulam WWTWs than those at Buffelsdrift and Umbilo WWTWs (χ^2=4.62, df=3, P=0.2, Dunn's Test z=-1.79, P=0.04 and z=-1.83, P=0.04, respectively; Figure 10), whereas the order Clostridiales occurred in a significantly lower load in bats at Inkunzi than those at Buffelsdrift and Umbilo WWTWs (χ^2=5.21, df=3, P=0.16, Dunn's Test z=1.95,

P=0.03 and z=-1.93, P=0.03, respectively; Figure 10). The family Clostridiaceae occurred in significantly greater numbers in bats at Buffelsdrift than those at Inkunzi and Verulam WWTWs (χ^2=4.98, df=3, P=0.17, Dunn's Test z=1.75, P=0.04 and z=1.92, P=0.03, respectively; Figure 10). Within this family, the genus *Candidatus Arthromitus* was significantly more abundant in bats at Buffelsdrift than those at Verulam WWTWs (χ^2=6.44, df=3, P=0.09, Dunn's Test z=2.43, P=0.008; Figure 10). Clostridiales not assigned to a family were significantly more abundant in males than females ($W_{(38)}$=152, P=0.05), and in bats at Verulam WWTWs compared to those at Buffelsdrift (χ^2=4.21, df=3, P=0.24, Dunn's Test z=-1.90, P=0.03).

Table 6: Taxonomic classification of the intestinal bacteria found within the class Clostridia in *Neoromicia nana* caught at wastewater treatment works and reference sites in KwaZulu-Natal, South Africa.

Class	Order	Family
Clostridia	Clostridiales	Christensenellaceae
		Clostridiaceae
		Lachnospiraceae
		Peptococcaceae
		Peptostreptococcaceae
		Ruminococcaceae
		Veillonellaceae
		Tissierellaceae
		Other
	OPB54	

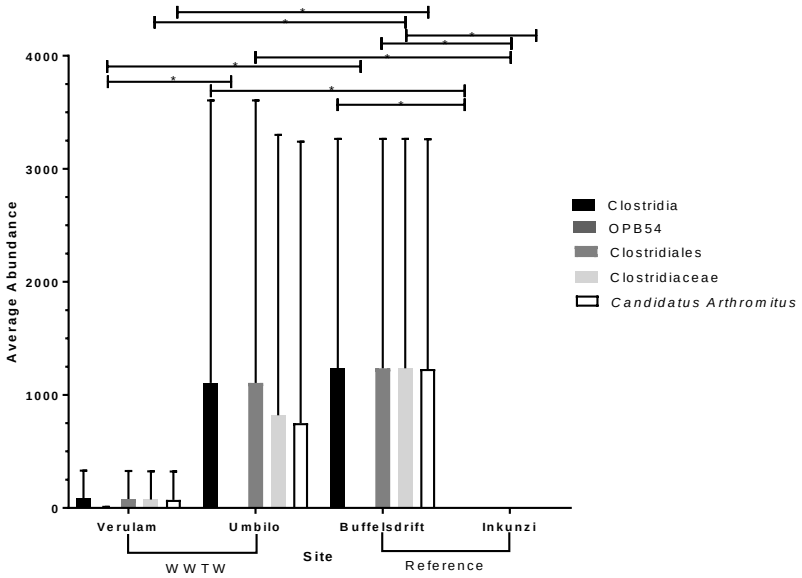

Figure 10: Significant differences found in the average intestinal Clostridia load in *Neoromicia nana* caught at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa. Asterisks indicate a significant difference between sites ($p<0.05$). Error bars = Standard deviation.

3.7.3 Class Erysipelotrichi

Bacteria belonging to the Class Erysipelotrichi were only found in bats caught at Umbilo WWTWs (Table 7).

Table 7: Taxonomic classification of the intestinal bacteria found within the class Erysipelotrichi in *Neoromicia nana* caught at wastewater treatment works and reference sites in KwaZulu-Natal, South Africa**.**

Class	Erysipelotrichi
Order	Erysipelotrichales
Family	Erysipelotrichaceae

3.7.4 Class Bacilli

Bats caught at Inkunzi, Umbilo WWTWs and Verulam WWTWs had a significantly greater load of Bacilli than bats caught at Buffelsdrift (χ^2=11.38, df=3, P=0.01, Dunn's Test z=-2.39, P=0.009, z=-3.09, P=0.001 and z=-1.69, P=0.05, respectively). Three orders were found within the Class Bacilli, namely Bacillales, Lactobacillales and Turicibacterales (Table 8).

Table 8: Taxonomic classification of the intestinal bacteria found within the class Bacilli in *Neoromicia nana* caught at wastewater treatment works and reference sites in KwaZulu-Natal, South Africa.

Class	Bacilli			
Order	Other	Bacillales	Lactobacillales	Turicibacterales

3.7.4.1 Order Bacillales

Bacillales were found in significantly greater loads in bats at Inkunzi than those at Buffelsdrift and Verulam WWTWs (χ^2=12.90, df=3, P=0.000, Dunn's Test z=-2.52, P=0.006, z=1.89, P=0.03, respectively), and in bats at Umbilo WWTWs than those at Buffelsdrift and Verulam WWTWs (χ^2=12.90, df=3, P=0.000, Dunn's Test z=-3.06, P=0.001, z=2.18, P=0.01, respectively). The most abundant family in this order, Bacillaceae, were found in significantly greater loads in bats at Inkunzi than those at Buffelsdrift and Verulam WWTWs (χ^2=13.09, df=3, P=0.000, Dunn's Test z=-2.56, P=0.005, z=1.94, P=0.03, respectively; Table 9), and bats at Umbilo WWTWs than those at Buffelsdrift and Verulam WWTWs (χ^2=13.09, df=3, P=0.000, Dunn's Test z=-3.06, P=0.001, z=2.20, P=0.01, respectively). The family Listeriaceae was significantly more abundant in bats at Umbilo WWTWs than those at Buffelsdrift and Verulam WWTWs (χ^2=4.62, df=3, P=0.2, Dunn's Test z=-1.79, P=0.04, z=1.83, P=0.03, respectively; Figure 11) and the family Staphylococcaceae was significantly more abundant in bats at Buffelsdrift than those at Umbilo WWTWs and Verulam WWTWs (χ^2=9.12, df=3, P=0.03, Dunn's Test z=2.98, P=0.002, z=1.99, P=0.02, respectively). Staphylococcaceae were also significantly more abundant in bats at references sites than those at WWTWs when the sites were grouped ($W_{(38)}$=246.5, P=0.01). Bacillales not assigned to one of these families were also found in greater loads in bats at WWTWs than those at reference sites ($W_{(38)}$=110, P=0.03). This difference was mainly driven by the significantly greater load in bats at Verulam WWTWs than those at Buffelsdrift (χ^2=5.98, df=3, P=0.11, Dunn's Test z=-2.40, P=0.008).

The genus *Staphylococcus* (family: Staphylococcaceae) were significantly more abundant in reference sites bats ($W_{(38)}$=245, P=0.01), mainly due to the significantly higher load in bats at Buffelsdrift than those at Umbilo and Verulam WWTWs (χ^2=8.76, df=3, P=0.03, Dunn's Test z=2.92, P=0.002, z=1.90, P=0.03, respectively; Figure 11) and the significantly greater load of Staphylococcaceae not assigned to a genus in bats at Inkunzi compared to those at Umbilo and Verulam WWTWs (χ^2=4.61, df=3, P=0.2, Dunn's Test z=1.89, P=0.03, z=1.89, P=0.03,

respectively). Bats caught at Inkunzi had a significantly greater load of the genus *Planomicrobium* (family: Planococcaceae) than bats caught at other sites (χ^2=8.75, df=3, P=0.03, Dunn's Test Buffelsdrift: z=-2.67, P=0.004, Umbilo: z=2.70, P=0.003 and Verulam: z=2.70, P=0.003; Figure 11). The same trend was seen in the genus *Planococcus* (family: Planococcaceae) (χ^2=8.75, df=3, P=0.03, Dunn's Test Buffelsdrift: z=-2.67, P=0.0037, Umbilo: z=2.70, P=0.003 and Verulam: z=2.70, P=0.003; Figure 11) and the genus *Marinibacillus* (family: Bacillaceae) (χ^2=12.27, df=3, P=0.01, Dunn's Test Buffelsdrift: z=-3.25, P=0.0006, Umbilo: z=3.29, P=0.0005 and Verulam: z=2.78, P=0.003; Figure 11).

Table 9: Taxonomic classification of the intestinal bacteria found within the order Bacillales in *Neoromicia nana* caught at wastewater treatment works and reference sites in KwaZulu-Natal, South Africa.

Order	Family
Bacillales	Alicyclobacillaceae
	Bacillaceae
	Listeriaceae
	Paenibacillaceae
	Planococcaceae
	Staphylococcaceae
	Exiguobacteraceae
	Other

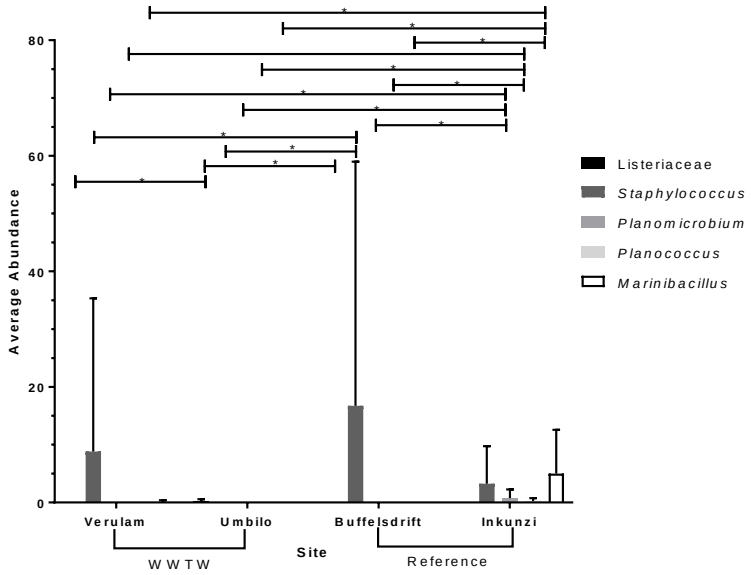

Figure 11: Significant differences found in the average intestinal Bacillales load in *Neoromicia nana* caught at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa. Asterisks indicate a significant difference between sites ($p<0.05$). Error bars = Standard deviation.

Bacillus, the most abundant genus from the family Bacillaceae, was significantly more abundant in bats at Inkunzi than those at Buffelsdrift and Verulam WWTWs (χ^2=13.28, df=3, P=0.000, Dunn's Test z=-2.54, P=0.006, z=1.90, P=0.03, respectively; Figure 12), and in those at Umbilo WWTWs than those at Buffelsdrift and Verulam WWTWs (χ^2=13.28, df=3, P=0.000, Dunn's Test z=-3.11, P=0.0009, z=2.24, P=0.01, respectively; Figure 12). Further, bacteria from the family Bacillaceae not assigned to a genus were significantly less abundant at reference sites ($W_{(38)}$=99, P=0.02), mainly due to their significantly lower load at Buffelsdrift than other sites (χ^2=8.86, df=3, P=0.03, Dunn's Test Verulam: z=-2.28, P=0.01, Umbilo: z=-2.74, P=0.003 and Inkunzi: z=-1.78, P=0.04).

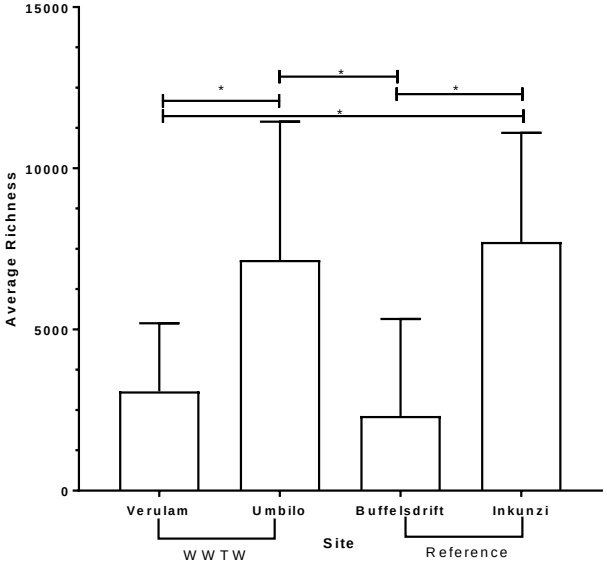

Figure 12: Average intestinal *Bacillus* load in *Neoromicia nana* caught at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa. Asterisks indicate a significant difference between sites ($p<0.05$). Error bars = Standard deviation.

3.7.4.2 Order Lactobacillales

Lactobacillales were found in significantly greater numbers at WWTWs sites than reference sites ($W_{(38)}=74$, P=0.002). More specifically, they were found in significantly greater numbers in bats caught at Verulam WWTWs than in bats at Buffelsdrift and Inkunzi ($\chi^2=10.18$, df=3, P=0.02, Dunn's Test z=-2.95, P=0.002 and z=-1.76, P=0.04, respectively) and in greater numbers in Umbilo WWTWs bats than in those at Buffelsdrift ($\chi^2=10.18$, df=3, P=0.02, Dunn's Test z=-2.15, P=0.02). Significant differences between sites were found in all families within this order. When grouped, bats caught at WWTWs had significantly greater numbers of Aerococcaceae and Carnobacteriaceae (Figure 14B) than bats at references sites ($W_{(38)}=57$, P=0.0001 and $W_{(38)}=58.5$, P=0.0004, respectively). Further, males had significantly more Enterococcaceae than females ($W_{(38)}=85$, P=0.003).

