The effects of metal nanoparticles on the microbiome and immune responses of earthworms

Elmer Swart

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A thesis presented for the degree of Doctor of Philosophy

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Summary

Metal nanomaterials are increasingly applied as an antimicrobial agent in consumer products, coatings and pesticides. Through environmental release, non-target microbes, including symbiotic microbiota associated with animals, may be at risk due to exposure to these nanomaterials. Here, the effects of biocidal nanomaterial exposures (i.e. copper oxide and silver) on the gut microbiome of three earthworm species (i.e. *Eisenia fetida*, *Lumbricus terrestris* and *Aporrectodea calignosa*) and associated soils are studied using a metabarcoding approach. Further, the consequences of a microbiome disruption by nanomaterials on the *E. fetida* immune responses and the resilience of *E. fetida* to an infection by the bacterium *Bacillus subtilis* are investigated. This thesis provides a unique and in-depth view of the gut bacterial microbiome of three environmentally relevant earthworm species and shows how metal pollutants can affect these host-associated bacterial communities. It is shown that the resident component of the earthworm gut microbiome is largely independent from the associated soil bacterial communities. Further, through high replication (within and across concentrations), this thesis shows that the earthworm resident gut microbiome is largely resilient to exposure to antimicrobial nanomaterials. However, a key earthworm symbiont (i.e. the *Mollicutes* '*Candidatus* Lumbricincola') that likely plays a role in earthworm digestion is negatively affected by exposure to copper oxide nanomaterial. These adverse effects on this symbiont were recorded in both short-term lab exposures and long-term outdoor soil mesocosm studies. Despite the impact of copper oxide nanomaterial on the earthworm gut microbiome, no evidence for an effect of nanomaterial exposure on host immunity or host susceptibility to a bacterial infection was found. This thesis demonstrates that pollutants can adversely affect crucial earthworm microbes, highlighting the need for further testing for effects of pollutants on microbiomes. The methodological approach adopted in this thesis should guide such future studies.

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Contents

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This thesis is the result of my own independent work, except where otherwise stated, and the views expressed are my own. Other sources are acknowledged by explicit references. The thesis has not been edited by a third party beyond what is permitted by Cardiff University's Use of Third Party Editors by Research Degree Students Procedure.

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Statement on research collaborations

The work and ideas contained in this thesis are my own, except for the following:

- **Chapter 3:** Soil texture characterization and determination of the water holding capacity of Chiltern, Woburn, North Wales and Lufa 2.2 soils were performed by the Department of Soil and Environment at the Swedish University of Agricultural Sciences.
- **Chapter 5:** The pilot experiment described in **5.2.2** was conducted by Dr. Petra Procházková at the Institute of Microbiology (Prague, Czech Republic). The histological analysis described in **5.2.8** and the sampling of the earthworms at the last time point of the recovery period as described in **5.2.6** was conducted by Dr. Jiří Dvořák at the Institute of Microbiology (Prague, Czech Republic). Denny Rigby (UK Centre for Ecology and Hydrology) conducted the soil metal measurements described in **5.2.9**. Szabolcs Hernádi (Cardiff University) kindly provided the transcriptomic information used for the design of primers as described in **5.2.13.**
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1

Introduction

1.1 General introduction

The environmental risk assessment of chemicals is aimed at protecting ecosystems and is based on the toxicity of chemicals on individual environmental organisms (e.g. soil invertebrates, aquatic invertebrates, plants, fish, etc.). However, organisms do not exist in isolation but in fact form close interactions with their environment and other organisms, including microbes (e.g. bacteria, microbial fungi and protists). Many of the interactions of animals and plants with microbes can be categorized as a symbiosis, which is a "persistent and intimate interaction" between a host and a microbe (Sachs et al., 2011). Symbiotic interactions include parasitic relationships, where one of the two members benefits but the other is negatively affected by the interaction. However, in many cases symbiosis takes the form of a mutualism, where both members benefit from the interaction by, for example, the mutual exchange of essential nutrients. A third possible symbiotic interaction is commensalism, where one member may benefit but the other member is not adversely affected by the interaction. In some cases the co-dependence of the host and the symbiont is so great, that the consortium of organisms together (sometimes referred to as the 'holobiont' [Rohwer et al., 2002]) can be considered as a single unit of evolutionary selection (Rosenberg et al., 2009; Zilber-Rosenberg & Rosenberg, 2008) or co-operation (Stencel & Wloch-Salamon, 2018). Owing to the integrated role of some microbial symbionts in host health (as discussed below), there is a need to consider the impact of pollutants on the whole consortium of symbionts to assess the full impact of environmental pollution on the health of environmental organisms that need to be protected (Dietert & Silbergeld, 2015).

Earthworms belonging to the Annelid family Lumbricidae are detritivorous animals that play a crucial role in the formation and functioning of the soil by mixing of mineral and organic soils, facilitating the degradation of organic matter and by improving the aeration and drainage capacity of soils (Darwin, 1881; Edwards & Bohlen, 1996). Through their continuous ingestion and egestion of soil and plant litter and the biochemical transformations of ingested materials inside the earthworm intestinal tract, earthworms can alter the physiochemical properties of the soil and shape the bulk soil bacterial and fungal communities (Bernard et al., 2012; Heděnec et al., 2020; Medina-Sauza et al., 2019). Owing to their importance in the functioning of the soil ecosystem as well as their ease of handling and sensitivity to environmental pollution, earthworms are important sentinels in ecotoxicological research. Previous research has shown that multiple earthworm tissues (i.e. the nephridia and the intestinal tract) are colonized by a unique consortium of bacteria, many of which are vertically transferred from parent to offspring. The loss of some of these symbionts can negatively affect the health of earthworms (Lund, Holmstrup, et al., 2010; Viana et al., 2018). This means that when environmental pollutants negatively impact earthworm

symbionts, adverse effects on the host animal can be expected. Previous studies have shown that the earthworm beneficial bacterial symbiont *Verminephrobacter* is particularly sensitive to heavy metal pollution (Pass, 2015). Adverse effects of pollution on the earthworm symbionts can further be expected for chemicals that are designed to target microbes.

Over the last decade, there has been an increase in the production and usage of nanomaterials (NMs), with a major application of NMs being as an antimicrobial agent. The release of these antimicrobials into the environment may lead to adverse effects in exposed animals and their symbionts. Yet the effects of antimicrobial NMs on earthworm symbionts and resulting effects on the possible functions provided by these symbionts (such as host immune defence) remains largely unstudied (Yausheva et al., 2016). Further, due to their particulate nature, NMs have an intrinsic potential to interact with the innate immune system of organisms (Bhattacharya et al., 2017). The host immune system and the host-associated microbiota do not operate in isolation but in fact are part of a single network of interactions: the host immune system is the main regulator of the microbiota, but in turn the beneficial or commensal microbiota have a fundamental role in the development and maintenance of host immunity. Owing to this 'two way street' of interactions, a disruption of the immune system is likely to affect the host associated microbes, and vice versa. Given the likely future increase of the production and usage of antimicrobial NMs, there is a clear need for further research on the impacts of NMs on host-symbiont interactions, including how NMs may alter the interaction between host immune system and host-associated microbes.

This thesis investigates the impact of two biocidal metal NMs on the gut bacterial communities of earthworms and the resulting effects on earthworm immune functioning. The following section introduces the roles that microbiota can play in host health and how the immune system of animals are able to regulate associated microbes (**1.2**). In the sections that follow, the release of antimicrobial NMs into the soil system and the subsequent effects on soil bacterial communities (**1.3**) and the biology of the earthworm microbiome are introduced (**1.4**). Lastly, the interactions between NMs and innate immunity as well as the biology of the earthworm immune system and its interactions with NMs are discussed (**1.5**).

1.2 The role of microbial symbionts in the health and functioning of invertebrate animals

Over the last decade, the development and increased accessibility of high-throughput sequencing has greatly enhanced our understanding of the complexity and functionality of microbial communities associated with animals. This community of microbial organisms associated to animals is commonly referred to as the 'microbiome', which can defined as a catalogue microbiota and their

genes found in a specific niche (Ursell et al., 2012). Research on humans, for example, has highlighted the vast microbial diversity, both in terms of microbial richness and metabolic capacity, of the intestinal microbiome and has established the integral link between host health and microbiome (Clemente et al., 2012) Compared to humans or mammals, the microbiome of invertebrates is less well studied and is often less diverse in terms of species richness (Engel & Moran, 2013). Nonetheless, research indicates that the microbiome also plays an essential role for invertebrates in the health and functioning of hosts. In fact, there are striking similarities between organisms across the tree of life in how the microbiome can contribute to its host's phenotype (**Table 1.1**). Due to the lower microbiome complexity and in particular the ability to specifically control and manipulate microbiomes, invertebrates are an ideal model to study host-microbiome interactions and to test ecological hypotheses on host-microbe symbioses which may also apply to humans (Clark & Walker, 2018).