Aerococcaceae were found in significantly greater numbers in bats at Verulam WWTWs and Umbilo WWTWs than those at Buffelsdrift and Inkunzi ($\chi^2=15.38$, df=3, P=0.000, Dunn's Test z=-3.01, P=0.001, z=-1.68, P=0.05 and z=-3.45, P=0.0003, z=-2.00, P=0.02, respectively). The genus *Facklamia* was found in significantly greater numbers in bats at WWTWs than those at reference sites ($W_{(38)}=65.5$, P=0.0003; Figure 14B). Further, they were

found in significantly greater numbers in bats at Verulam WWTWs than those at Buffelsdrift (χ^2=14.03, df=3, P=0.000, Dunn's Test z=-2.77, P=0.003; Figure 13), and in Umbilo WWTWs bats than those at Buffelsdrift and Inkunzi (χ^2=14.03, df=3, P=0.000, Dunn's Test z=-3.39, P=0.0003 and z=-1.91, P=0.03, respectively; Figure 13). Bacteria within this family not assigned to a genus were found in significantly greater numbers in bats at WWTWs than those at reference sites ($W_{(38)}$=75, P=0.0004). More specifically, they were found in significantly greater numbers in bats at Verulam WWTWs than those at Buffelsdrift (χ^2=14.38, df=3, P=0.00, Dunn's Test z=-2.11, P=0.02), and in bats at Umbilo WWTWs than those at Buffelsdrift and Inkunzi (χ^2=14.38, df=3, P=0.000, Dunn's Test z=-3.46, P=0.0003 and z=-2.50, P=0.006, respectively).

Significantly greater numbers of Carnobacteriaceae were found in Verulam WWTWs and Umbilo WWTWs bats than those at Buffelsdrift and Inkunzi (χ^2=12.60, df=3, P=0.01, Dunn's Test z=-2.88, P=0.002, z=-2.01, P=0.02 and z=-2.77, P=0.003, z=-1.93, P=0.03, respectively; Table 10). The genus *Granulicatella* was found in significantly greater numbers in bats caught at Verulam WWTWs than those caught at Buffelsdrift and Umbilo WWTWs (χ^2=4.62, df=3, P=0.2, Dunn's Test z=-1.79, P=0.04 and z=-1.83, P=0.03, respectively; Figure 13). The genus *Trichococcus* was significantly more abundant at WWTWs than at reference sites ($W_{(38)}$=126, P=0.04). More specifically, Buffelsdrift bats had significantly fewer *Trichococcus* than bats at Umbilo and Verulam WWTWs (χ^2=5.3, df=3, P=0.15, Dunn's Test z=-1.83, P=0.03 and z=-2.15, P=0.02, respectively; Figure 13). Bacteria within Carnobacteriaceae not assigned to one of these genera were also found in significantly greater numbers at WWTWs than at reference sites ($W_{(38)}$=58.5, P=0.0004). More specifically, Umbilo WWTWs bats had significantly greater numbers of these bacteria than bats at both Buffelsdrift and Inkunzi (χ^2=12.60, df=3, P=0.01, Dunn's Test z=-2.77, P=0.003 and z=-1.93, P=0.03, respectively) and Verulam WWTWs bats had significantly greater numbers than bats at Buffelsdrift and Inkunzi (χ^2=12.60, df=3, P=0.01, Dunn's Test z=-2.88, P=0.002 and z=-2.01, P=0.02, respectively).

Enterococcaceae were found in significantly greater numbers in bats at Verulam WWTWs than those at Umbilo WWTWs (χ^2=4.62, df=3, P=0.2, Dunn's Test z=-2.08, P=0.02; Table 10). Male bats had a significantly greater average load of the genera *Enterococcus* and *Vagococcus* than females ($W_{(38)}$=85, P=0.003 and $W_{(38)}$=111, P=0.02, respectively; Figure

14A). Moreover, *Enterococcus* were significantly more abundant in bats at Verulam WWTWs than those at Umbilo WWTWs (χ^2=4.41, df=3, P=0.22, Dunn's Test z=-2.05, P=0.02; Figure 13) and *Vagococcus* were significantly more abundant in bats at Inkunzi than those at Buffelsdrift (χ^2=4.57, df=3, P=0.24, Dunn's Test z=-1.71, P=0.04; Figure 13). Bacteria within Enterococcaceae not assigned to a genus were found in greater numbers in males than females ($W_{(38)}$=98.5, P=0.006). Further, bats at Verulam WWTWs had a significantly greater load of these bacteria than bats at Buffelsdrift and Umbilo WWTWs (χ^2=7.18, df=3, P=0.07, Dunn's Test z=-1.87, P=0.03 and z=-2.49, P=0.006, respectively).

Streptococcaceae were found in significantly lower numbers in bats at Umbilo WWTWs than in those at Inkunzi and Verulam WWTWs (χ^2=7.22, df=3, P=0.07, Dunn's Test z=1.77, P=0.04 and z=-2.43, P=0.008, respectively; Table 10). Within this family, the genus *Lactococcus* was found in significantly greater load in bats at Inkunzi than bats at other sites (χ^2=12.27, df=3, P=0.01, Dunn's Test Buffelsdrift: z=-3.25, P=0.0006, Umbilo: z=3.29, P=0.0005 and Verulam: z=2.78, P=0.003; Figure 13). The other genus found in this family, *Streptococcus*, was significantly more abundant in bats at Verulam WWTWs than in those at Inkunzi and Umbilo WWTWs (χ^2=6.05, df=3, P=0.11, Dunn's Test z=-1.83, P=0.03 and z=-2.18, P=0.01, respectively; Figure 13). Further, bacteria in this family not assigned to a genus were significantly more abundant in bats at Verulam WWTWs than bats at other sites (χ^2=9.74, df=3, P=0.02, Dunn's Test Buffelsdrift: z=-2.59, P=0.005, Umbilo: z=-2.65, P=0.004 and Inkunzi: z=-1.88, P=0.03).

Further, Lactobacillales not assigned to one of these four families were found in greater numbers in bats at Umbilo WWTWs than those at Buffelsdrift (χ^2=7.99, df=3, P=0.05, Dunn's Test z=-2.09, P=0.02), and in bats caught at Verulam WWTWs than those caught at Buffelsdrift and Inkunzi (χ^2=7.99, df=3, P=0.05, Dunn's Test z=-2.27, P=0.01 and z=-1.76, P=0.04, respectively). When sites were grouped, these bacteria were also found in greater loads at WWTWs than reference sites ($W_{(38)}$=83.5, P=0.005).

Table 10: Taxonomic classification of the intestinal bacteria found within the order Lactobacillales in *Neoromicia nana* caught at wastewater treatment works and reference sites in KwaZulu-Natal, South Africa.

Order	Lactobacillales				
Family	Other	Aerococcaceae	Carnobacteriaceae	Enterococcaceae	Streptococcaceae

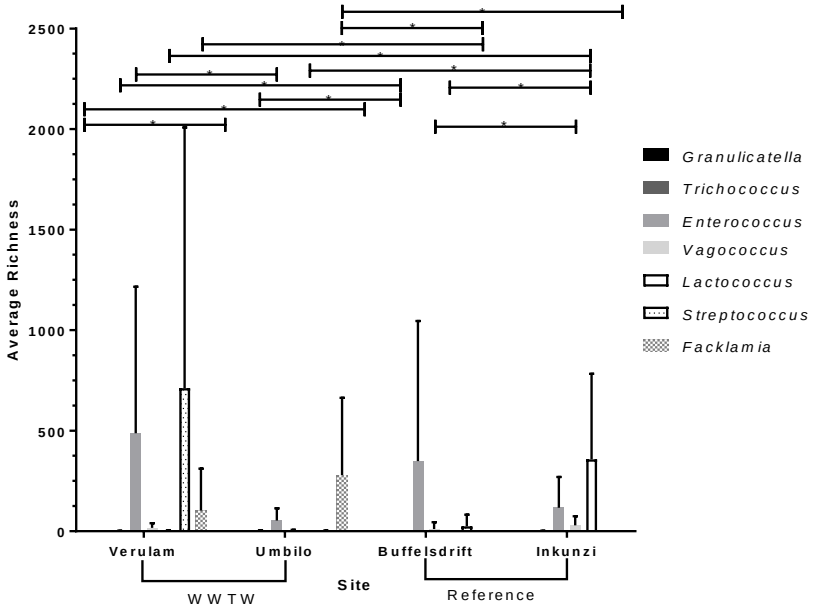

Figure 13: Significant differences found in the average intestinal Lactobacillales load in *Neoromicia nana* caught at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa. Asterisks indicate a significant difference between sites ($p<0.05$). Error bars = Standard deviation.

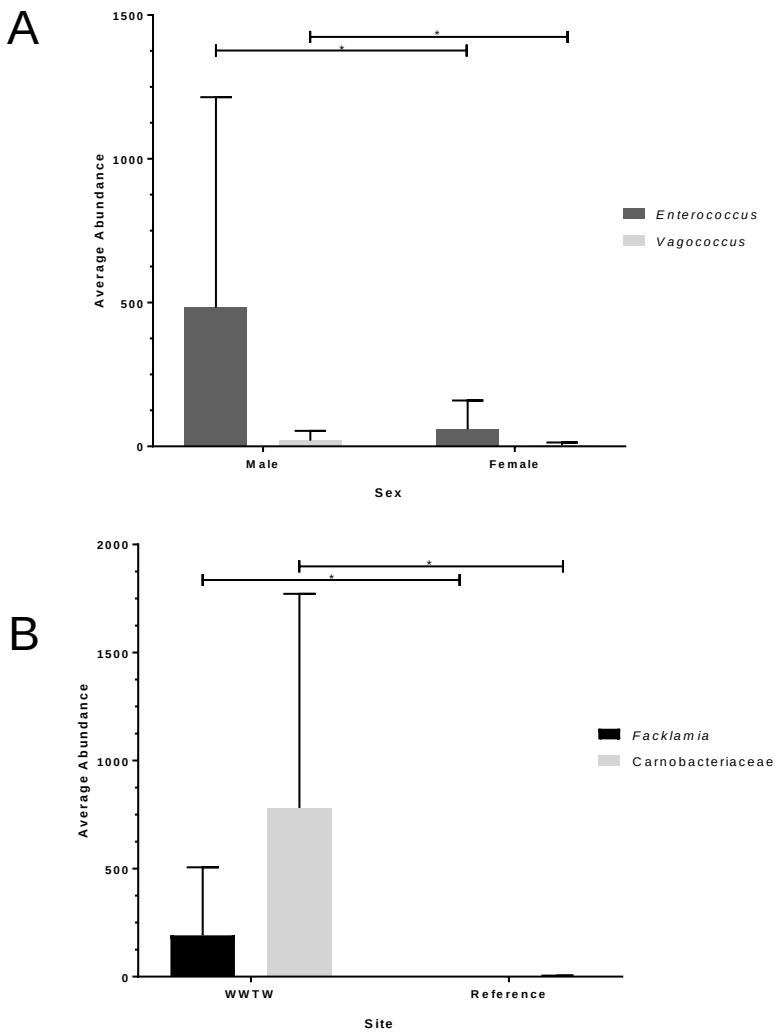

Figure 14: Significant differences found in the grouped average intestinal Lactobacillales loads by (A) site and (B) sex in *Neoromicia nana* caught at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa. Asterisks indicate a significant difference between sites ($p<0.05$). Error bars = Standard deviation.

3.7.4.3 Order Turicibacterales

Turicibacterales were found in a greater load in bats at Inkunzi than bats at Buffelsdrift, Umbilo and Verulam WWTWs (χ^2=8.75, df=3, P=0.03, Dunn's Test z=-2.67, P=0.004, z=2.70, P=0.003 and z=2.70, P=0.003, respectively). These differences were due to differences in the genus *Turicibacter* (Table 11; Figure 16C).

Bacteria within Bacilli not assigned to one of these orders (3.7.4.1–3.7.4.3) were significantly more abundant in bats at Verulam than those at other sites (χ^2=12.50, df=3, P=0.01, Dunn's Test Buffelsdrift: z=-2.94, P=0.002, Umbilo: z=-3.00, P=0.001 and Inkunzi: z=-2.12, P=0.02).

Table 11: Taxonomic classification of the intestinal bacteria found within the order Turicibacterales in *Neoromicia nana* caught at wastewater treatment works and reference sites in KwaZulu-Natal, South Africa.

Order	Turicibacterales
Family	Turicibacteraceae
Genus	*Turicibacter*

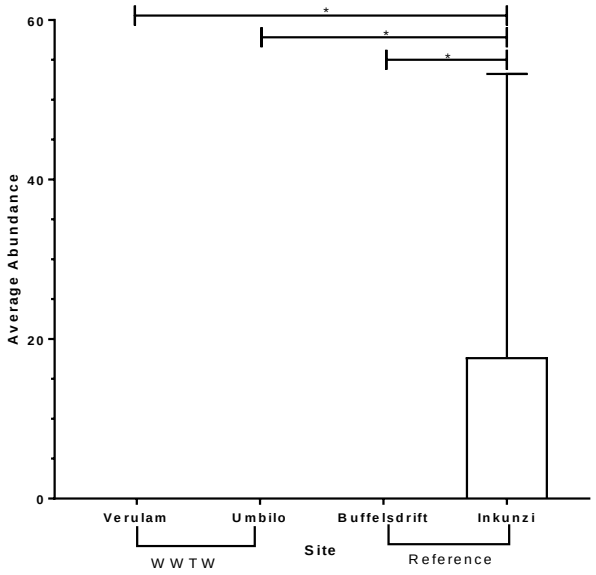

Figure 15: Average intestinal *Turicibacter* load in *Neoromicia nana* caught at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa. Asterisks indicate a significant difference between sites ($p<0.05$). Error bars = Standard deviation.

load

Proteobacteria (Table 12) accounted for ~19.9%, 46.6%, 33.0% and 34.8% of all intestinal bacterial diversity in bats caught at Umbilo WWTWs, Inkunzi, Verulam WWTWs and Buffelsdrift, respectively (Figure 4). Inkunzi bats had a significantly higher load of Proteobacteria than Umbilo WWTWs bats (χ^2=5.69, df=3, P=0.13, Dunn's Test z=-2.28, P=0.01). Proteobacteria was the most genus rich phylum found.