1.2.1 Common beneficial roles of microbes in invertebrate health

Beneficial roles of microbial symbionts in invertebrate heath can be diverse (**Figure 1.1**). In invertebrate animals, like in mammals, one of the most common roles of the microbiome is to aid in digestion and the supply of essential nutrients (Engel & Moran, 2013). A well-described example of symbiont-facilitated digestion is that of termites. Termites feed on lignocellulose from wood, and although termites do possess endogenous cellulases (Lo et al., 2011), microbial symbionts in the hindgut of these animals are crucial for the efficient digestion of lignocellulose (Brune, 2014). Degradation of wood is mediated by either protists or bacteria, which ultimately leads to the release of host accessible short-chain fatty acids (Köhler et al., 2012). Loss of symbionts reduces cellolytic potential in the termite gut (Peterson et al., 2015) and, accordingly, leads to reduced host longevity and fecundity (Rosengaus et al., 2011). Microbes can further contribute to host functioning by supplying nutrients that are absent from host food. Pea aphids (*Acyrthosphion pisum*), for example, feed on plant phloem sap that is deficient in essential amino acids. Nutritional deficiency is however compensated by endosymbiotic bacteria (*Buchnera aphidicola)* which enable the synthesis of the missing amino acids, allowing the host to live on a nutritionally limited food source (Douglas, 1998; Shigenobu & Wilson, 2011). Similar cases of nutritional symbiosis between insects and bacteria have been reported in mealybugs (Husnik et al., 2013), whiteflies (Thao & Baumann, 2004) and Carpenter ants (Stoll et al., 2010) and are particularly common among insects feeding on plant phloem (see Skidmore & Hansen, 2017). Symbioses like these have allowed animals to expand their niches (Henry et al., 2013) and thereby symbiosis is an important driver for the evolutionary diversification of insects (Joy, 2013).

Table 1.1: Similarities in the roles and characteristics of the microbiomes of humans, plants, honey bees and earthworms. Underlined text in the last column indicates a gap of knowledge.

Role or characteristic	Human gut microbiome[a]	Plant rhizosphere microbiome[‡]	The honey bee hindgut microbiome	The earthworm gut microbiome[b]
Nutrition and digestion	Breakdown of dietary products and supply of essential nutrients, in return for the supply of carbon sources	Rhizosphere microbiome assists with phosphorus and nitrogen uptake, weathering of minerals and organic matter degradation	Likely role in the breakdown of saccharides and the supply of short chain fatty acids. [1,2]	*Transient:* anaerobic fermentation of carbon sources [3], uptake of microbial lipid acids [4] and direct source for food. [5] *Resident:* earthworm gut wall associated *Mollicutes* have possible role in degradation of structural carbohydrates [3], benefits to host unknown.
Colonization resistance	Competition for nutrients and niches, production of antimicrobial compounds. Dysbiosis associated increased susceptibility to pathogens.	Competition for nutrients and niches and production of antimicrobial compounds, which contribute to resistance. Removal of microbial communities increases pathogen susceptibility.	The loss of some core symbionts compromises the honey bee ability to fight off pathogenic infections [2], mechanism not fully understood.	Unknown
Degradation of xenobiotics	Microbiota in the human gut are involved in the transformation of drugs. [6,7]	Some evidence for enhanced degradation of xenobiotics by rhizosphere microbes. [8,9]	The expression of Cytochrome P450 by the host upon exposure to pesticides in microbiota deficient honey bees is lower than that of animals with a normal gut community [10]	Intestinal environment of earthworms a site of degradation of xenobiotics [11,12], role of microbiome unknown
Modulation of host immune system	Priming of the immune system which boosts the susceptibility to disease.	Priming of the host immune system which boosts the systemic resistance of plants.	Native microbiota stimulate the expression of AMPs by honey bees. [13]	High doses of soil bacteria induce the host expression of host PRRs and AMPs [14,15], interactions between earthworm symbionts and host immunity system unknown
Microbiome density and diversity	High density (10^{11}–10^{12} cells per ml intestinal fluid) with 500-1000 bacterial species.	Higher density (10^{8}–10^{9} cells per gram) but lower diversity than bulk soil community.	10^{8}–10^{9} bacterial cells in the hindgut with five core bacterial species ubiquitous in nearly all adult workers worldwide. [16,17]	*Transient:* High density (~10^{10} cells per gram). [18] Gut passage has a negative impact on the diversity [19,20] *Resident:* Density relatively low. [21, 22] ~300 species identified. [23]

[a] Adapted from Berendsen et al. (2012) except where explicitly referenced otherwise; [b] Two microbial communities can be distinguished: the transient microbiome (the community that is associated to ingested soil and litter that passes through the gut) and the resident microbiome (the community that remains in the gut after the gut contents have been cleared). References: [1] Lee et al. (2015), [2] Raymann & Moran (2018), [3] Zeibich et al. (2019a) [4] Sampedro et al. (2006), [5] Edwards & Bohlen (1996), [6] Sousa et al. (2008),[7] Haiser & Turnbaugh (2013), [8] Anderson & Coats (2015), [9] Ye et al. (2017),[10] Wu et al. (2020), [11] Sanchez-Hernandez et al. (2009), [12] Sanchez-Hernandez et al. (2014), [13] Kwong et al. (2017), [14] Dvořák et al. (2013), [15] Dvořák et al. (2016), [16] Powell et al. (2014), [17] Kwong & Moran (2016), [18] Drake & Horn (2007), [19] Schönholzer et al. (1999), [20] Aira et al. (2015), [21] Singleton et al. (2003), [22] Jolly et al. (1993) and [23] Yausheva et al. (2016).

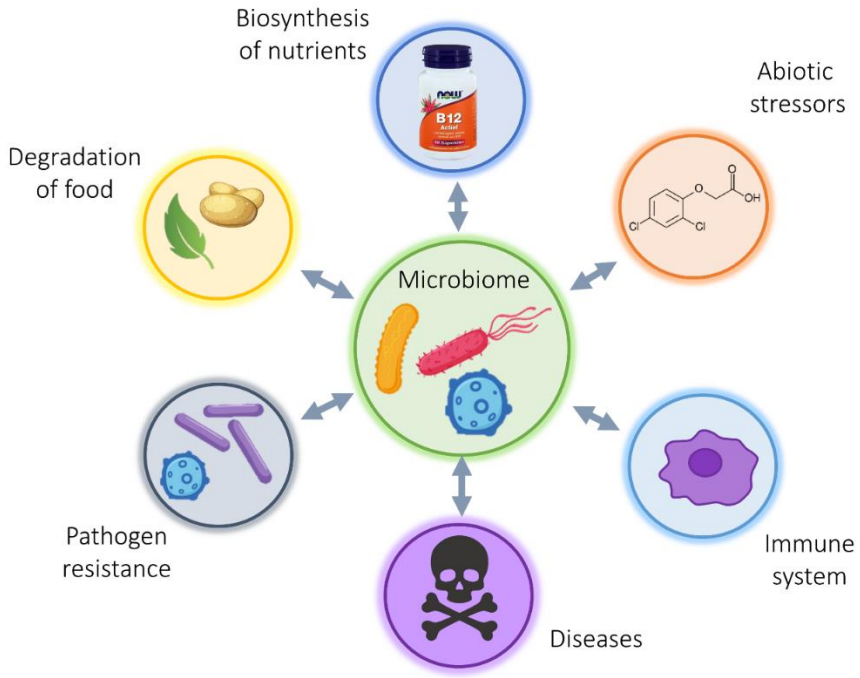


Figure 1.1: schematic overview of some of known the interactions between the microbiome and its host.