Table 12: Taxonomic classification of the intestinal bacteria found within the phylum Proteobacteria in *Neoromicia nana* caught at wastewater treatment works and reference sites in KwaZulu-Natal, South Africa.

Phylum	Class
Proteobacteria	Alphaproteobacteria
	Betaproteobacteria
	Deltaproteobacteria
	Epsilonproteobacteria
	Gammaproteobacteria
	Other

3.8.1 Class Alphaproteobacteria

Buffelsdrift bats had a significantly greater average load of Alphaproteobacteria than bats at all other sites (χ^2=6.92, df=3, P=0.07, Dunn's Test Verulam: z=1.82, P=0.03, Umbilo: z=2.05, P=0.02 and Inkunzi: z=2.21, P=0.01). Bats at Inkunzi had a significantly greater abundance of the order Caulobacterales (Table 13) than bats at all other sites (χ^2=11.98, df=3, P=0.01, Dunn's Test Verulam: z=2.73, P=0.003, Umbilo: z=3.25, P=0.0006 and Buffelsdrift: z=-3.21, P=0.0007). This was also seen in the family Caulobacteraceae and the genera *Phenylobacterium* (χ^2=8.75, df=3, P=0.03, Dunn's Test Verulam: z=2.70, P=0.003, Umbilo: z=2.70, P=0.003 and Buffelsdrift: z=-2.67, P=0.004; Figure 16A), *Caulobacter* (χ^2=8.75, df=3, P=0.03, Dunn's Test Verulam: z=2.70, P=0.003, Umbilo: z=2.70, P=0.004 and Buffelsdrift: z=-2.67, P=0.004; Figure 16A) and *Brevundimonas* (χ^2=4.62, df=3, P=0.2, Dunn's Test Umbilo: z=1.94, P=0.03 and Buffelsdrift: z=-1.92, P=0.03; Figure 16A), therein. The same trend was also seen in Caulobacteraceae that were not assigned to a genus (χ^2=8.75, df=3, P=0.03, Dunn's Test Verulam: z=2.70, P=0.003, Umbilo: z=2.70, P=0.003 and Buffelsdrift: z=-2.67, P=0.004).

The order Rhizobiales (Table 13), one of the most abundantly found Alphaproteobacteria, was found in significantly greater numbers in bats at Verulam WWTWs compared to those at Umbilo WWTWs (χ^2=3.90, df=3, P=0.27, Dunn's Test z=1.97, P=0.02; Figure 16B). The same trend was seen in the orders Rhodobacterales and Sphingomonadales (χ^2=4.31, df=3, P=0.23, Dunn's Test z=-1.90, P=0.03 and χ^2=4.13, df=3, P=0.25, Dunn's Test z=-1.95, P=0.03, respectively; Figure 16B).

Table 13: Taxonomic classification of the intestinal bacteria found within the class Alphaproteobacteria in *Neoromicia nana* collected at wastewater treatment works and reference sites in KwaZulu-Natal, South Africa.

Class	Order
Alphaproteobacteria	Caulobacterales
	Rhizobiales
	Rhodobacterales
	Rhodospirillales
	Rickettsiales
	Sphingomonadales
	Other

3.8.1.1 Order Rickettsiales

The order Rickettsiales (Table 14) was found in significantly higher loads in bats at Verulam WWTWs compared to those at Inkunzi (χ^2=15.52, df=3, P=0.000, Dunn's Test z=-2.09, P=0.02; Figure 16B), and in those at Buffelsdrift compared to those at all other sites (χ^2=15.52, df=3, P=0.000, Dunn's Test Verulam: z=1.68, P=0.0466, Umbilo: z=3.20, P=0.0007 and Inkunzi: z=3.27, P=0.0005; Figure 16A). Bacteria within the order Rickettsiales not assigned to a family were significantly more abundant in bats at Verulam WWTWs than those at Inkunzi (χ^2=16, df=3, P=0.000, Dunn's Test z=-2.09, P=0.02) and in those at Buffelsdrift compared to those at all other sites (χ^2=16, df=3, P=0.000, Dunn's Test Verulam: z=1.72, P=0.04, Umbilo: z=3.28, P=0.0005 and Inkunzi: z=3.30, P=0.0005).

Table 14: Taxonomic classification of the intestinal bacteria found within the order Rickettsiales in *Neoromicia nana* collected at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa.

Order	Rickettsiales		
Family	Other	Anaplasmataceae	Mitochondria
Genus		*Neorickettsia*	

3.8.1.2 Order Sphingomonadales

The family Sphingomonadaceae (Table 15) were significantly more abundant in bats at Verulam WWTWs than those at Umbilo WWTWs (χ^2=4.36, df=3, P=0.23, Dunn's Test z=-1.95, P=0.03). The same trend was seen in the genus *Sphingomonas* (family: Sphingomonadaceae) (χ^2=4.36, df=3, P=0.23, Dunn's Test z=-1.95, P=0.03; Table 15; Figure 16B). Sphingomonadaceae not assigned to a genus were also significantly more abundant in

bats at Verulam WWTWs than those at Buffelsdrift and Umbilo WWTWs (χ^2=4.624, df=3, P=0.2, Dunn's Test z=-1.79, P=0.04 and z=-1.83, P=0.03, respectively).

Table 15: Taxonomic classification of the intestinal bacteria found within the order Sphingomonadales in *Neoromicia nana* collected at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa.

Order	Sphingomonadales				
Family	Other	Erythrobacteraceae	Sphingomonadaceae		
Genus			Other	*Novosphingobium*	*Sphingomonas*

3.8.1.3 Order Rhodobacterales

The family Rhodobacteraceae (Table 16) were significantly more abundant in bats at Verulam WWTWs than those at Umbilo WWTWs (χ^2=4.23, df=3, P=0.24, Dunn's Test z=-1.86, P=0.03). The same trend was seen in the genus *Rhodobacter* (χ^2=4.46, df=3, P=0.22, Dunn's Test z=-1.97, P=0.02; Figure 16B).

Table 16: Taxonomic classification of the intestinal bacteria found within the order Rhodobacterales in *Neoromicia nana* collected at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa.

Order	Rhodobacterales			
Family	Hyphomonadaceae	Rhodobacteraceae		
Genus		Other	*Paracoccus*	*Rhodobacter*

3.8.1.4 Order Rhizobiales

The family Bartonellaceae (Table 17) was significantly more abundant in bats at Inkunzi and Umbilo WWTWs than those at Buffelsdrift and Verulam WWTWs (χ^2=9.4, df=3, P=0.02, Dunn's Test z=-2.46, P=0.007, z=2.48, P=0.007 and z=-1.79, P=0.04, z=1.83, P=0.03, respectively). The same trend was also seen in the genus *Bartonella* (family: Bartonellaceae; Figure 16C), while Bartonellaceae not assigned to a genus were significantly more abundant in bats at Inkunzi than those at all other sites (χ^2=9.74, df=3, P=0.02, Dunn's Test Verulam: z=2.81, P=0.003, Umbilo: z=1.88, P=0.03 and Buffelsdrift: z=-2.78, P=0.003). The family Bradyrhizobiaceae was also found in significantly greater numbers in bats at Inkunzi than those at Verulam WWTWs (χ^2=4.06, df=3, P=0.25, Dunn's Test z=1.65, P=0.05; Figure 16C). However, within this family, the genus *Bosea* was significantly more abundant in bats at Umbilo WWTWs than those at Buffelsdrift and Verulam WWTWs (χ^2=4.62, df=3, P=0.2,

Dunn's Test z=-1.79, P=0.04 and z=1.83, P=0.04, respectively). Phyllobacteriaceae were significantly more abundant in bats at Verulam WWTWs than those at Buffelsdrift and Umbilo WWTWs (χ^2=4.62, df=3, P=0.2, Dunn's Test z=-1.79, P=0.04 and z=0.04, P=0.04, respectively; Figure 16C). The genus *Hyphomicrobium* (family: Hyphomicrobiaceae) was significantly more abundant in bats at Verulam WWTWs than those at Umbilo WWTWs (χ^2=6, df=3, P=0.11, Dunn's Test z=-2.28, P=0.01; Figure 16C). Within the same family, the genus *Rhodoplanes* was significantly more abundant in Umbilo WWTWs bats than bats at Inkunzi and Verulam WWTWs (χ^2=7.01, df=3, P=0.07, Dunn's Test z=-1.65, P=0.05 and z=2.33, P=0.01, respectively; Figure 16C) and significantly more abundant in bats at Buffelsdrift than those at Verulam WWTWs (χ^2=7.01, df=3, P=0.07, Dunn's Test z=1.73, P=0.04; Figure 16C). Bacteria within the same family not assigned to a genus were significantly more abundant in bats at Verulam WWTWs than those at Buffelsdrift and Umbilo WWTWs (χ^2=7.11, df=3, P=0.07, Dunn's Test z=-2.22, P=0.01 and z=-2.27, P=0.01, respectively). Within the family Rhizobiaceae, the genus *Agrobacterium* was significantly more abundant at Umbilo WWTWs than Inkunzi (χ^2=3.82, df=3, P=0.28, Dunn's Test z=-1.75, P=0.04; Figure 16C), and the genus *Rhizobium* was significantly more abundant at Umbilo WWTWs than Buffelsdrift (χ^2=4.17, df=3, P=0.24, Dunn's Test z=1.7, P=0.05; Figure 16C). Within the same family, bacteria not assigned to a genus were significantly more abundant in bats at Umbilo WWTWs than those at Buffelsdrift and Verulam WWTWs (χ^2=11.3, df=3, P=0.01, Dunn's Test z=-2.9, P=0.002 and z=2.57, P=0.005, respectively) and significantly more abundant in bats at Buffelsdrift than those at Inkunzi (χ^2=11.3, df=3, P=0.01, Dunn's Test z=-1.78, P=0.04). Bacteria within Rhizobiales not assigned to a family were significantly more abundant in bats at Umbilo WWTWs than those at Buffelsdrift (χ^2=4.67, df=3, P=0.2, Dunn's Test z=-1.99, P=0.02). These bacteria were also significantly more abundant in females than males ($W_{(38)}$=261.5, P=0.05).

Table 17: Taxonomic classification of the intestinal bacteria found within the order Rhizobiales in *Neoromicia nana* collected at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa.

Order	Family
Rhizobiales	Aurantimonadaceae
	Bartonellaceae
	Bradyrhizobiaceae
	Hyphomicrobiaceae
	Methylobacteriaceae
	Phyllobacteriaceae
	Rhizobiaceae
	Xanthobacteraceae
	Other

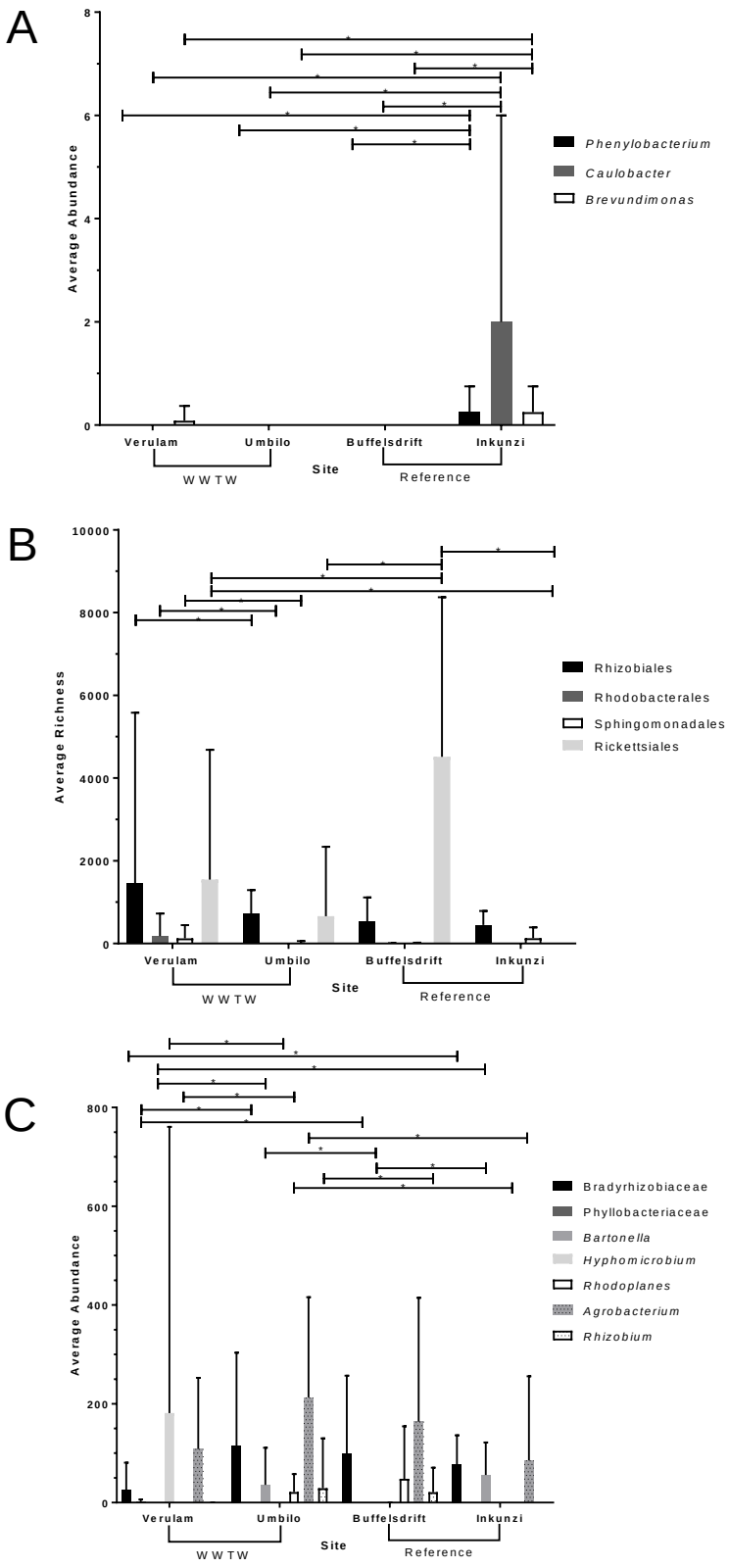

Figure 16: (A) Significant differences in the average intestinal Alphaproteobacteria load, (B) intestinal Alphaproteobacterial order loads, and (C) intestinal Rhizobiales loads in *Neoromicia nana* caught at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa. Asterisks indicate a significant difference between sites ($p<0.05$). Error bars = Standard deviation.