In many organisms, the intestinal tract is colonized by a dense community of microbiota, yet many of these microbiota are not involved in specialized mutualistic interactions like nutrient exchange. However, a crucial role played by the community of microbiota as a whole is in defence against invading pathogens (Brownlie & Johnson, 2009; Haine, 2008). Microbiota-mediated colonization resistance against pathogens has been described in both invertebrate as well as human models and although exact mechanisms are not fully resolved, three mechanisms have so far been recognized, as reviewed elsewhere (Buffie & Pamer, 2013; Stecher & Hardt, 2011). One of these is direct competition for nutrients and niches between resident intestinal bacteria and pathogens, which can prevent successful infection by pathogens (Sorbara & Pamer, 2019). A second, relatively well-described mechanism, is the release of anti-microbial molecules by the resident microbial community. In fungus-growing ants, for example, bacterial symbionts inhabiting the cuticle of these animals produce a peptide that inhibits the growth of a fungal pathogen (Oh et al., 2009). These antifungal peptides, however, have no activity against the fungal cultivars. The bacterial symbiont in this case protects against infection from a pathogenic fungi without harming the beneficial fungal cultivar (Scott et al., 2008). In malaria-transmitting mosquitos, an *Enterobacter* symbiont can prevent infection of the host by the malaria parasite *Plasmodium* through the release of reactive oxygen species (ROS) (Cirimotich et al., 2011). Similar examples of microbe-mediated defence against pathogenic infection through the release of antimicrobial molecules have been observed in fruit flies (Teixeira et al., 2008) and pea aphids (Scarborough et al., 2005). Lastly, intestinal

microbiota may further mediate resistance against infection through the direct stimulation of host immune responses and the subsequent release of humoral factors by the host. This process has been well described in vertebrate models (Buffie & Pamer, 2013), but similar examples have been recorded in invertebrates as well (J. K. Kim et al., 2015; Kwong et al., 2017; B.L. Weiss et al., 2012). In some cases, the diversity of the microbial community is linked to enhanced resistance against infection (Dillon et al., 2005). However, a higher diversity of the intestinal microbiome is not always associated with increased pathogen resistance. In fact, in bumblebees, a higher diversity of gut microbiota is associated with higher pathogens loads (Näpflin & Schmid-Hempel, 2018). Colonization resistance may therefore be more about the presence of a specific microbial symbiont and not so much about total microbial load or diversity.

In addition to biotic stressors, microbiota can also aid in dealing with abiotic stressors, like the exposure to chemicals. Gut microbiota have diverse metabolic capacities and are therefore able to metabolize a broad selection of chemicals, including drugs, polycyclic aromatic hydrocarbons, and metals, as reviewed elsewhere (Claus et al., 2016). The degradation and transformation of drugs in human intestinal models has been well studied (Haiser & Turnbaugh, 2013; Sousa et al., 2008). However, an increasing body of evidence suggests that, gut microbiota play also an important role for invertebrate animals in the biotransformation of chemicals (Cheng et al., 2017; De Almeida et al., 2017; Kikuchi et al., 2012). The bean bug *Riptortus pedestris*, for example, houses a mutualistic bacterial symbiont belonging to the genus *Burkholderia* (Kikuchi et al., 2005). This symbiont is taken up from the environment and is housed in specialized organs in the insect gut. The symbionts found in bean bug populations in natural environments, exhibit no resistance to the organophosphate insecticide fenitrothion (Kikuchi et al., 2012). However, in bean bug populations from agricultural regions where this pesticide is applied, *Burkholderia* symbionts are able to degrade fenitrothion in the gut and thereby provide host resistance to this pesticide. The microbiome of animals therefore not only contributes to host health by providing nutrients or resistance to pathogens, it also supports animals in dealing with abiotic stressors by extending the metabolic capacity of the host.

1.2.2 Regulation of the microbiome

The innate immune system of animals provides a first line of defence against pathogens and is capable of recognizing and eliminating pathogenic invaders. At the same time, the immune system needs to recognize and maintain the microbiota that are beneficial to host health. A crucial element of the regulation of intestinal microbiota is the recognition of microorganism associated molecular patterns (MAMPs), such as lipopolysaccharide (LPS) and peptidoglycan (PGN), by host pattern recognition receptors (PRRs). Two well described and evolutionarily conserved PRRs are Toll-like

receptors (TLRs) (Satake & Sekiguchi, 2012) and peptidoglycan recognition receptor proteins (PGRPs) (Royet et al., 2011). The control of microbiota is mediated through the recognition of MAMPs by PRRs. Upon recognition, PRRs induce cellular and humoral responses. These humoral responses include the production of antimicrobial peptides (AMPs) and other antimicrobial molecules such as ROS. The control of the microbiome by the host through these humoral factors has been described in many invertebrate species including leeches, squid and insects, as review by Nyholm & Graf (2012).

However, MAMPs are not exclusively produced by pathogens but also by beneficial and commensal symbionts. The exact mechanism of how the animal immune system prevents inflammation upon recognition of MAMPs that are derived from a non-pathogenic symbiont, is not fully understood (Chu & Mazmanian, 2013). A possible mechanism that may prevent such inflammation is through the linkage of MAMP signalling with other indicators of infections such as cellular damage or cytosolic MAMP recognition. Such coupling may prevent hyper-reactivity of the immune system and may promote the association between symbiotic partners (Chu & Mazmanian, 2013; Nyholm & Graf, 2012). Inflammation in response to beneficial or commensal microbiota may further be prevented by host-mediated degradation of MAMPs. The squid *Euprymna scolopes*, for example, forms an association with the bioluminescent bacterial symbiont *Aliivibrio fischeri*, a symbiosis which is thought to provide camouflage to the host (Visick & Ruby, 2006). Some of the squid PGRPs have amidase activity which results in the degradation of the symbiont released PGNs by the host PGRPs (Troll et al., 2010). Also in *Drosophilla melanogaster* some PGRPs have amidase activity that can degrade the PGNs released by intestinal microbiota (Paredes et al., 2011). In both squid and fruit flies, the receptor mediated degradation of inflammatory molecules prevents a further inflammation response upon interaction with commensal PGNs (Chu & Mazmanian, 2013). Lastly, inflammation may further be prevented by the physical separation of microbes and the host cells (Nyholm & Graf, 2012). In many invertebrates, symbiotic bacteria are housed in specialized organs (e.g. woodlice, bobtail squid and bean bugs) which could further contribute to the control of symbiotic bacteria and prevent an excessive host immune response.

It has become increasingly clear that a stable symbiosis is mediated by various feedback loops that link recognition by PRRs of MAMPs to specific AMP responses (Chu & Mazmanian, 2013; Thaiss et al., 2014). Binding of PGN to PGRPs in *Drosophila*, for example, induces the expression of *Caudal*, a gene that encodes for a regulator of the signalling pathway that induces the release of AMPs (Ryu et al., 2008). Suppression of *Caudal* subsequently leads to enhanced AMP release and accordingly a shift in the bacterial community composition. Symbiosis may be further supported by the lower sensitivity of beneficial and commensal bacteria to host AMPs. In humans, some commensal

bacterial are much more resistant against host secreted AMPs than pathogens (Cullen et al., 2015). In this specific case, the resistance against AMPs by commensals is caused by a modification of the commensal LPS. This modification of the LPS structure neutralizes the bacterial cell membrane and, accordingly, reduces the ability of cationic AMPs to disrupt the membrane (Cullen et al., 2015). However, symbioses are not only controlled by the host. In fact, the establishment of symbioses between animals and microbes are the result of a co-evolved network of signals and pathways (Visick & Ruby, 2006). The insect-infecting nematode *Steinernema carpocapsae* forms an association with the bacterium *Xenorhabdus nematophila*, a symbiosis that enhances host virulence and host reproductive success (Cowles & Goodrich-Blair, 2008). The host-symbiont association is partly mediated by the bacterial symbiont via two genes (e.g. *nilB* and *nilC*), which encode for membrane localized proteins that facilitate host colonization (Heungens et al., 2002). Other related *Xenorhabdus* that do not possess these genes, are not able to colonize the host. Transgenic expression of *nilB* and *nilC* in these non-symbiotic *Xenorhabdus*, however, enables these strains to also colonize the host (Heungens et al., 2002). The symbiosis between animals with microbes thus often requires specific genetic regulation from both members.

1.2.3 Consequences of the microbiome disruption (dysbiosis)

Disruption of the microbiome of animals (commonly referred to as 'dysbiosis') can be caused by external stressors such as the exposure to chemicals (Rosenfeld, 2017). Dysbiosis can lead to enhanced pathogen susceptibility through, for example, changes in microbe-microbe competition. However, enhanced host susceptibility under dysbiosis can also be driven by changes in the stimulation by the resident microbiota of the host PRRs and accordingly altered immune-reactivity of the host. In the tsetse fly, for example, treatment with antibiotics can result in the loss of symbionts making the host vulnerable to infection by bacteria that are non-pathogenic under normal conditions (B.L. Weiss et al., 2012). Here, the loss of symbionts also results in lower expression of humoral factors and reduced number of circulating haemocytes. Interestingly, in the case of symbiont loss in this fly, inoculation with extracts from the symbionts can restore host immunity, possibly due to the stimulation of the host immune system by symbiont-derived molecules (B.L. Weiss et al., 2012). Similarly, in bean bugs, removal of bacterial symbionts through antibiotic treatment, results in lower expression of AMPs, and accordingly, in enhanced susceptibility to pathogens (J. K. Kim et al., 2015). Dysbiosis in response to exposure to pesticides has been well-described in honeybees (Blot et al., 2019; Motta et al., 2018; Paris et al., 2020). Glyphosate exposure in honey bees, for example, strongly reduces the growth of the core honey bee symbiont *Snodgrassella alvi* (Blot et al., 2019). Such pesticide-induced dysbiosis can subsequently lead to enhanced susceptibility to pathogens, even at concentrations of pesticides

that are non-toxic to honey bees themselves (Motta et al., 2018; Paris et al., 2020). The environmental risk assessment of a chemical is based its direct toxicity on model organisms. However, the integral link between microbiome, host immune functioning and host health makes it important to also study the effects of chemicals on microbiomes, immunity and pathogen susceptibility. Such research is particularly relevant for chemicals that are designed to target microbes and may help to understand effects of chemicals on field populations (Motta et al., 2018).