3.8.2 Class Betaproteobacteria

The order Methylophilales (Table 18) were significantly more abundant in males than females ($W_{(38)}$=142.5, P=0.02; Figure 17A). Bacteria in the family Methylophilaceae not assigned to a genus were significantly more abundant in bats at Verulam WWTWs than those at Buffelsdrift and Umbilo WWTWs (χ^2=4.62, df=3, P=0.2, Dunn's Test z=-1.79, P=0.04 and z=-1.83, P=0.04, respectively).

Table 18: Taxonomic classification of the intestinal bacteria found within the class Betaproteobacteria in *Neoromicia nana* collected at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa.

Class	Order	Family
Betaproteobacteria	Burkholderiales	Alcaligenaceae
		Burkholderiaceae
		Comamonadaceae
		Oxalobacteraceae
		Other
	Methylophilales	Methylophilaceae
	Neisseriales	Neisseriaceae
	Procabacteriales	Procabacteriaceae
	Rhodocyclales	Rhodocyclaceae

3.8.2.1 Order Burkholderiales

The family Oxalobacteraceae was significantly more abundant at WWTWs than reference sites ($W_{(38)}$=125, P=0.04). This was predominantly due to the significantly greater load at Umbilo WWTWs than at Buffelsdrift (χ^2=7.75, df=3, P=0.05, Dunn's Test z=-2.77, P=0.003; Figure 17B). Further, the genus *Ralstonia* within this family, was significantly more abundant in bats at Verulam WWTWs than those at Umbilo WWTWs (χ^2=3.7, df=3, P=0.3, Dunn's Test z=-1.7, P=0.04; Figure 17B). Further, the bacteria in this family not assigned to a genus were significantly more abundant in bats at Umbilo WWTWs than those at Buffelsdrift and Verulam WWTWs (χ^2=10.4, df=3, P=0.02, Dunn's Test z=-2.7, P=0.003 and z=2.77, P=0.003, respectively). Burkholderiales not assigned to a family were significantly more abundant in females than males ($W_{(38)}$=241.5, P=0.03). Further, these bacteria were significantly more abundant in bats at Umbilo WWTWs than those at Buffelsdrift and Verulam WWTWs (χ^2=7.63, df=3, P=0.05, Dunn's Test z=-2.53, P=0.006 and z=2.09,

P=0.02, respectively). The genus *Curvibacter* (family: Comamonadaceae) was significantly more abundant in bats at Verulam WWTWs than those at Buffelsdrift and Umbilo WWTWs (χ^2=7.11, df=3, P=0.07, Dunn's Test z=-2.22, P=0.01 and z=-2.27, P=0.01, respectively; Figure 17B). Further, the bacteria within the family Comamonadaceae not assigned to a genus were significantly more abundant in bats at Verulam WWTWs than those at Umbilo WWTWs (χ^2=4.3, df=3, P=0.23, Dunn's Test z=-1.89, P=0.03).

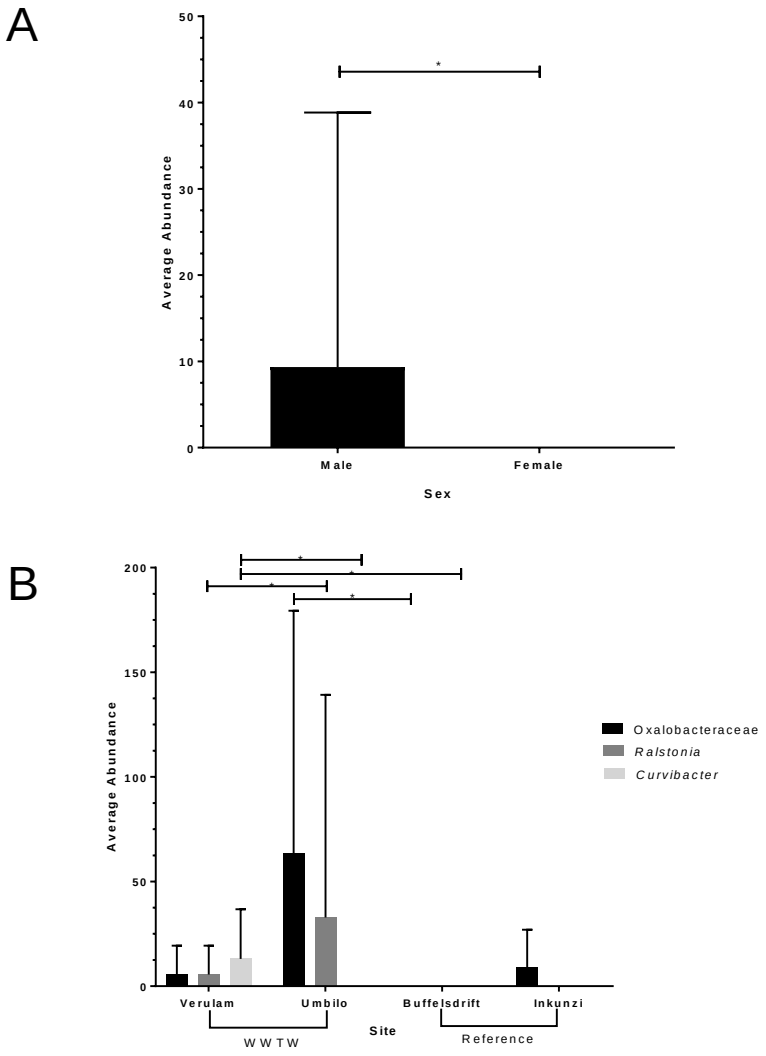

Figure 17: (A) Average intestinal Methylophilaceae load in male and female *Neoromicia nana* and (B) significant differences in the average intestinal Burkholderiales load in *N. nana* caught at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa. Asterisks indicate a significant difference between sites ($p<0.05$). Error bars = Standard deviation.

3.8.3 Class Epsilonproteobacteria

Inkunzi bats had a significantly greater load of Epsilonproteobacteria than bats at all other sites (χ^2=6.92, df=3, P=0.07, Dunn's Test Verulam: z=1.98, P=0.02, Umbilo: z=2.08, P=0.02

and Buffelsdrift: z=-2.6, P=0.005). This trend was also found in the order Campylobacterales and the family Helicobacteraceae (Table 19). *Helicobacter* were found in significantly greater numbers in bats at Inkunzi than those at Buffelsdrift (χ^2=5.29, df=3, P=0.15, Dunn's Test z=-2.21, P=0.01; Table 19; Figure 18) and *Flexispira* were found in significantly greater numbers in bats at Inkunzi compared to bats at all other sites (χ^2=13.68, df=3, P=0.000, Dunn's Test Verulam: z=2.94, P=0.002, Umbilo: z=3.13, P=0.0009 and Buffelsdrift: z=-3.64, P=0.0001; Table 19; Figure 18). The bacteria not assigned to a genus were also found in significantly greater numbers in bats at Inkunzi compared to those at all other sites (χ^2=6.82, df=3, P=0.08, Dunn's Test Verulam: z=1.978, P=0.02, Umbilo: z=2.08, P=0.02 and Buffelsdrift: z=-2.6, P=0.005).

Table 19: Taxonomic classification of the intestinal bacteria found within the class Epsilonproteobacteria in *Neoromicia nana* collected at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa.

Class	Epsilonproteobacteria		
Order	Campylobacterales		
Family	Helicobacteraceae		
Genus	Other	*Flexispira*	*Helicobacter*

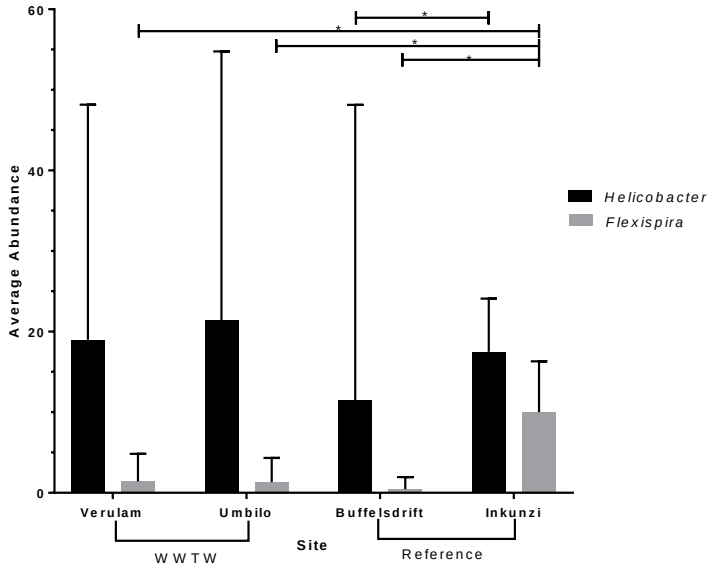

Figure 18: Significant differences found in the average intestinal Epsilonproteobacteria load in *Neoromicia nana* caught at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa. Asterisks indicate a significant difference between sites (p<0.05). Error bars = Standard deviation.

3.8.4 Class Gammaproteobacteria

Bats at Inkunzi had a significantly greater load of Gammaproteobacteria (Table 20) than those at Umbilo WWTWs (χ^2=3.17, df=3, P=0.37, Dunn's Test z=1.77, P=0.04).

Table 20: Taxonomic classification of the intestinal bacteria found within the class Gammaproteobacteria in *Neoromicia nana* collected at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa.

Class	Order	Family
Gammaproteobacteria	Aeromonadales	Aeromonadaceae
		Succinivibrionaceae
		Other
	Enterobacteriales	Enterobacteriaceae
	Legionellales	Coxiellaceae
		Legionellaceae
		Other
	Oceanospirillales	Alcanivoracaceae
	Pasteurellales	Pasteurellaceae
	Pseudomonadales	Moraxellaceae
		Pseudomonadaceae
	Xanthomonadales	Sinobacteraceae
		Xanthomonadaceae
	Other	

3.8.4.1 Order Enterobacteriales

The order Enterobacteriales (Table 21) were found in significantly greater numbers in bats at Inkunzi than those at Buffelsdrift and Umbilo (χ^2=5.5243, df=3, P=0.14, Dunn's Test z=-1.83, P=0.03 and z=1.87, P=0.03, respectively). The same trend was evident in the family Enterobacteriaceae (Table 20). Significantly greater numbers of the genus *Citrobacter* were found in bats at Inkunzi than those at Buffelsdrift (χ^2=4.93, df=3, P=0.18, Dunn's Test z=-2.11, P=0.02; Figure 19A). The genus *Morganella* was significantly more abundant in males than females ($W_{(38)}$=141, P=0.04; Figure 19B) and in bats at Verulam WWTWs than in those at Umbilo WWTWs (χ^2=3.46, df=3, P=0.33, Dunn's Test z=-1.65, P=0.05; Figure 19A). Bats at Inkunzi had significantly greater numbers of the genera *Serratia* (χ^2=13.41, df=3, P=0.000, Dunn's Test Verulam: z=3.52, P=0.0002, Umbilo: z=2.45, P=0.007 and Buffelsdrift: z=-3.12, P=0.0009; Figure 19A) and *Providencia* (χ^2=8.75, df=3, P=0.03, Dunn's Test Verulam: z=2.7, P=0.003, Umbilo: z=2.7, P=0.003 and Buffelsdrift: z=-2.67, P=0.004; Figure 19A) than bats at all other sites. The genus *Proteus* was significantly more abundant in bats at Inkunzi than

those at Buffelsdrift and Umbilo WWTWs (χ^2=6.83, df=3, P=0.08, Dunn's Test z=-1.88, P=0.03 and z=2.3, P=0.01, respectively; Figure 19A) and significantly more abundant in bats at Verulam WWTWs than those at Umbilo WWTWs (χ^2=6.83, df=3, P=0.08, Dunn's Test z=-1.74, P=0.04; Figure 19A). Enterobacteriaceae not assigned to a genus were significantly more abundant in bats at Inkunzi than those at Buffelsdrift and Umbilo (χ^2=5.8, df=3, P=0.12, Dunn's Test z=-1.96, P=0.03 and z=1.67, P=0.05, respectively) and significantly more abundant in bats at Verulam WWTWs than those at Buffelsdrift (χ^2=5.81, df=3, P=0.12, Dunn's Test z=-1.74, P=0.04).

Table 21: Taxonomic classification of the intestinal bacteria found within the order Enterobacteriales in *Neoromicia nana* collected at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa.