1.3 Biocidal metal nanoparticles in the soil environment

Nanomaterials (NMs) are engineered materials with at least one dimension in the nanoscale (1-100 nm) and can be made from carbon as well as metals. Nanoparticles (NPs) can be defined as particles with all of their dimensions in the nanoscale. Over the recent decades, NMs have been increasingly produced and used in various applications including coatings, pesticides, textiles and electronics (Keller et al., 2013; Sun et al., 2016). The potential risks of nanotechnology to human health and the environment have long been established (Colvin, 2003; Handy et al., 2008; Moore, 2006). Initial concerns regarding NM safety related mainly to uncertainty as to their toxicity, exposure routes and future exposure levels, but also to the suitability of existing methods to test these aspects and whether existing risk assessments were appropriate for nanomaterials (Hristozov et al., 2012). Accordingly, much research over the last decade has focussed on filling these gaps of knowledge, which has resulted in improved toxicity testing methods (Handy et al., 2012), the availability of models to predict the release and fate of NMs in the environment (Giese et al., 2018; Keller et al., 2017; Meesters et al., 2016) and a vast amount of toxicity data that has allowed for comparative hazard and risk assessment of NMs (Bondarenko et al., 2013; Courtois, Rorat, Lemiere, Guyoneaud, et al., 2019; Lead et al., 2018; Notter et al., 2014; Rousk et al., 2012).

1.3.1 The antimicrobial activity of Ag-NPs and Cu-based-NPs

A major application of metal NMs is as an antimicrobial agent. Two of the most common biocidal NMs are Ag-NPs and Cu-based-NPs. Due to its broad antibacterial activity, silver (in both salt and nanoparticle form) is widely used in various applications including textiles and medical equipment. Ag-NPs have received much attention as an alternative to existing antibiotics and as a possible solution to the increasing prevalence of multi-drug resistance in the environment (Prabhu & Poulose, 2012; You et al., 2012). Some studies have shown that Ag-NPs can act as a synergist to conventional antibiotics, and thereby may reduce application rates of antibiotics (Fayaz et al., 2010; Panácek et al., 2016). The mechanism of toxicity of Ag-NPs towards bacteria has been relatively well studied, as reviewed elsewhere (Franci et al., 2015; Hajipour et al., 2012; Marambio-Jones & Hoek, 2010; Mijnendonckx et al., 2013). A critical mechanism for the toxicity of Ag-NPs is the

association of the particles with the bacterial surface leading to cell membrane damage and subsequent movement of Ag-NPs across the damaged membrane (Franci et al., 2015). Once inside the cell, Ag-NPs or the released Ag-ions can interact with DNA and enzymes, in particular enzymes that are rich in thiol-containing cysteine such as respiratory chain enzymes (Holt & Bard, 2005; Marambio-Jones & Hoek, 2010; Mijnendonckx et al., 2013; Park et al., 2009). Ag-NPs may also induce toxicity by extracellular ion release and subsequent uptake of Ag-ions by bacterial cells. Lastly, Ag-NPs and Ag-ions can generate ROS via redox reactions, but also through the impairment of respiratory enzymes and superoxide scavengers (Park et al., 2009). Cu-based-NPs are predominantly applied in agriculture as a fungicide to control mould and other pathogenic fungi, however, copper (either in salt or nanoparticle form) also has antibacterial activity (Ingle et al., 2014; Yoon et al., 2007). Although the exact mechanism of toxicity of Cu-based-NPs has been less well studied than that of Ag-NPs, similar mechanisms of toxicity are likely to apply (Hajipour et al., 2012; Ingle et al., 2014). The antibacterial activity of copper ions is likely related to the generation of ROS through redox reactions, but also to the binding of copper ions to sulphur groups of enzymes leading to the destabilization of essential clusters of catalytic enzymes (Dupont et al., 2011; Ladomersky & Petris, 2015; Macomber & Imlay, 2009).

Generally speaking, one of the most critical elements for the biocidal activity of NPs is the interaction of NPs with the cell surface (Nel et al., 2009). This interaction can be driven by various factors, including NP surface charge and NP coating (El Badawy et al., 2011; Handy et al., 2012; Levard et al., 2012; Silva et al., 2014). When NPs and cell surfaces have an opposite charge, or when the difference in charge is small, NPs and cells are more likely to closely associate, which can enhance NP toxicity. The outer membrane of Gram-negative bacteria is negatively charged which allows positively charged Ag-NPs to closely associate to the cell surface. Gram-positive bacteria, on the other hand, have a thick cell wall composed out of peptidoglycan, which may prevent Ag-NPs from associating with the cell surface. This difference in the interaction of Ag-NPs with the surface of Gram-negative and Gram-positive bacteria may explain the differential sensitivity to Ag-NPs of these bacterial groups (Fayaz et al., 2010; Franci et al., 2015). NP size is another major driver of NP toxicity. Generally, smaller particles are more toxic than larger particles (Lok et al., 2007; Marambio-Jones & Hoek, 2010; Silva et al., 2014). Ivask and colleagues, for example, tested the toxicity of $AgNO_3$ and a size range of Ag-NPs on six different organisms/cells, including two bacterial species (Ivask et al., 2014). The authors found that the EC_{50} (the calculated concentration at which 50% of a population is negatively affected for the measured endpoint) of Ag-NPs was positively related to NP size. However, when corrected for Ag dissolution, toxicity was equal for all tested Ag forms, except for the smallest Ag-NPs which were more toxic than all other treatments including $AgNO_3$.

The size dependent effect of Ag-NPs in this case may be linked to interactions between the NPs and the cell surface, which could enhance bioavailability or facilitate cellular uptake (Ivask et al., 2014).

1.3.2 Route of exposure and transformations of antimicrobial NPs in soils

Common applications of silver NPs as antibacterial agents are in textiles, medical equipment or coatings (Giese et al., 2018; Keller et al., 2013). Ag-NPs that are released from such products or production processes can enter wastewater and, accordingly, wastewater treatment plants (WWTP), where they tend to deposit to solids (Kaegi et al., 2011; R. Ma et al., 2014). Ag-NPs can subsequently enter the soil system through the application of sewage sludge onto agricultural soils (Sun et al., 2016). For Cu-based-NPs, the most likely route of entry into the soil system is through the direct application as a fungicide onto agricultural fields (Keller et al., 2017). Route of entry to soils is an important driver for the transformation, behaviour and accordingly the toxicity of NPs. When NPs enter the environment, they are likely to undergo transformations and may dissolve, aggregate, form homo- and/or hetero-agglomerates or react with other chemicals (e.g. sulphides, chlorides, etc.) and materials (e.g. organic matter) present in the environment. The stability and transformation of metal NPs is dependent on the NP properties, such as NP size, surface charge and coating, but also on the properties of the environment, such as pH, ionic strength and the presence of organic matter (Levard et al., 2012; Tourinho et al., 2012). Transformation can be an important factor determining the toxicity of NPs (Reinsch et al., 2012). For example, Ag-NPs going through wastewater treatment are most likely to react with available sulphide and form Ag_2S (Kaegi et al., 2011; Lombi et al., 2013; C. Meier et al., 2016). Ag_2S particles are highly stable and much less toxic to invertebrates and bacteria than Ag salts or pristine Ag-NPs, likely due to reduce release of toxic ions from Ag_2S particles (Doolette et al., 2016; Levard et al., 2012; Reinsch et al., 2012). Cu-based-NPs can exist in three different speciation forms. These include Cu(0), Cu(I) (often in the form of Cu_2O-NP) and Cu(II) (as CuO-NPs) (Keller et al., 2017). The short-term fate of Cu-based-NPs is dependent on copper form as well as environment, in particular pH, ionic strength and organic matter (Conway et al., 2015; Sekine et al., 2017). In aqueous media and under aerobic conditions Cu-based-NPs ultimately oxidize to form CuO-NP. Under anaerobic conditions Cu-based NPs ultimately transform to Cu_XS (Kent & Vikesland, 2016; Mudunkotuwa et al., 2012). Although both CuO-NPs and Cu_xS-NP are relatively stable, these NPs can still dissolve and release toxic Cu^{2+}, especially under low copper saturated conditions (Kent & Vikesland, 2016; Z. Wang et al., 2013). Sekine and colleagues showed that the long term fate of Cu-based-NPs in soils is independent from form and soil properties and that ultimately Cu-based-NPs in soils will either inorganically bind to iron oxyhydroxide or bind to natural organic matter (Sekine et al., 2017). Also for Cu-based-NPs, environmental transformation tends to reduce toxicity towards organisms (Z. Wang et al., 2013).

Until now, most ecotoxicity testing has been conducted using pristine NPs, yet with environmental transformation being a major driver for toxicity, the effects of aged NPs on organisms will have to be considered in the environmental risk assessment of NPs (Svendsen et al., 2020).

1.3.3 Effects of antimicrobial NPs on soil bacterial communities

The enhanced availability of high-throughput sequencing methods has enabled the study of patterns in soil microbial composition and structure under environmental stressors in high resolution (Rocca et al., 2019). Increasingly, these techniques have been employed to study the impact of NPs on soil microbial communities (Parada, Rubilar, Fernández-Baldo, et al., 2019; Simonin & Richaume, 2015). Studies have shown that Ag-NPs, for example, can alter soil bacterial diversity, community composition, community structure and enzymatic activity (McGee et al., 2017; 2018; Peyrot et al., 2014; Samarajeewa et al., 2017; Sillen et al., 2015; Zhai et al., 2016). Further, several studies have reported that specifically nitrifying communities can be negatively affected by Ag-NPs exposure (Doolette et al., 2016; McGee et al., 2017, 2018; J. Wang et al., 2017). However, many of these studies have tested Ag-NPs at relatively high concentrations and using relatively short exposure periods. Grun and colleagues, on the other hand, studied the effects of low concentrations of Ag-NPs and Ag salts (i.e. between 0.1-1 mg Ag/kg soil) on soil bacterial communities over a one-year period. The authors showed that the relative abundance of some bacteria taxa (e.g. *Acidobacteria*, *Bacteroidetes* and *Betaproteobacteria*) are significantly negatively affected by exposure to silver forms (Grün & Emmerling, 2018). At similar concentrations, Colman and colleagues, found that Ag-NPs not only alter the bacterial community composition, but also reduce extracellular enzymatic activity and increase N_2O flux (Colman et al., 2013). Compared to Ag-NPs, the impact of Cu-based-NPs on soil microbial communities is less well studied. However, previous studies have established that Cu-based-NPs can also affect growth of soil bacteria (Parada, Rubilar, Sousa, et al., 2019; Rousk et al., 2012) as well as soil enzymatic activity (Asadishad et al., 2018; Frenk et al., 2013; Simonin et al., 2018). Few studies, however, have assessed the impact of Cu-based-NPs on the soil microbial community composition and structure using next-generation sequencing techniques. Using metabarcoding techniques, Asadishad and colleagues studied the impact of CuO-NPs spiked biosolids on the soil bacterial community and found that *Firmicutes* specifically were negatively affected by the exposure (Asadishad et al., 2018). The relative abundance of the nitrogen fixing bacterial taxa *Bradyrrhizoabiaceae* and *Rhizomicrobium* were, however, positively affected by CuO-NPs exposure, but the impact of exposure on nitrification in soils was not further tested. Effects of biocidal NPs, or antimicrobial compounds in general, on the microbiomes of soil invertebrates are not well studied (**Table 1.2**). Zhu and colleagues, investigated the impact of a 28 day exposure of Ag-NPs on the depurated gut microbiome of *Folsomia candida*

using a metabarcoding technique (D. Zhu et al., 2018). The authors recorded significant changes in the bacterial community composition and community structure at 200 mg/kg food. In *Enchytraeus crypticus*, a 21 day exposure of CuO-NPs in soils (at 100 mg/kg) led to a change in the bacterial community composition and structure of this soil invertebrate (J. Ma, Chen, et al., 2019). Biocidal NPs that are released into the soil system thus have adverse effects on soil bacterial communities, yet few have also considered their effects on the microbiome of soil invertebrates which may provide functions to their host or ecosystems.

Table 1.2: Details and main results of experimental studies that have employed metabarcoding techniques to investigate the effects of pollutants on the microbiome of soil invertebrates. '# Conc.' and '# Reps.' indicate the number of test concentrations (excluding controls) and the number of replicates per treatment, respectively.

Pollutant	Organism	Test conc.	# Conc.	# Reps.	Duration (days)	Effect of pollutant on microbiome composition and α-diversity	Ref.
Ag-NPs	*Folsomia candida*	200 mg/kg food	1	3	28	Increase in *Proteobacteria* Decrease in *Firmicutes* and *Actinobacteria* A-diversity not affected.	(1)
CuO-NPs	*Enchytraeus crypticus*	100 mg/kg soil	1	4	21	Increase in *Bacteroidetes* and Actinobacteria Decrease in *Proteobacteria* Increase in the Shannon index	(2)
Zn-NPs	*Eisenia fetida*	1000 mg/kg soil	1	3	7	Increase in *Proteobacteria* Decrease in *Firmicutes* Decrease in the taxa richness	(3)
Cadmium	*Lumbricus terrestris*	10 and 50 mg/kg soil	2	30[a]	28	Increase in *Actinobacteria*, *Proteobacteria* and *Bacteroidetes* Decrease in *Tenericutes* Alpha-diversity not affected.	(4)
Arsenic	*Metaphire sieboldi*	70–280 mg/kg soil	3	3	28	Increase in *Bacteroidetes* Decrease in *Firmicutes* Dose dependent change in α-diversity	(5)
Tetracycline	*Enchytraeus crypticus*	10 mg/kg food	1	5	14	Increase in *Proteobacteria* and *Actinobacteria* Decrease in *Firmicutes* Decrease in the Shannon index	(6)
Oxy-tetracycline	*Enchytraeus crypticus*	10 mg/kg soil	1	6	21	Increase in *Planctomycetes* Decrease in *Proteobacteria* Increase in the Shannon index	(7)
Tetracycline	*Metaphire guillelmi*	7–55 µg/cm^2 filter paper	5	3	1 and 2	Increase in *Planctomycetes* and *Firmicutes* Decrease in *Proteobacteria* α-diversity not reported	(8)
Tetracycline	*Enchytraeus crypticus*	10 mg/kg food	1	5	14	Changes in the ration between *Firmicutes* and *Bacteroidetes*	(9)
Triclosan	*Eisenia fetida*	10–1000 mg/kg soil	4	4	7	Decrease in taxa richness	(10)

Table 1.2 continued:

Pollutant	Organism	Test conc.	# Conc.	# Reps.	Duration (days)	Effect of pollutant on microbiome composition and α-diversity	Ref.
Azoxystrobin	*Enchytraeus crypticus*	0.1–5 mg/kg soil	5	4	28	Increase in *Proteobacteria* Decrease in *Firmicutes* and *Bacteroidetes* Decrease in Shannon index at highest concentration	(11)
Polystyrene-NPs + sulfa-methoxazole	*Folsomia candida*	1% food (PS) + 116 g/kg SMZ	1	4	28	Increase in *Alphaproteobacteria* Decrease in *Bacteroidetes* Increase in α-diversity	(12)
Polystyrene-NPs	*Enchytraeus crypticus*	1000 mg/kg food	1	5	14	Changes in the ration between *Firmicutes* and *Bacteroidetes*	(9)
Microplastics	*Folsomia candida*	0.5% soil	1	4	28	Decrease in *α-Proteobacteria* Decrease in α-diversity	(13)
Microplastics	*Folsomia candida*	1000 mg/kg soil	1	3	56	Increase in *Firmicutes* Decrease in *Bacteroidetes* Increase in α-diversity	(14)

References: (1) D. Zhu et al. (2018), (2) J. Ma, Chen, et al. (2020), (3) Yausheva et al. (2016), (4) Šrut et al. (2019), (5) H. T. Wang et al. (2019), (6) J. Ma, Zhu, Chen, et al. (2019), (7) J. Ma, Zhu, Sheng, et al. (2019), (8) Chao et al. (2020), (9) (J. Ma, Sheng, et al. (2020), (10) L. Ma et al.(2017), (11) Q. Zhang et al. (2019), (12) Xiang et al. (2019), (13) Ju et al. (2019) and (14) B. K. Zhu et al. (2018). [a] Srut et al. (2019) used 10 pseudo-replicates for each of the three treatments.