Order	Family	Genus
Enterobacteriales	Enterobacteriaceae	*Citrobacter*
		Cronobacter
		Erwinia
		Escherichia
		Klebsiella
		Morganella
		Plesiomonas
		Proteus
		Providencia
		Serratia
		Trabulsiella
		Xenorhabdus
		Other

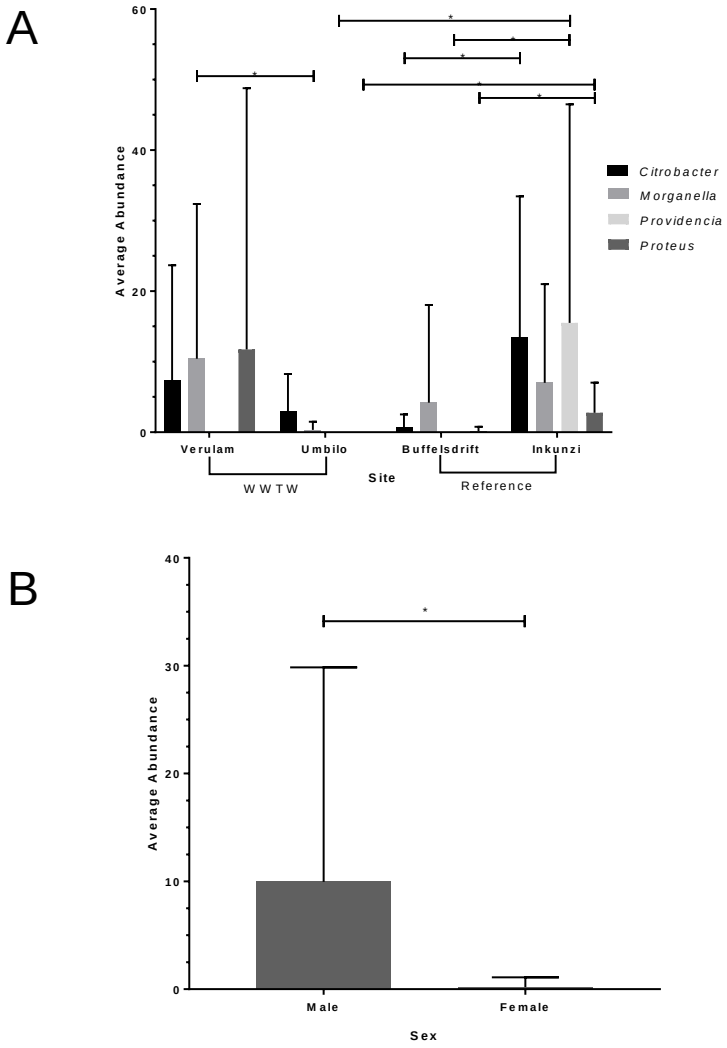

Figure 19: (A) Significant differences found in the average intestinal Enterobacteriales load in *Neoromicia nana* and (B) average intestinal *Morganella* load in male and female *N. nana* caught at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa. Asterisks indicate a significant difference between sites ($p<0.05$). Error bars = Standard deviation.

3.8.4.2 Order Legionellales

The order Legionellales (Table 22) were found in significantly greater numbers in bats at Verulam WWTWs than those at Buffelsdrift and Umbilo WWTWs (χ^2=10.53, df=3, P=0.01, Dunn's Test z=-2.73, P=0.003 and z=-2.79, P=0.003, respectively; Figure 20). The family Legionellaceae followed the same trend (χ^2=7.11, df=3, P=0.07, Dunn's Test z=-2.22, P=0.01 and z=-2.27, P=0.01, respectively; Table 22). Bacteria within this family not assigned to a genus were significantly more abundant at Verulam WWTWs than at Buffelsdrift and Umbilo WWTWs (χ^2=4.62, df=3, P=0.2, Dunn's Test z=-1.79, P=0.04 and z=-1.83, P=0.04, respectively). Within the family Coxiellaceae, the genus *Rickettsiella* was found in

significantly greater numbers in bats at Inkunzi than those at all other sites (χ^2=8.75, df=3, P=0.03, Dunn's Test Verulam: z=2.7, P=0.003, Umbilo: z=2.7, P=0.003 and Buffelsdrift: z=-2.67, P=0.004; Figure 20).

Table 22: Taxonomic classification of the intestinal bacteria found within the order Legionellales in Neoromicia nana collected at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa.

Order	Legionellales					
Family	Other	Coxiellaceae			Legionellaceae	
Genus		Other	*Aquicella*	*Rickettsiella*	Other	*Legionella*

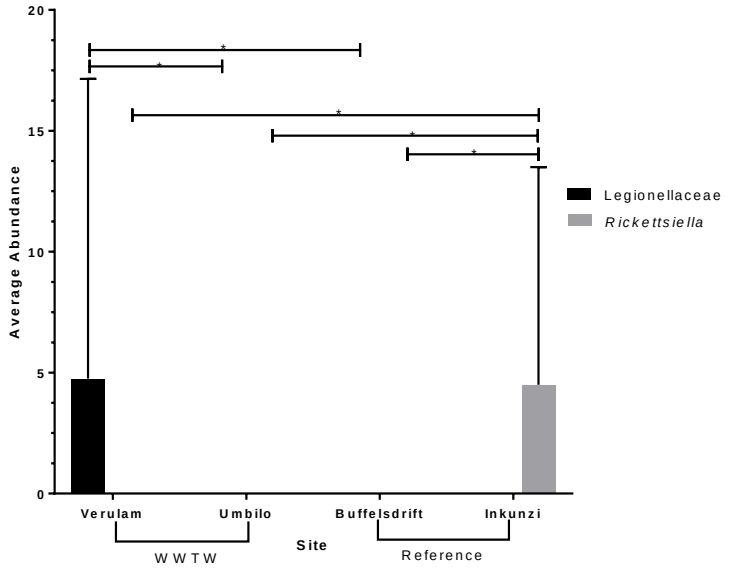

Figure 20: Significant differences found in the average intestinal Legionellales load in *Neoromicia nana* caught at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa. Asterisks indicate a significant difference between sites (p<0.05). Error bars = Standard deviation.

3.8.4.3 Order Pasteurellales

The order Pasteurellales (Table 23) were found in significantly greater numbers at reference sites than at WWTWs ($W_{(38)}$=286, P=0.002). More specifically, bats at the reference sites Inkunzi and Buffelsdrift had significantly more Pasteurellales than bats at both WWTWs sites (χ^2=9.97, df=3, P=0.02, Dunn's Test Verulam: z=2.25, P=0.01, Umbilo: z=1.85, P=0.03 and Verulam: z=2.56, P=0.005, Umbilo: z=2, P=0.02, respectively). The same trend was seen in the family Pasteurellaceae (Table 23; Figure 21). The genus *Actinobacillus* was significantly more abundant at references sites than WWTWs ($W_{(38)}$=282, P=0.003). Further, these bacteria

were found in a significantly greater load in bats at Inkunzi than those at Verulam and Umbilo WWTWs (χ^2=10.42, df=3, P=0.02, Dunn's Test z=2.54, P=0.006 and z=1.77, P=0.04, respectively; Figure 21) and were significantly more abundant in bats at Buffelsdrift than those at Verulam WWTWs (χ^2=10.42, df=3, P=0.02, Dunn's Test z=2.65, P=0.004; Figure 21). Pasteurellaceae not assigned to a genus were found in significantly greater numbers at Inkunzi than at Umbilo WWTWs (χ^2=3.69, df=3, P=0.3, Dunn's Test z=1.79, P=0.04).

The genus *Stenotrophomonas* (order: Xanthomonadales, family: Xanthomonadaceae) were significantly more abundant in females than males ($W_{(38)}$=240, P=0.02). Bacteria within the order Aeromonadales that were not assigned to a family were found in greater loads at Verulam WWTWs than at Umbilo WWTWs (χ^2=4.64, df=3, P=0.2, Dunn's Test z=-2, P=0.02).

Table 23: Taxonomic classification of the intestinal bacteria found within the order Pasteurellales in *Neoromicia nana* collected at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa.

Order	Pasteurellales				
Family	Pasteurellaceae				
Genus	Other	*Actinobacillus*	*Aggregatibacter*	*Haemophilus*	*Pasteurella*

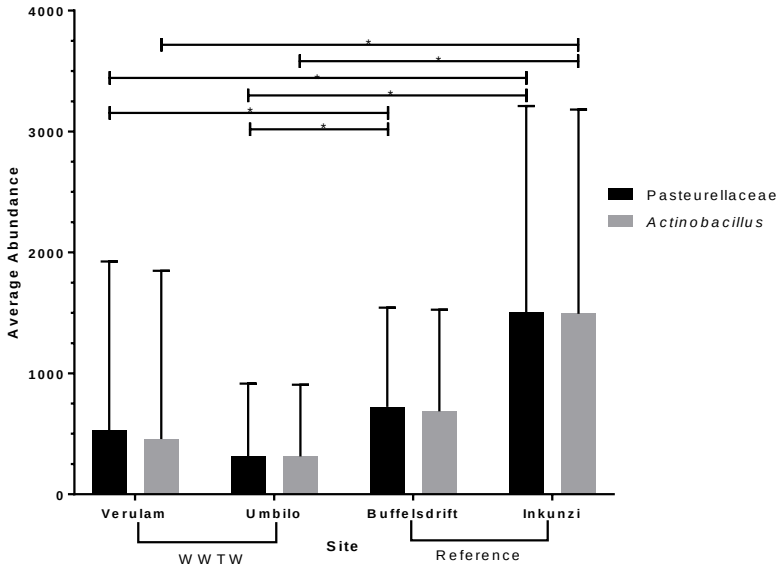

Figure 21: Significant differences found in the average intestinal Pasteurellales load in *Neoromicia nana* caught at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa. Asterisks indicate a significant difference between sites (p<0.05). Error bars = Standard deviation.

3.9 Phylum Bacteroidetes

All four classes found within this phylum showed significant differences between sites (Table 24). Bats caught at Verulam WWTWs had a significantly greater load of the class Bacteroidia than those caught at Buffelsdrift (χ^2=4.2, df=3, P=0.24, Dunn's Test z=-1.90, P=0.03). Within Bacteroidales (Table 24), bats caught at Verulam WWTWs had a significantly greater load of the family Porphyromonadaceae than those caught at Buffelsdrift and Umbilo WWTWs (χ^2=4.62, df=3, P=0.2, Dunn's Test z=-1.79, P=0.04 and z=-1.83, P=0.04, respectively; Figure 22A). Bats caught at Inkunzi had a significantly greater load of bacteria belonging to the class Sphingobacteriia than bats at Umbilo WWTWs and Verulam WWTWs (χ^2=4.94, df=3, P=0.18, Dunn's Test z=1.99, P=0.02 and z=1.99, P=0.02, respectively). This difference was solely due to the genus *Sphingobacterium* (order: Sphingobacteriales, family: Sphingobacteriaceae; Table 24; Figure 22A). Bacteria from the class Saprospirae were found in significantly greater numbers in Verulam WWTWs bats compared to bats at Buffelsdrift and Umbilo WWTWs (χ^2=7.11, df=3, P=0.07, Dunn's Test z=-2.22, P=0.01 and z=-2.27, P=0.01, respectively). The biggest contributor to this difference was the genus *Sediminibacterium* (order: Saprospirales, family: Chitinophagaceae; Table 24) (χ^2=4.62, df=3, P=0.2, Dunn's Test z=-1.79, P=0.04 and z=-1.83, P=0.04, respectively; Figure 22A).

Table 24: Taxonomic classification of the intestinal bacteria found within the phylum Bacteroidetes in *Neoromicia nana* collected at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa.

Phylum	Bacteroidetes			
Class	Bacteroidia	Flavobacteriia	Sphingobacteriia	Saprospirae
Order	Bacteroidales	Flavobacteriales	Sphingobacteriales	Saprospirales

3.9.1 Class Flavobacteriia

Flavobacteriales (Table 25) were found in significantly larger numbers in bats caught at Inkunzi compared to those caught at Verulam WWTWs (χ^2=3.9, df=3, P=0.27, Dunn's Test z=1.73, P=0.04). Bats caught at Inkunzi had a significantly greater average load of Flavobacteriaceae than bats caught at Buffelsdrift, Umbilo and Verulam WWTWs (χ^2=8.51, df=3, P=0.04, Dunn's Test z=-2.39, P=0.009, z=2.89, P=0.002 and z=2.4, P=0.008, respectively). Of the two genera found within Flavobacteriaceae (*Flavobacterium* and

Myroides; Table 25), the only significant difference was in *Myroides*, where bats at Inkunzi showed a significantly higher load than those at Buffelsdrift, Umbilo WWTWs and Verulam WWTWs (χ^2=17.96, df=3, P=0.000, Dunn's Test z=-3.83, P=0.0001, z=3.87, P=0.0001 and z=3.87, P=0.0001, respectively; Figure 22B). Further, Buffelsdrift bats had a significantly higher load of Weeksellaceae than those at Verulam WWTWs (χ^2=4.85, df=3, P=0.18, Dunn's Test z=1.86, P=0.03; Table 25). This was also the case in the genus *Chryseobacterium* (χ^2=4.74, df=3, P=0.19, Dunn's Test z=2.02, P=0.02; Table 25; Figure 22B).

Table 25: Taxonomic classification of the intestinal bacteria found within the class Flavobacteriia in *Neoromicia nana* collected at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa.