1.4 The earthworm microbiome

The microbiome of earthworms has been an active field of research for more than 50 years with the earliest work having been conducted in the 1960s (see Parle, 1963). So far, two microbial communities have been well described: the nephridial and the gut communities. However, most studies have only investigated the bacterial communities, and relatively few have also assessed fungal or eukaryotic microbial communities (L. Ma et al., 2017; Procházková et al., 2018; Petra Prochazkova et al., 2019).

1.4.1 The nephridial bacterial community

The nephridia are the excretory organs of earthworms that are located laterally along the length of the animal, with two nephridia in each segment. Each nephridium has an internal opening into the coelom which connects to the exterior via a looped series of canals. The main function of the nephridia is the excretion of nitrogen. However, metal pollutants can also be removed through these organs (Courtois et al., 2019; Diez-Ortiz et al., 2015b). The nephridia house a small but unique consortium of bacteria, which has been well studied in many earthworm species (Davidson et al., 2010, 2013). Although the community composition can be diverse between different earthworm species, a bacterial symbiont that is present in nearly all Lumbricidae earthworms is *Verminephrobacter* (Davidson et al., 2013). Some of the nephridial symbionts, can be transferred from parent to offspring through an ingenious vertical transmission mechanism (Davidson & Stahl, 2008). Earthworms are hermaphrodites, but in most species require mating with conspecifics for

successful reproduction. After copulation, the clitellum forms an external mucus sheet that eventually hardens and subsequently slides off over the earthworm's head to form a cocoon. During this process, sperm, ova and nephridial symbionts are discarded into pre-cocoon. After fertilization (which occurs in the formed cocoon), the developing embryo opens up a specialized canal which connects the developing nephridia to the interior of the cocoon (Davidson & Stahl, 2008). At this point, the bacterial symbionts are able to migrate through the canal and colonize the developing nephridia (Davidson & Stahl, 2008; Dulla et al., 2012). The specialized migratory canal subsequently closes and degenerates. It is only during this point in the earthworm's development that symbionts can successfully colonize the nephridia; juveniles that have been cleared of their symbionts through an antibiotic treatment during the cocoon development stage can no longer take up nephridial symbionts (Davidson et al., 2006). The ability to clear earthworms from their nephridial symbionts through antibiotic treatment enables the study of the role of the symbionts in the earthworm health. So far, the beneficial role of *Verminephrobacter* has been demonstrated in two earthworm species (i.e. *Eisenia fetida* and *Aporrectodea tuberculata*). In both species earthworms without this symbiont (i.e. 'apo-symbiotic' earthworms) have slower developmental rates and lower reproductive output compared to symbiotic earthworms (Lund, Holmstrup, et al., 2010; Viana et al., 2018). In *A. tuberculata* the effects of the loss of the symbionts were greater in earthworms that were reared on a low nutrient diet compared to earthworms fed with a high nutritional food source, suggesting a nutritional role for the nephridial symbionts (Lund, Holmstrup, et al., 2010). However, *Verminephrobacter* symbiont does not affect the riboflavin content of earthworms, and therefore, this symbiont is likely not involved in vitamin B12 provision (Viana et al., 2018). Moreover, the loss of symbionts does not affect the total nitrogen, organic carbon or amino-acid content in cocoons (Lund, Holmstrup, et al., 2010). Thus, the mechanism by which earthworm nephridial symbionts contribute to host health is still not fully understood (Møller et al., 2015).

1.4.2 The transient and resident gut bacterial community

The gut bacterial microbiome can be further divided into two compartments: a transient community (the community associated to ingested soil and litter that passes through the intestinal tract) and a resident bacterial community (which remains in the gut after all gut contents have been cleared). So far, most research has been done on the bacterial community of the gut contents, which is largely composed of transient members (Drake & Horn, 2007; Zeibich et al., 2019b). Although food source is a major driver of the bacterial community present in the gut contents (Knapp et al., 2008; 2009), the bacterial community composition and structure of the gut contents are in most cases different to those of the bulk soil or food associated bacterial communities (Aira et al., 2015; Egert et al., 2004; Parle, 1963; Pass et al., 2015; Sampedro & Whalen, 2007). These

differences are largely driven by the selective activation and filtering of bacteria during gut passage, which causes the bacterial community to change along the length of the tract (Fischer et al., 1997; Pass, 2015; Sampedro & Whalen, 2007). Through physical grinding, ingested microbes can be lysed in the crop and gizzard (Schönholzer et al., 1999; 2002). Released proteins and nucleotides are subsequently quickly consumed by the remaining microbes (Zeibich et al., 2018). The earthworm gut is an anoxic environment (Horn et al., 2003). Further, the moisture content in the gut is higher than that of soils (Karsten & Drake, 1995). The earthworm intestinal environment is thereby an ideal habitat for the anaerobic digestion of carbon sources (Drake & Horn, 2007) which specifically activates bacteria that are able to perform such fermentation reactions (A. B. Meier et al., 2018; Wüst et al., 2011). *In vitro* supplementation of various carbon sources greatly enhances the production of fermentation products in earthworm gut contents, indicating that the earthworm gut is a powerful digester (Wüst et al., 2009; 2011; Zeibich et al., 2019a) (**Figure 1.2**). Fermentation products are taken up by the earthworm (Sampedro et al., 2006) and so the bacteria-mediated fermentation facilitates the trophic transfer of nutrients to the host. The specific environmental conditions in the earthworm gut also stimulate denitrifying bacteria which results in enhanced production of N_2O in the gut lumen compared to soils (Horn et al., 2003; Ihssen et al., 2003; Karsten & Drake, 1995; Wüst et al., 2009). The earthworm gut system thereby plays an important role in carbon and nitrogen cycling of soils.

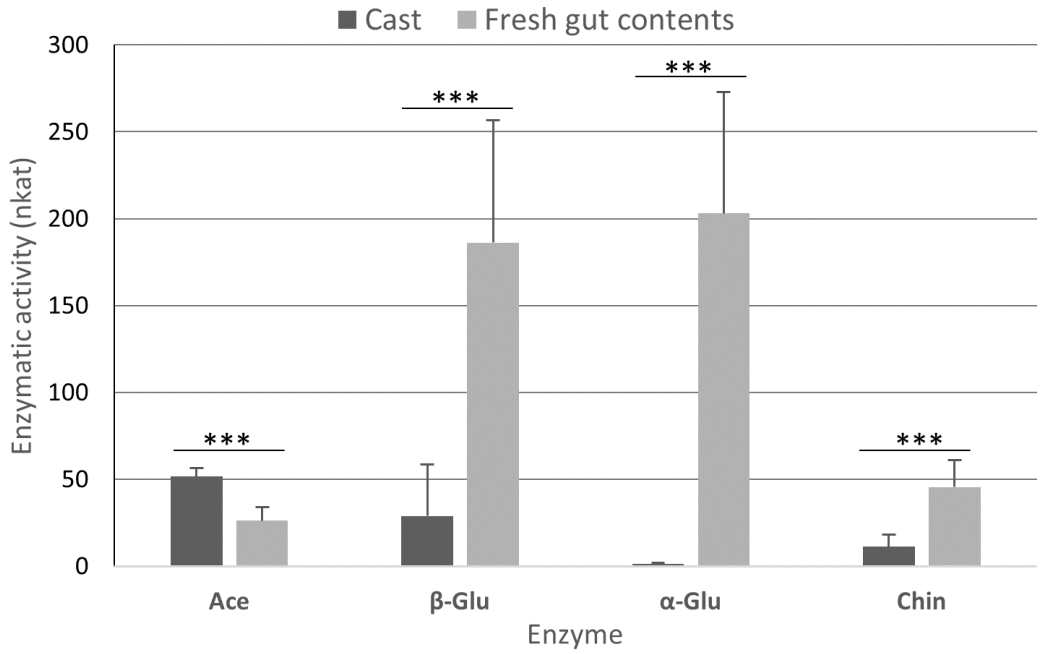


Figure 1.2: Extracellular enzymatic activity of four enzymes in cast and freshly collected gut content samples. The graphs shows that the extracellular enzymatic activity of polysaccharide degrading enzymes is up to an 2 orders of magnitude higher in the gut contents than in cast, indicating the high capacity of the gut as digester of carbon sources. (*Description continues on the next page*).

Figure 1.2: (*description continued*) Cast was collected from *Lumbricus terrestris* earthworms that were depurated for 24-hours. Fresh gut contents were collected by dissection of sedated animals. Extracellular enzymatic activities were determined by measuring the degradation of substrates conjugated with a fluorescent marker through fluorescence spectrophotometry. N=5 for all enzymes. Ace: acetylesterase, hydrolysis of ester bonds, an indicator for global enzymatic activity; β-Glu: β-1,4-glucosidase, releases glucose from cellulose; α-Glu: α-1,4-glucosidase, starch degrading enzyme; Chin: β-1,4-N-acetyl-glucosaminidase, hydrolysis of chitin derived oligomers; nkat: nanomol substrate degraded per minute, normalized per gram dry sample. Triple asterisks indicate statistically significant differences in enzymatic activity between cast and fresh gut content samples (*t*-test: $p<0.001$, for all comparisons).

The resident gut microbiome of earthworms has not been studied extensively (Jolly et al., 1993; Singleton et al., 2003; Thakuria et al., 2010; Toyota & Kimura, 2000). Through transmission electron microscopy, Jolly and colleagues investigated the microbes that were associated to the gut wall of *Lumbricus rubellus* after the gut contents had been removed through a washing procedure (Jolly et al., 1993). In particular the midgut region of these earthworms was densely colonized by a consortium of bacteria including cocci and segmented filamentous bacteria. The higher bacteria density in the midgut region coincided with a higher occurrence of intestinal folds in this region of the intestinal tract compared to the foregut and hindgut region. The presence of a community closely associated to the gut wall of earthworms has also been demonstrated by several other studies (Singleton et al., 2003; Thakuria et al., 2010). However, the community of gut wall-associated bacteria appears to be relatively small. Singleton and colleagues, for example, investigated the *L. rubellus* gut wall microbiome through culturing studies, 16S-rRNA gene clone library technique and fluorescent in situ hybridization (FISH) and found that the gut wall community of this earthworm was largely composed of a small group of mostly *Acidobacteria*, *Firmicutes* or *β-Proteobacteria* phylotypes (Singleton et al., 2003). Most of the retrieved phylotypes were either absent in cast samples or present in the cast but in much lower abundances, indicating that the community composition of the resident gut microbiome is not equivalent to that of the total gut content which is largely dominated by transients. Moreover, the resident microbes are of little importance to the denitrification process in the earthworm gut (Ihssen et al., 2003), which further supports the idea that the transient and the resident microbiome are genetically and metabolically distinct communities. Thakuria and colleagues investigated the gut wall bacterial communities of several Lumbricidae earthworm species collected from fields under arable or pasture land-use using DNA fingerprinting techniques (Thakuria et al., 2010). The authors showed that land-use was a larger driver for the community composition than species, which indicates that the resident gut microbiome of earthworms is partly driven by the environment. However, there is a component of the resident and gut content microbiome that appears to be earthworm specific (Sampedro et al., 2006). The bacterium '*Candidatus* Lumbricincola' is a *Tenericutes* that is exclusively found associated to earthworm tissues, but so far has not been detected in soils or compost (Nechitaylo

et al., 2009). Recent studies indicate that this bacterium positively responds to the supplementation of the rigid polysaccharides xylan and chitin (Zeibich et al., 2019a). There is currently no evidence for vertical transmission of this symbiont through the known vertical transmission route (Davidson et al., 2013; Lund, Davidson, et al., 2010), and thus this symbiont is either taken up from the environment (where it may be present in low abundances) or it may be transmitted to offspring via a yet unknown mechanism. The resident gut microbiome of most earthworms has, however, not been investigated with the latest next-generation sequencing techniques (Pass, 2015), and so there is still a significant gap of knowledge regarding the community composition and structure of this microbiome which may provide functions to the host.

1.4.3 The earthworm microbiome under chemical stress

Several authors have investigated the impact of pollutants on the microbiome of earthworms (**Table 1.2**). These studies have shown that both the resident gut microbiome and the gut content microbiome can be affected by exposure to pollutants including antibiotics (Chao et al., 2020; L. Ma et al., 2017) and metals (Rieder et al., 2013; Šrut et al., 2019; H. T. Wang et al., 2019; Yausheva et al., 2018). Most of these studies reported on the bacterial community composition and structure. Using flow-cytometry, Kim and colleagues investigated the cell-viability of the bacteria in gut contents of earthworms that were exposed to copper and nickel for seven days (S. W. Kim et al., 2016). Both copper (at 100 mg/kg soil) and nickel (at 200 mg/kg) negatively affected the cell viability of gut associated microbes. In a field study, Pass and colleagues investigated the impact of heavy metal pollution on the total earthworm microbiome and found that the earthworm symbiont *Verminephrobacter* was below detectable levels in polluted sites (Pass et al., 2015). Given the beneficial role of this symbiont in the health of earthworms, the loss of this symbiont may have adverse consequences for the host. However, it remains unknown whether the loss of symbionts or the alteration of the gut microbiome composition and structure as a result of pollutant exposure have any further implications for host health. Further, except for one paper that studied the impact of Zn-NPs at lethal concentrations for earthworms (i.e. 1000 mg/kg) (Yausheva et al., 2016), hardly anything is known about the impacts of biocidal NMs on the earthworm bacterial communities (**Table 1.2**)

1.5 Innate immunity in earthworms and the interaction with NPs

The innate immune system is the first line of host defence that protects organisms against infection and damage by foreign agents. In most eukaryotes, innate immunity is the only type of immunity. The exception being higher vertebrates which have also acquired adaptive immunity. Innate immunity is largely evolutionarily conserved with many animals relying on a similar set of

recognition proteins, signal transduction cascades and effector molecules for host defence (Buchmann, 2014). Previous research on the effects of NPs on human health and the environment has mostly been focussed on organismal or cytotoxicological effects. Owing to their particulate nature, NMs are likely to induce immune responses in exposed immune cells and thereby are an immuno-safety risk. Accordingly, the need for additional immuno-safety screening of nanotechnology has been recognized (Alijagic & Pinsino, 2017; Auguste, Lasa, et al., 2019; Boraschi et al., 2011, 2020; Dobrovolskaia & McNeil, 2007). Because of its basal and evolutionary conserved function in host defence, it has been suggested that the innate immune system should be the prime focus of nano-immuno-safety assessments (Boraschi et al., 2020).

1.5.1 NPs and the innate immune system

Innate immunity is provided by immune cells (e.g. macrophages) and humoral factors (e.g. opsonins and antimicrobial molecules). Crucial for immunity is the recognition by PRRs of MAMPs or damage associated molecular patterns (DAMPs), which can indicate infection or damage by a pathogen. Upon recognition, immune cells can eliminate an invader through various mechanisms including endocytosis, but also through the release of antimicrobial peptides or ROS. Innate immunity is supported by the complement system, a set of circulating small proteins that can associate with foreign bodies and subsequently trigger inflammation responses. Initial research on nano-immuno-safety has mainly focussed on two aspects: 1) the characterization of inflammation responses induced by NMs and 2) the NM uptake mechanisms by immune cells. It has now become increasingly clear that NMs can be both recognized and internalized by the innate immunity of organisms. The interactions between the innate immune system and NMs is likely dependent on the characteristics of NMs as well as the environmental medium, as reviewed elsewhere (Alsaleh & Brown, 2018; Boraschi et al., 2017; Fadeel, 2019; Pallardy et al., 2017).

A crucial factor driving the interaction between NMs and the innate immune system is the surface properties of NMs (Monopoli et al., 2012; Moyano et al., 2016). Upon entry into a biological system (e.g. bloodstream or coelomic cavity), proteins and other macromolecules can quickly adsorb to the surface of NMs which can form a so-called corona (commonly referred to as protein corona, even though the corona that is formed on the NM surface is a complex of biomolecules) (Hayashi, Miclaus, et al., 2013; Tenzer et al., 2013). The adsorption of complement proteins and other macromolecules can subsequently facilitate the recognition by PRRs and cellular uptake (F. Chen et al., 2017; Hayashi, Miclaus, et al., 2013; Tenzer et al., 2013). NP coatings such as polyethylene glycol (PEG) may reduce the adsorption potential of biomolecules and, accordingly, the reactivity of the NMs with the immune system (Liu et al., 2018; Moyano et al., 2016). The potential to keep NMs

'under the radar' of the immune system through surface modifications of NMs is particularly studied in light of the development of nano-medicines (Liu et al., 2018). Different types of NMs may also induce different immune responses. Schanen and colleagues studied *in vitro* effects of TiO_2-NP and CeO_2-NP exposure on human dendritic cells (Schanen et al., 2013). The authors found that the two tested NPs have opposing inflammatory effects, with TiO_2-NPs triggering a pro-inflammatory response while CeO_2-NPs induced an anti-inflammatory immune profile. Similar results were found in a recent screening in which human macrophages were exposed to 19 different NPs (Bhattacharya et al., 2017). Based on the cytokines and chemokines production profile in response to the NP exposures, the authors were able characterize each NP as either pro-inflammatory or anti-inflammatory. Generally speaking, cellular uptake of NMs can be mediated through either phagocytosis or pinocytosis (Dobrovolskaia & McNeil, 2007). Both endocytosis mechanisms can be further subcategorized by the receptor that facilitates the uptake. Several mechanisms of NM uptake by immune cells have so far been observed (Kuhn et al., 2014). The specific mechanism by which NMs are taken up by immune cells is likely dependent on the size of the NM as well as the immune cell type (Dobrovolskaia & McNeil, 2007; França et al., 2011; Kuhn et al., 2014). Thus, NMs interact with the innate immune system, but the responses by the immune system are dependent on both the environment as well as the NM.