Class	Flavobacteriia			
Order	Flavobacteriales			
Family	Flavobacteriaceae		Weeksellaceae	
Genus	*Flavobacterium*	*Myroides*	Other	*Chryseobacterium*

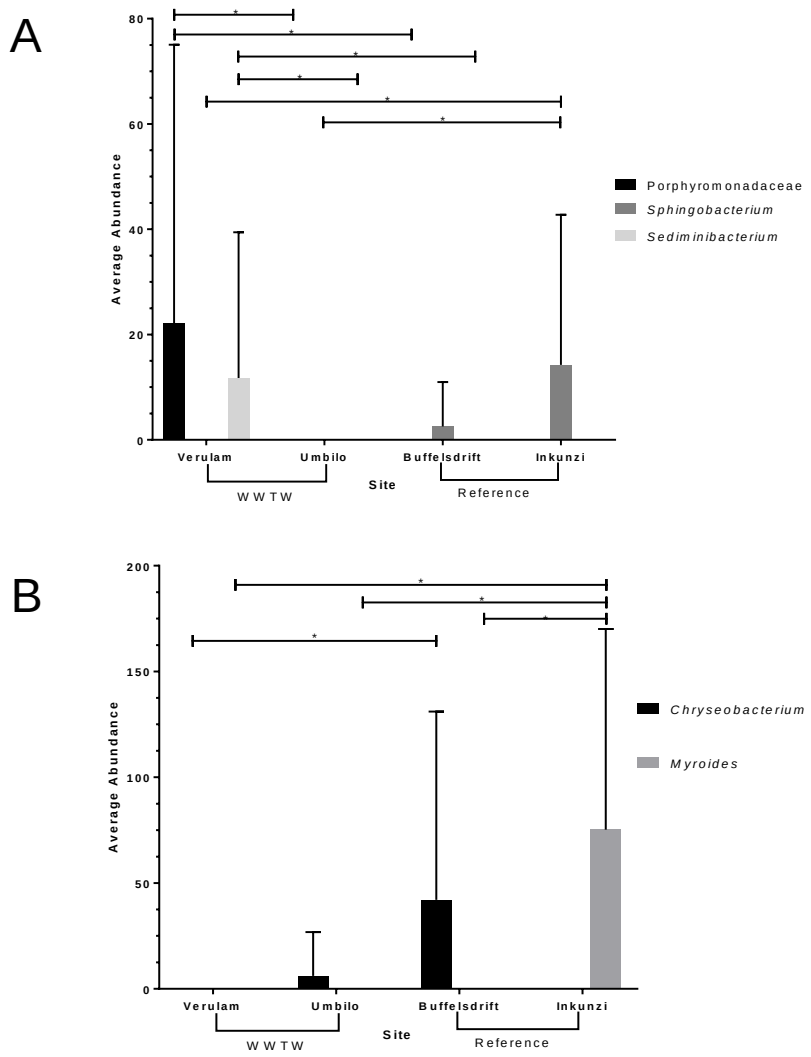

Figure 22: Significant differences found in the average intestinal (A) Bacteroidetes and (B) Flavobacteriia load in *Neoromicia nana* caught at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa. Asterisks indicate a significant difference between sites ($p<0.05$). Error bars = Standard deviation.

3.10 Phylum Cyanobacteria

Within the phylum Cyanobacteria, the order Stramenopiles (class: Chloroplast; Table 26) was found in significantly greater numbers in bats at Verulam WWTWs than those caught at Inkunzi and Umbilo WWTWs (χ^2=4.85, df=3, P=0.18, Dunn's Test z=-1.86, P=0.03 and z=-1.76, P=0.04, respectively; Figure 23).

Table 26: Taxonomic classification of the intestinal bacteria found within the phylum Cyanobacteria in *Neoromicia nana* collected at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa.

Phylum	Cyanobacteria			
Class	4C0d-2	Chloroplast	Oscillatoriophycideae	Synechococcophycideae

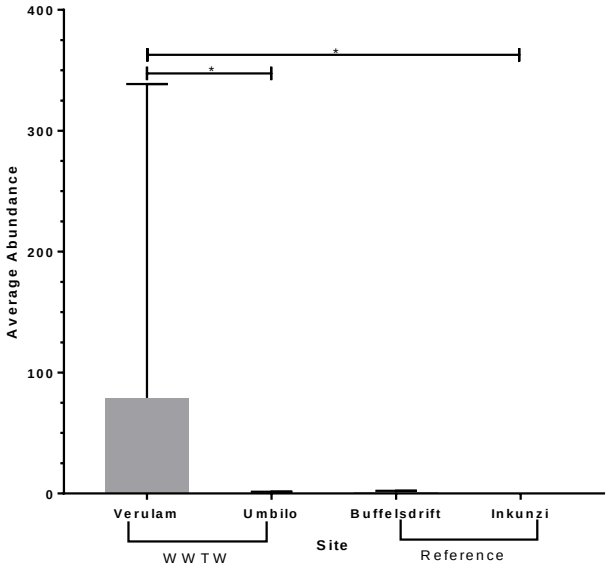

Figure 23: Average intestinal Stramenopiles load in *Neoromicia nana* caught at wastewater treatment works (WWTWs) and reference sites in KwaZulu-Natal, South Africa. Asterisks indicate a significant difference between sites ($p<0.05$). Error bars = Standard deviation.

3.11 Similarity in intestinal bacterium communities among individual bats

NMDS revealed that male and female bats showed a lot of overlap based on the number of bacteria per phylum (Figure 24). The polygon defining the female microbiome is extended far to the left because of one individual at Verulam. NMDS further revealed that, based on the number of bacteria per phylum, bats at different sites showed a lot of overlap (Figure 25). The Verulam WWTWs polygon is extended far to the left because of the same individual extending the female polygon. This effect was enhanced as resolution increased from phylum to genus.

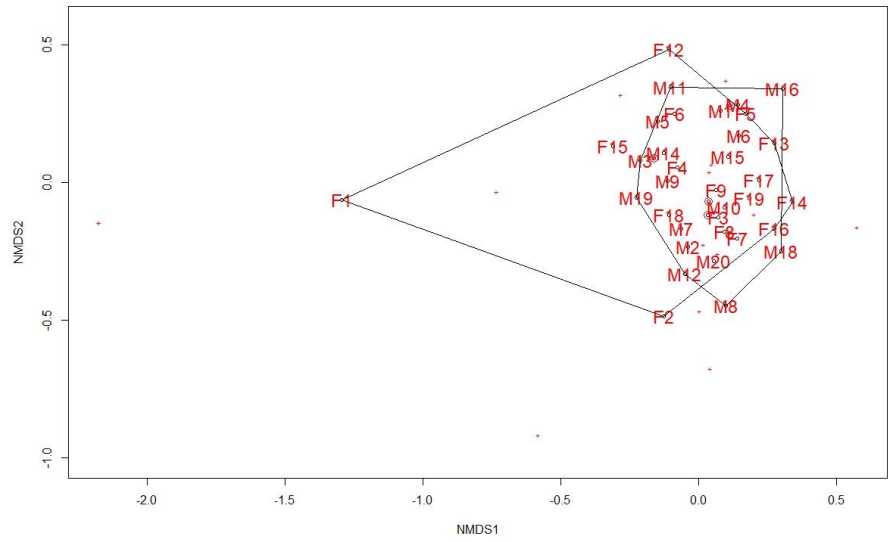

Figure 24: Non-metric Multi-Dimensional Scaling (NMDS) showing the similarity of male and female *Neoromicia nana*, based on their intestinal bacterium phyla caught at wastewater treatment works and reference sites in KwaZulu-Natal, South Africa. Points labelled F1-F19 and M1-M20 represent female and male bats, respectively. Polygons outline the region that males and females occupy within the two-dimensional field.

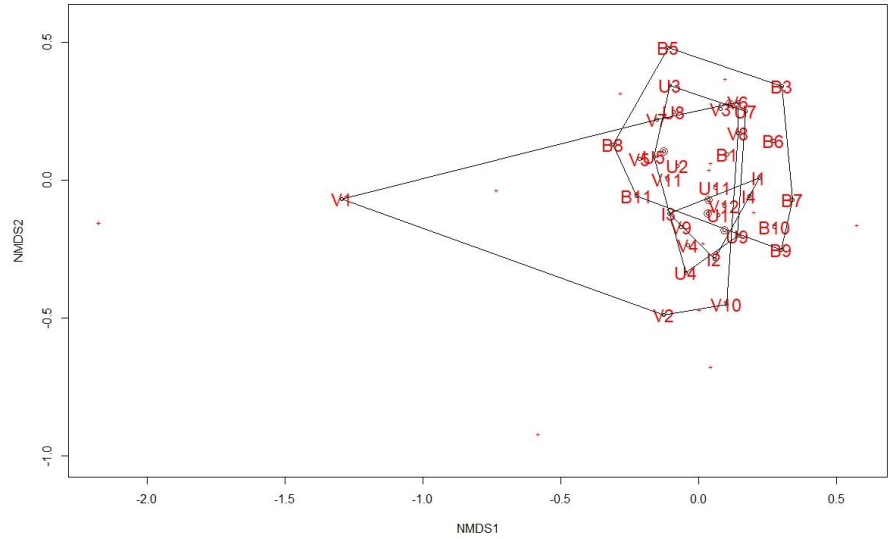

Figure 25: Non-metric Multi-Dimensional Scaling (NMDS) showing the similarity of *Neoromicia nana* bats based on their intestinal bacterium phyla caught at wastewater treatment works and reference sites in KwaZulu-Natal, South Africa. Points labelled V, U, B and I represent bats caught at Verulam WWTWs, Umbilo WWTWs, Buffelsdrift and Inkunzi, respectively. Polygons outline the region that bats at these sites occupy within the two-dimensional field.

Heirarchical clustering of differences in microbiome composition (at phylum level), as well as differences in bat body size, sex and locality resulted in three clusters with high confidence

(Figure 26). All clusters had a confidence level > 80%, unless specified. V1, far removed from the other individuals, is the same individual that extended the female and Verulam WWTWs polygons in Figures 24 and 25. The dendrogram (Figure 26) reveals a significant difference in the sexes: two clusters formed at the first split (left cluster=females, right cluster=males). When clusters were produced at lower taxonomic levels, the differences between sexes became less distinct (Figures 27 and 28). Although some clusters comprised predominantly one sex, increased taxonomic resolution resulted in overlap between the sexes (Figure 28).

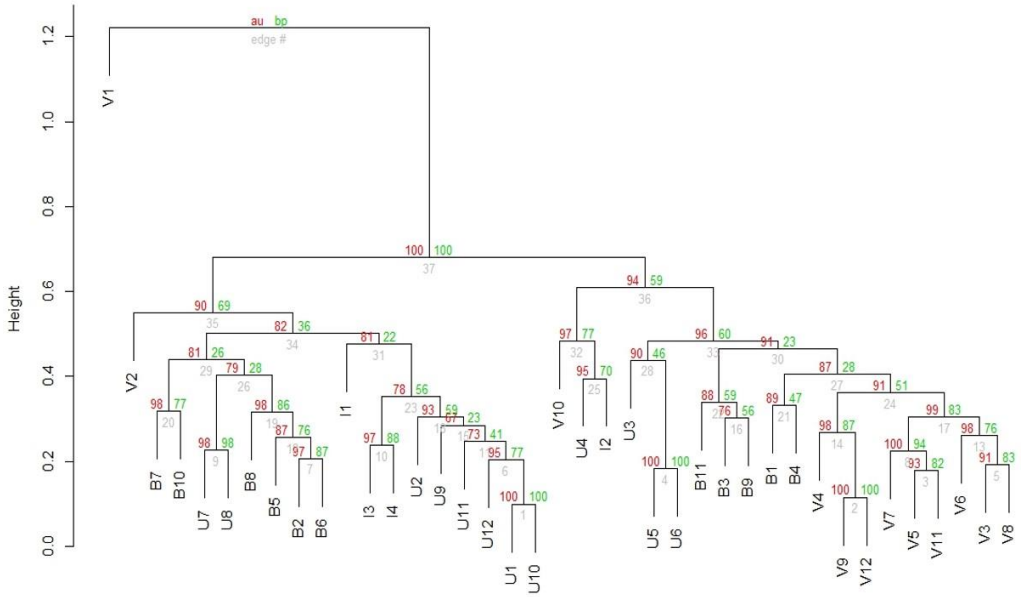

Figure 26: Hierarchical cluster analysis showing the similarity among *Neoromicia nana* caught at wastewater treatment works and reference sites in KwaZulu-Natal, South Africa based on bacterium phyla and extrinsic and intrinsic factors that may influence bacterium phyla and extrinsic and intrinsic factors that may influence bacterium abundance. Red (au) values represent the bootstrap value of each branch. Each leaf represents an individual bat (U=Umbilo, V=Verulam, I=Inkunzi, B=Buffelsdrift).

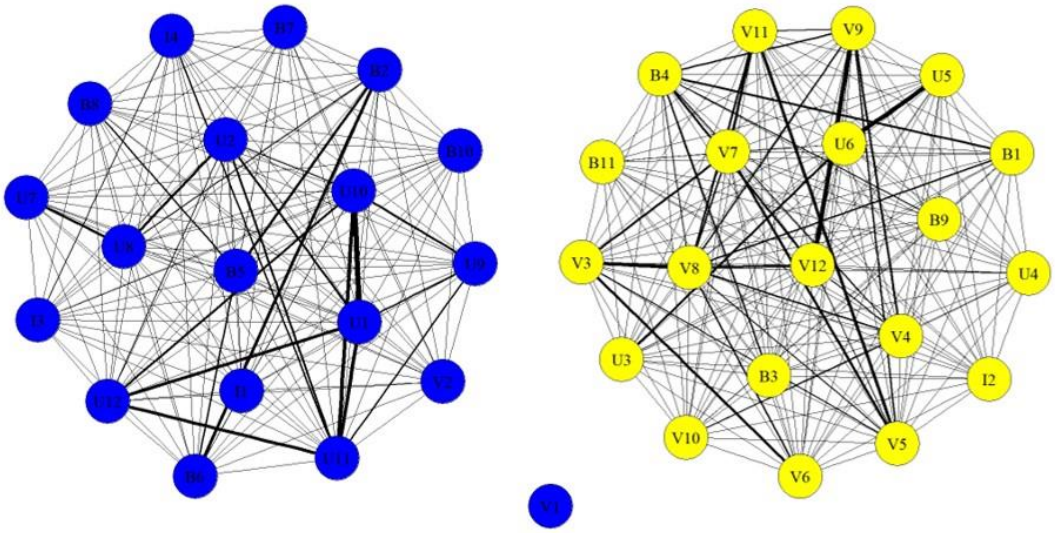

Figure 27: Subgraphs of the network showing the similarity among *Neoromicia nana* caught at wastewater treatment works and reference sites in KwaZulu-Natal, South Africa, based on bacterium phyla and extrinsic and intrinsic factors that may influence bacterium abundance. Nodes represent individual bats (U=Umbilo, V=Verulam, I=Inkunzi, B=Buffelsdrift; Blue=Female, Yellow=Male). Edges represent the similarity between individuals based on the abundance of bacterium phyla, sex, site, forearm length and body mass. Edge width represents the strength of the similarity between nodes. Each subgraph represents a cluster determined by a hierarchical cluster analysis. Edges connecting clusters are not shown.

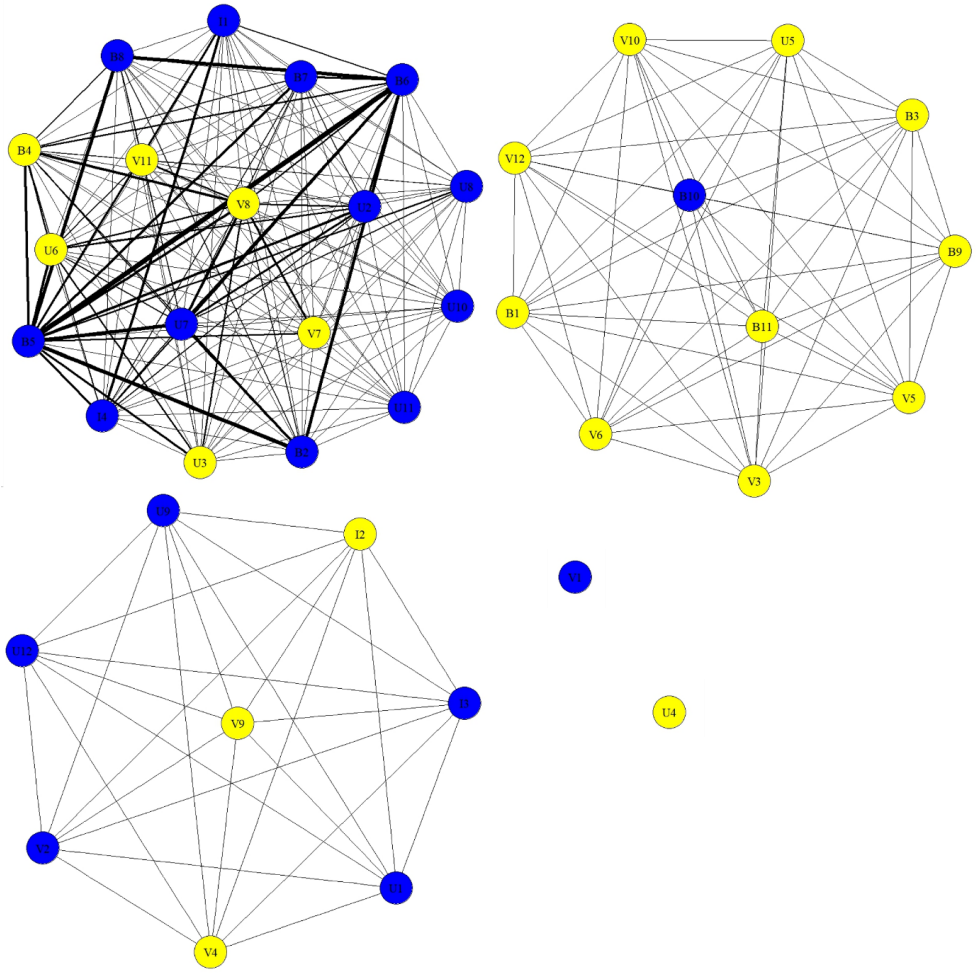

Figure 28: Subgraphs of the network showing the similarity among *Neoromicia nana* caught at wastewater treatment works and reference sites in KwaZulu-Natal, South Africa, based on bacterium genera and intrinsic and extrinsic factors that may influence bacterium abundance. Nodes represent individual bats (U=Umbilo, V=Verulam, I=Inkunzi, B=Buffelsdrift; Blue=Female, Yellow=Male). Edges represent the similarity between individuals based on the abundance of bacterium genera, sex, site, forearm length and body mass. Edge width represents the strength of the similarity between nodes. Each subgraph represents a cluster determined by a hierarchical cluster analysis. Edges connecting clusters are not shown. U4, which formed its own cluster, had a confidence level of 74%.

CHAPTER 4: DISCUSSION

4.1 Differences in microbial taxa load

In accordance with predictions, bats at WWTWs had greater gut bacterial diversity than those at reference sites. This difference was most significant in bats at the Verulam WWTWs and may be due to differences in several factors such as sex, locality, diet, exposure to chemical and biological pollutants and ectoparasites. Moreover, bats at WWTWs had greater numbers of unique taxa than those at reference sites. Further, bats at Buffelsdrift has a greater number of taxa in common with those at WWTWs than those at Inkunzi, probably because this site was in closer proximity to urban settlements and urban influences than Inkunzi. Because each microbial taxon may be affected in different ways by these factors, and their presence and abundance may have positive (pollution detoxification and the breakdown of organic substances) or adverse (pathogens) effects on the hosts health (King, 2014), statistically significant differences in medically and environmentally important taxa are discussed below.

Chlamydia species were only found in bats at Inkunzi. Some *Chlamydia* species are widely known as human pathogens (Koren et al., 2011). The higher load of these bacteria in these bats may be due to ectoparasites such as ticks, fleas, lice and mites which are known carriers of pathogens such as *Chlamydia* (Eddie et al., 1969) and other bacteria from the Chlamydiaceae family (Hokynar et al., 2016). Further, bats can naturally harbour *Chlamydia* species acquired from their prey as well as their own *Chlamydiales* species (Hokynar et al., 2017), such as those from the genus *Waddlia*, which cause lesions in the spleen and lungs of their hosts (Pierlé et al., 2015). In bats such as *Molossus rufus*, ectoparasite prevalence may be higher in bats roosting in natural environments than those in urban environments (Esbérard et al., 2005). Similarly, ectoparasite prevalence may be significantly lower in birds from urban environments than those from natural environments (Delgado-V and French, 2012). Bacteria, introduced into the blood stream by ectoparasites, are able to invade the biliary tract (which is typically sterile in healthy individuals) and therefore the intestinal environment via the duodenum (Sung et al., 1992). Because Inkunzi is further removed from urban influences than the other sites, the resident bats may be more exposed to ectoparasites than bats at urban sites or WWTWs.

An unexpected result was the high occurrence of the phylum Chloroflexi in Verulam WWTW bats. In their natural environment, these bacteria are typically found in cave roosting bats (Mérida et al., 2016) and bat guano collected from caves (De Mandal et al., 2015). The presence of these bacteria in *N. nana* is therefore unexpected as these bats roost in the rolled-up leaves of banana plants and not caves (van der Merwe and Stirnemann, 2009). Chloroflexi, filamentous bacteria, can be found in high loads in wastewater due to their biological nutrient removal capability (Björnsson et al., 2002). The abundance of these bacteria in soil is positively correlated with increasing metal pollution levels (Azarbad et al., 2015). If this trend holds true for aquatic ecosystems, we would expect a high relative load of these bacteria in wastewater, thereby increasing the risk of exposure to bats foraging at WWTW sites.

Wastewater exposure may also be responsible for the significantly greater number of bacteria from the family Pirellulaceae (phylum: Planctomycetes) and the genus *Gemmata* in Verulam WWTW bats. Planctomycetes, associated with WWTWs due to their important role in nitrogen cycling (Kartal et al., 2010), are highly resistant to ammonium, nitrite and nitrate concentrations (Flores et al., 2014). Because these bacteria are resistant to the harsh wastewater conditions, I expected a high load of these bacteria at WWTWs and therefore a greater risk of resident bats being exposed to these bacteria.

Fusobacteria, typically found in low loads (Willing et al., 2010) or not at all (McLellan et al., 2011) in the human GI tract, were significantly more abundant in bats at Verulam WWTW. These bacteria, found in large numbers in wastewater (McLellan et al., 2011), are indicative of intestinal distress in humans (Willing et al., 2010). Moreover, the significantly higher load of the genus *Fusobacterium* in these bats may indicate intestinal distress given that this genus has been linked to intestinal inflammation, tumour formation and cancer formation in the GI tract of humans (Sheflin et al., 2014).

The polyunsaturated fatty acid (PUFA) (Ghioni et al., 1996; Hill et al., 2016) rich diet of bats at WWTWs (Huang et al., 2014; Zhang et al., 2016), may also have played a role in the lower load of Tenericutes at WWTW bats compared to those at Buffelsdrift (Zhang et al., 2016). Although not significantly different, Tenericutes load varied greatly between sites: 17%, 17%, 21% and 3% of all GI bacterial diversity in bats at Verulam, Umbilo, Buffelsdrift and Inkunzi, respectively. Tenericutes, one of the most abundant phyla found in organisms such as

lampreys (Tetlock et al., 2012), amphibians (Zhang et al., 2016), humans (Costello et al., 2013), dogs and cats (Suchodolski, 2011), are typically commensal but several species are disease causing (Taylor-Robinson, 1996). The majority of the Tenericutes found in this study belonged to the genus *Mycoplasma*, comprising host-specific bacteria found in the mouth, respiratory tract and genitourinary tracts of animals and humans (Taylor-Robinson, 1996). The short-chain fatty acid diet of bats at Inkunzi (DeFoliart, 1991; Hildebrandt et al., 2009) may result in dysbiosis of intestinal bacterial communities and may alter the natural diurnal patterns in the load of these bacteria (Leone et al., 2015), particularly Cyanobacteria (Voight et al., 2016). Although the low (~0.1%) relative load of these bacteria found in bats at Inkunzi is consistent with naturally occurring loads in healthy individuals (Costello et al., 2013), the lower load compared to bats at Verulam WWTW may be due to differences in dietary fat content.

The high load of certain Bacteroidetes classes in Verulam WWTW bats may partially be attributed to their resistance to metal toxicity (Zhang et al., 2016). However, the phylum Bacteroidetes are a specialised group of bacteria involved in the breakdown of complex polysaccharides (Koenig et al., 2011). The high load of the family Chitinophagaceae in Verulam WWTW bats may be due to differences in diet. These bacteria are responsible for the breakdown of chitin (Banskar et al., 2016), found at higher levels in chironomid midges (Diptera), the main food source of bats foraging at WWTWs, than in lepidopterans (Cauchie, 2002), which comprise a high percentage of the diet of *N. nana* bats at sites not polluted by WWTWs (Naidoo et al., 2013; Schoeman and Jacobs, 2011).

Differences in diet may also explain the significant greater load of the phylum Spirochaetes at reference sites (De Filippo et al., 2010,). *Spironema*, the only genus found within this phylum, are responsible for fibre digestion and short-chain fatty acid production, protecting the gut against inflammation (De Filippo et al., 2010). However, because these bacteria (some of which are pathogenic) are also transmitted by ticks (Venzal et al., 2008), their increased load in bats at reference sites is more likely due to exposure to ectoparasites. Ectoparasite loads are significantly lower in urban populations of volant animals such as the bat *Molossus rufus* (Esbérard et al., 2005) and the bird *Turdus merula* (Gregoire et al., 2002) compared to populations in natural habitats, yet the mechanisms driving these differences are not yet understood.

Ectoparasites may be responsible for differences within the phylum Actinobacteria between sites. Inkunzi bats had the highest load of almost all Actinobacteria taxa (Nocardioidaceae, Micrococcaceae, *Mycobacterium*, *Microbispora* and *Microbacterium*). The phylum Actinobacteria is generally found in low loads in the GI tract (Ley et al., 2005) and used to produce most of the antibiotics humans use today (Hamm et al., 2017; Kumari et al., 2013). Of significant importance is the high load of the genus *Mycobacterium* in Inkunzi bats. Although these species are commonly found in the intestine, some species within this genus are pathogenic, for example *Mycobacterium tuberculosis* is known to cause tuberculosis (Alirol et al., 2010). Several species of *Mycobacterium* are common in wildlife (Alexander et al., 2002; de Lisle et al., 2001), including bats, and can be transmitted to humans and domestic pets (Marinkelle and Grose, 1972). Further, these bacteria are adequately removed from wastewater influent using filtration, UV treatment and chlorination (Bohrerova and Linden, 2006), thereby reducing the risk of exposure. Adequate removal through treatment may therefore have caused significantly lower than natural loads of this bacterium.

4.1.1 Factors influencing Proteobacteria load

Proteobacteria was one of the most abundant and genus-rich bacterial phyla in *N. nana*. This is consistent with other studies on the bat microbiome (Banskar et al., 2016). One reason for the significant richness of this phylum may be because they are well studied and well categorised (Björnsson et al., 2002). This group of bacteria are prominent pathogens in vertebrates. They may enter the circulatory system from the intestine if the gut barrier is impaired (Moon and Stappenbeck, 2012). Caves inhabited by bats harbour an abundance of Proteobacteria, most of which are pathogenic and some are antibiotic resistant (Mérida et al., 2016). However, the significant difference in Proteobacteria load between bats at Inkunzi and WWTWs may be due to an inhibition of Proteobacteria by PCB (Choi et al., 2013) and metal (Sandaa et al., 1999) exposure at WWTWs, and/or stimulation of Proteobacteria by a diet high in saturated fats at reference sites (Krajmalnik-Brown et al., 2012). Because of the high species richness of this phylum, only the most medically important taxa are discussed further.

Gamma– and Betaproteobacteria were found in greater loads in wastewater than in faecal samples, suggesting they proliferate in this environment (McLellan et al., 2011). This is not

surprising because this class contains the species *Vibrio cholerae*, the bacteria responsible for Cholera in humans and prominent in human sewage (Broza et al., 2005). Emerging Chironomid midges, exposed to *V. cholerae* from egg to adult stages, are able to transfer viable pathogenic bacteria from wastewater to clean waterbodies (Broza et al., 2005). *V. cholerae* were not found in bats at any of the sites. Further, despite the typically high load of *Escherichia coli* in some wastewater (McLellan et al., 2011), these bacteria were only observed in five individuals, an observation that will be further explored in section 4.1.2.

High load of the genus *Bartonella* (Alphaproteobacteria) was found in Inkunzi bats. Some *Bartonella* species are disease-causing in humans (Bitam et al., 2010) including trench fever, cat-scratch disease and Oroya fever as well as skin lesions (Spach and Koehler, 1998). *Bartonella* is typically transmitted via ectoparasites (Bitam et al., 2010; Breitschwerdt and Kordick, 2000) including mites, flies, fleas, ticks and bat flies (Wilkinson et al., 2016), and are found in bats (Lilley et al., 2015; Olival et al., 2014; Veikkolainen et al., 2014). Therefore, the significantly greater load of these bacteria in bats at Inkunzi may be due to greater exposure to natural parasites. By contrast, the order Rickettsiales, which was significantly more abundant in Verulam WWTW bats, contains the genus *Neorickettsia* which form a symbiosis with the endoparasites Platyhelminthes and Trematoda (Lawrence and Poulin, 2016), common in human waste (Crompton and Savioli, 1993). These bacteria can be passed to humans and animals via these endoparasites where they may cause diseases (Lawrence and Poulin, 2016). Bat endoparasites have been linked to the transmission of Potomac Horse Fever; this disease is caused by a species from the genus *Neorickettsia* (Gardner and Jimenez-Ruiz, 2009).

The family Legionellaceae (Gammaproteobacteria), found in significantly greater loads in bats at Verulam WWTW, are highly abundant in wastewater and wastewater contaminated ecosystems (Ortiz-Roque and Hazent, 1987). From this family, other than *Legionella pneumophila*, the bacteria responsible for Legionnaire's disease, many species from the same genus are disease-causing in humans (Ortiz-Roque and Hazent, 1987). Ectoparasites may also be responsible for the high load of Gammaproteobacteria in bats at Inkunzi. A high diversity of these bacteria can be found in bat ectoparasites, specifically Streblidae and Nycteribiidae (Morse et al., 2013). Further, the load of the genus *Rickettsiella* was highest in bats at Inkunzi.

These bacteria are intracellular pathogens in arthropods (Leclerque and Kleespies, 2008) and are therefore likely derived from the diet of these bats.

4.1.2 Factors influencing Firmicutes load

Firmicutes are responsible for the production of important metabolites involved in maintaining a healthy intestinal ecosystem. These bacteria, often found in high loads in sewage (McLellan et al., 2011), were one of the most abundant phyla found at all sites. In humans, infants are generally inoculated with the commensal order Lactobacillales via the mother's nipples and breastmilk, thereafter exposure to these bacteria may come through the consumption of food and liquids (Adlerberth and Wold, 2009). Lactobacillales load may be reduced because of environmental cadmium pollution (Dietert and Dietert, 2015), environmental and dietary stresses (Tannock and Savage, 1974), PCB exposure (Choi et al., 2013) and in IBD patients suffering from diarrhoea (Thomas and Ockhuizen, 2012). Lactobacillales are used as probiotics because of their ability to persist through antibiotic administration and ward off invading bacteria through decreased environmental pH brought on by their production of short-chain fatty acids (Woodmansey, 2007). These bacteria also reduce depressive and anxious behaviour in mice (Clarke et al., 2013).

Even though one would expect low load of the order Lactobacillales at WWTWs as a result of possible cadmium and PCB exposure, this order was significantly more abundant in bats at Verulam WWTW. These bacteria increase in abundance when hosts have elevated PUFA levels in the liver (Kalavathy et al., 2006) and pectoral muscles (Jahromi et al., 2016). Therefore, the high PUFA levels in the WWTWs bats (Hill et al., 2016) may be partially responsible for this difference. Further, these bacteria also significantly increase with increased exercise levels (Choi et al., 2013). Mehl et al. (2016) found evidence of increased lactic acid concentrations in the pectoral muscles of WWTWs bats, similar to what you would expect with recovery after exercise (Fournier et al., 2005). The high load of these lactic acid producing bacteria (Oude Elferink et al., 2001) may further indicate increased exercise levels in these individuals.

Although cadmium and PCBs can inhibit Lactobacillales, the genus *Enterococcus* is found in high loads in wastewater influent (Cai et al., 2014). *Enterococcus* were also found in

significantly greater loads in Verulam WWTW bats and specifically in male bats. In healthy GI tracts, bacteria of the genus *Enterococcus* are responsible for vitamin K_2, vitamin B_{12}, folate and biotin production (Suchodolski, 2011). This genus is also known to produce a variety of enterocins (proteins that inhibit the growth of other bacteria) which act against pathogenic species of *Listeria*, *Clostridum*, *Staphylococcus aureus* (Wang et al., 2016) and *Escherechia coli* (Benyacoub et al., 2003). This may be partially responsible for the significantly lower load of the families Listeriaceae, Clostridiaceae and Staphylococcaceae in bats at Verulam WWTW. In mice, increased *E. faecium* load resulted in increased faecal immunoglobulin (Ig) A and increased circulating IgA and IgB levels (Benyacoub et al., 2003), thereby stimulating immunocompetence. The higher load of these bacteria in bats at the Verulam WWTW may, therefore, suggest high immunocompetence in these bats. However, not all *Enterococcus* species are advantageous. For example, *E. faecalis* have been linked with cancer promotion (Sheflin et al., 2014) as a result of chromosome instability and double-strand DNA breaks due to their extracellular superoxide production (Schwabe and Jobin, 2013). These bacteria, frequently found in bats, may also cause infectious lesions, septicaemia, meningitis (Banskar et al., 2016a), diarrhoea (Suchodolski, 2011) and increased gut permeability in mammals (Shen et al., 2014).

Likewise, the significantly greater load of the genus *Streptococcus* (Lactobacillales) in Verulam WWTW bats may be disadvantageous. Although typically commensal, some *Streptococcus* species are pathogenic (Banskar et al., 2016a) and cancer causing (Sheflin et al., 2014). For example, *S. pneumoniae* is the leading cause of pneumonia and meningitis in children (O'Brien et al., 2009).

Bats at Inkunzi had significantly lower load of the class Clostridia than those at other sites. These bacteria are the predominant producers of intestinal butyrate, a chemical that plays an important role in maintaining a healthy intestinal ecosystem by promoting colonic epithelial cell development and energy metabolism (Pryde et al., 2002). Diets rich in short-chain fatty acids have been linked with a higher load of these bacteria (Krajmalnik-Brown et al., 2012). Butyrate, the preferred energy source for colonic cells, has anti-inflammatory and anti-cancer properties (Pryde et al., 2002). Further, Clostridia species may also play a regulatory function of the immune system through the induced production of T cells (Faith et al., 2014). However, some species cause intestinal inflammation (Libby and Bearman, 2009), while the

species *Clostridium botulinum* causes the food-borne disease botulism (Melling et al., 1988). In humans, Clostridia load appears to be partially sex-related where a greater load is found in males than in females (Li et al., 2008). This may explain the low load of these bacteria in (mainly female) bats at Inkunzi.

Bats caught at WWTWs had high loads of advantageous Firmicutes. This may be due to their PUFA rich diets (Kalavathy et al., 2006) and may protect these individuals from pathogens (Benyacoub et al., 2003). The high load of these bacteria may in fact be responsible for the low concentrations of pathogenic bacteria found in this and other phyla. Differences in specific taxa can be explained by differences in the host diet (Cauchie, 2002), toxicant exposure (Choi et al., 2013), ectoparasite exposure (Olival et al., 2014) and sex (Bolnick et al., 2014). However, all these factors have significant impacts on the load of bacterial taxa, thus it is important to determine the predominant influencing factor on the overall community composition. Therefore, the entire bacterial community, as well as host factors that may influence these communities, must be considered.

4.2 Sex and site as determining factors of intestinal bacterium load

Hierarchical analysis revealed significant differences between *N. nana* sexes in terms of gut microbiota community composition at phylum level. Similarly, Phillips et al. (2012) found significantly greater gut microbial diversity in female phyllostomid bats than in males. The authors attributed these differences to reproductive females requiring more nutrients for reproduction than males and non-reproductive females. Despite differences in body condition between males and females, intestinal bacteria diversity showed no correlation with body condition in either sex, suggesting that some other sex-related difference is driving bacterial diversity. Although not in the reproductive season, differences in sex hormone concentrations may have influenced gut microbiota (Bolnick et al., 2014; Sankaran-Walters et al., 2013). Further, diet and exposure to stresses may affect bacterial diversity in sexes differently (Bolnick et al., 2014). Moreover, within the male and female clusters, individuals clustered by site. This suggests that site also mediated gut microbiota phyla in these individuals, perhaps due to locality-specific differences in the environment. The most obvious difference was that bats at WWTWs were exposed to environmental pollutants absent at reference sites. Further, the diet of bats at WWTWs may be distinctly different from those at reference sites (Naidoo

et al., 2013). Although reproductive individuals (scrotal, lactating and pregnant) have shown significantly greater microbial diversity than non-reproductive individuals (Phillips et al., 2012), these bats were all adults sampled outside the reproductive season. Further, Mehl et al. (2016) found no significant differences in energy storage between sexes or sites in these bats.

With increased taxonomic resolution, clustering by sex becomes less clear. At genus level, three major clusters comprising both sexes were observed. This may be because of the way distance was calculated between individuals. With increasing taxonomic resolution, the number of factors that contribute to the overall dissimilarity between individuals also increases. Because these factors carried equal weight, the contribution of sex and site towards the dissimilarity decreases. Further, a female bat at Verulam WWTW was distinctly different from all other bats and formed its own cluster. This individual harboured 47 bacteriual genera, including the phyla Lentisphaerae and Deinococcus-Thermus, that were not found in any other individual. Although quite rare, these phyla are known to occur in the intestinal tract of animals (Stearns et al., 2011).

Although some studies have shown differences in intestinal bacterial diversity and the load of certain taxa between sexes (Bolnick et al., 2014; Gomez et al., 2012; Sankaran-Walters et al., 2013), little is known about the interaction between environmental stresses and sex related differences on the GI microbial community. When differences between hosts and their intestinal bacterial communities were considered, bats clustered primarily by sex, whether they were associated with WWTWs or not. Further, within the sexes, bats clustered by site irrespective of body condition despite no significant differences in body condition by site.

4.3 The implications of dysbiosis of intestinal bacterial communities

In humans, the load of bacteria such as Proteobacteria, Bacteroidetes and Clostridia may be partially determined by the host's sex (Li et al., 2008). Differences between sexes in the upregulation of genes involved in immune function and inflammation of the gut mucosal immune system may cause females to be predisposed to diseases of the gut (Sankaran-Walters et al., 2013). Differences in immunity between sexes is probably facilitated by sex hormones (Sankaran-Walters et al., 2013). In early-life, when mice from either sex are inoculated with commensal bacteria, their circulating testosterone levels increase, they have an increased

resistance to diabetes and increased immunocompetence (Markle et al., 2013). Further, dysbiosis of the gut microbiome is related to anxiety, depression, autism, allergies, autoimmune diseases (Sylvia et al., 2017), and abnormalities in memory, emotions and behaviour (Dinan and Cryan, 2012). Dysbiosis due to recurrent antibiotic exposure has been linked with decreased aggression in hamsters, which did not return to normal in females after microbiome recovery (Sylvia et al., 2017). Moreover, dysbiosis of the gut microbiome can possibly alter the social structure of bats. Bats are social animals; communication and individual identification is very important (González-Quiñonez et al., 2014). In addition to the gut microbiome, the bat skin microbiome, responsible for odour production through substrate metabolism, differs significantly between individuals and sexes (González-Quiñonez et al., 2014).

4.4 Future research

Because many of the bacteria associated with the WWTW bats can cause histopathological lesions/cancers, the histopathological lesion found in the detoxification organs of *N. nana* caught at WWTWs (Naidoo et al., 2016) should be examined for a possible link between the bacteria and lesion formation. This could be done through the excision of these lesions and the subsequent sequencing of the bacteria within. Further, bacterial communities from the water and emerging insects at WWTWs should be studied to assess the precise route by which pathogens are being passed from wastewater to these bats. Moreover, bacterial communities within the ectoparasites of these bats and bat immunocompetence also warrant further investigation.

CHAPTER 5: CONCLUSION

Significant differences in the load of many intestinal bacteriual phyla were observed between bats at WWTWs and at reference sites. Despite the recent focus on the transmission of diseases from bats to humans (Halpin et al., 2007; Wang et al., 2011), few studies examine the converse situation. The high diversity and high load of certain taxa (such as Chloroflexi, Planctomycetes and Fusobacteria) in bats at WWTWs are likely due to their exposure to wastewater (Björnsson et al., 2002) and the associated difference in diet (as in the case of Chitinophagaceae). Concurrent stressors such as PUFA rich diets, diurnal bacterium patterns (Leone et al., 2015), pollutant exposure, increased lactic acid production (Choi et al., 2013) and pathogen laced prey (Broza et al., 2005) experienced by bats at WWTWs may cause dysbiosis of GI communities which may affect the host's metabolism (Leone et al., 2015), immune function (Benyacoub et al., 2003; Faith et al., 2014) and behaviour (González-Quiñonez et al., 2014). Despite the prevalence of pathogenic bacteria in wastewater, bats at WWTWs had lower pathogenic bacterial loads in several taxa, including *Chlamydia* and *Bartonella*. This may be attributable to the high exposure to naturally occurring pathogen carrying ectoparasites at unpolluted sites (Eddie et al., 1969; Lilley et al., 2015; Veikkolainen et al., 2014). These bacteria likely pass from the circulatory system, through the biliary system and into the GI tract (Sung et al., 1992). Network analyses showed that sex, rather than extrinsic factors such as wastewater pollution or intrinsic factors such as body condition, was the best predictor of intestinal microbiota composition in these animals. Differences in diet and exposure to toxicants at WWTWs may have sex-specific effects on microbiome composition (Bolnick et al., 2014) which in turn may have significant effects on the health and behaviour of the hosts. With faecal indicator bacteria present in treated water at some of South Africa's drinking water sources (Mulamattathil et al., 2014), the effects of wastewater exposure on organism health is increasingly relevant. This study provides insight into possible implications for human communities surrounding WWTWs if exposed to contaminated water sources. Exposure to these wastewater-derived pathogens is especially important to immunocompromised individuals such as those suffering from HIV/AIDS in Sub-Saharan Africa. This further emphasises the need for more stringent water treatment techniques and the implementation of settlement-free buffer zones around and downstream from these sites to minimise human exposure.

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