In vitro testing of NM-immune system interactions can form the basis for the immuno-safety assessments of NMs (Boraschi et al., 2011). However, immuno-modulation does not necessarily indicate that the immune system is being compromised. In fact, inflammation is part of a healthy response by the immune system towards a foreign object. It is therefore crucial for the immuno-safety assessment of NMs to also study chronic effects and the effects of co-exposures with pathogens (Boraschi et al., 2011). Recent studies indicate that Au-NPs can interfere with innate immune memory towards live bacteria (Swartzwelter et al., 2020). So far, most of the NM-immune research has been based on *in vitro* models. However, *in vivo* exposure to NMs also modulates the immune system of organisms (Auguste, Balbi, et al., 2019; Barmo et al., 2013; H. Chen et al., 2017; Williams et al., 2015). NMs that are released into the environment are likely to undergo transformations, as discussed above. It remains unclear how *in vivo* effects of pristine NMs relate to the effects in more realistic exposure scenarios. In order to test whether NMs can compromise immunity, future studies should focus on *in vivo* effects of NMs in co-exposure with pathogens. With the additional need to reduce the usage of vertebrate animals in research (Scholz et al., 2013), invertebrate models provide a suitable model for future studies on NM-immune interactions under more environmentally realistic exposure scenarios. Such invertebrate based research could help to validate *in vitro* studies and may improve the current immuno-safety assessment of NMs.

1.5.2 Innate immunity in earthworms

The earthworm immune system has been relatively well described. Cellular immunity in earthworms is provided by immune cells called coelomocytes which circulate the coelomic cavity. Three different subpopulations of coelomocytes have so far been recognized: eleocytes, and granular and hyaline amoebocytes. Each of these subpopulations have their own role in cellular immunity and exhibit unique transcriptional profiles (Adamowicz, 2005; Bodó et al., 2018). The coelomic cavity of earthworms is connected to the outer environment through dorsal pores and excretory organs. Microbes are able to enter the coelomic cavity through these openings and therefore the coelomic cavity is typically aseptic. Bacterial infections in the coelom are, however, controlled by coelomocytes which can outnumber coelomic bacteria by a factor of 10 (Dales & Kalaç, 1992). A crucial element of immunity is the recognition of MAMPs by PRRs. A well described PRR in earthworms is coelomic cytolytic factor (CCF). Upon binding to specific MAMPs, this PRR can induce the prophenoloxidase pathway which ultimately leads to the production of antimicrobial factors (Beschin et al., 1998; Bilej et al., 2001; Šilerová et al., 2006). CCF is conserved within the earthworm *Lumbricidae* family and is composed of a central and a C-terminal recognition domain. The central domain can interact with both lipopolysaccharide (LPS) (Gram-negative bacteria) and β-1.3-glucan (yeast) whereas the C-terminal domain can interact with peptidoglycan (Gram-positive bacteria) (Bilej et al., 2010). CCF found in *Eisenia fetida*, however, has a broader recognition potential than CCF found other Lumbricids (Silerová et al., 2006). Recently, homologs of LPS-binding protein (LBP), bacterial permeability increasing protein (BPI) (together referred to as LPS/BPI) and TLR have been identified in *Eisenia andrei* (P. Prochazkova et al., 2020; Škanta et al., 2016). In *E. andrei*, LBP/BPI and TLR are highly expressed in the seminal vesicles. The reproductive organs of earthworms are often infected with parasites which may explain the expression profile of these PRRs. Earthworm pathogens are also controlled by the lysozyme, an evolutionarily conserved enzyme that can hydrolyse components of the cell wall of Gram-positive bacteria (Josková et al., 2009). In the earthworms *Eisenia fetida* and *E. andrei* immunity is further supported by the humoral factors called lysenin and fetidin (Cooper & Roch, 2003; Lassegues et al., 1997). The exact antimicrobial mechanism of these peptides is not fully understood (Bruhn et al., 2006; Lassegues et al., 1997). Both lysenin and fetidin can exert haemolysis through the binding to cell membrane sphingomyelin resulting in the formation of pores in the cell envelope. Bacterial membranes lack sphingomyelin, therefore the antibacterial mode of action of these peptides is likely to be different than their haemolytic activity (Bruhn et al., 2006). Recent work shows that changes in gene expression of some of these immune factors can be used as a marker of immune-modulation in earthworms (Dvořák et al., 2013, 2016a; Josková et al., 2009).

1.5.3 The interactions of NMs with the earthworm immune system

Previous *in vitro* research has shown that NMs are quickly taken up by earthworm coelomocytes, predominantly by the amoebocytes (Bigorgne et al., 2012; Hayashi et al., 2012). It has been estimated that each earthworm amoebocyte may accumulate up to 5000 NPs within the first 24 hours of exposure (Hayashi et al., 2016). Fetidin proteins, which are present in high quantities in the coelomic fluid of earthworms, have a strong affinity to associate with Ag-NPs (Hayashi et al., 2012; Hayashi, Miclaus, et al., 2013). The binding of these proteins to NPs can enhance cellular uptake by earthworm amoebocytes, suggesting that in earthworm immunity these proteins have the role of opsonins (Hayashi, Miclaus, et al., 2013). Furthermore, *in vitro* NM exposure can alter the expression of immune genes in coelomocytes (Bigorgne et al., 2012; Hayashi et al., 2016). By measuring the expression levels of mRNA, Hayashi and colleagues showed that both lysenin and CCF are downregulated in response to *in vitro* exposure to Ag-NPs (Hayashi et al., 2016). The *in vivo* effects of NMs on earthworm immunity have been less well studied, but recent work shows that the transcriptional profiles following *in vitro* exposure to Ag-NP do not always match those of *in vivo* exposures (Hayashi, Heckmann, et al., 2013). As earthworms are most likely to be exposed to environmentally transformed NMs, future studies should focus on establishing effects of NMs on host immunity in more environmentally relevant exposure scenarios.

1.6 Research question and hypotheses

The microbiome of organisms can contribute to their host phenotype through the provision of functions such as nutrient supply and colonization resistance. The ways in which microbiota can contribute to host health appears to be conserved among the tree of life, with similar roles being recognized in many different organisms including mammals, plants and invertebrates (**Table 1.1**). Earthworms provide essential ecosystem services, many of which may be driven by earthworm associated microbes. Yet, the ways in which earthworms interact with their microbiota remains largely unresolved (**Table 1.1**). However, the dependence of many animals on mutualistic interactions with microbes to provide health critical functions, such as immune defence, means that when environmental pollutants negatively affect host-associated microbes, adverse effects on the host may be expected, even in the absence of direct toxicity of the pollutant on the host. Further, due to their particulate nature, NMs have an increased potential to interact with the innate immune system of organisms. Therefore, the release of NMs into soils may further disturb soil organisms through altering of their immune status. Due to the integrate links between hosts, microbiomes and innate immunity (**Figure 1.3**), studies investigating the impact of NMs should concurrently include an analysis of the microbiome as well as the host immune status. With the

likely future increase in production and subsequent environmental release of antimicrobial NMs, there is therefore a need to investigate the effects of antimicrobial NMs on both invertebrate microbiomes and host innate immunity.

The work presented in this thesis revolves around the central research question:

> *Does exposure to metal nanomaterials have an effect on the gut microbiome and the immune functioning of earthworms?*

In order to answer this question, in the following chapters (**Figure 1.3**) the following research hypothesis are tested:

1. The earthworm gut bacterial microbiome has a resident component that is unaffected by associated soil bacterial communities (**Chapter 3**).
2. Both short-term and chronic exposure to NMs cause effects on the earthworm gut microbiome (**Chapter 4 and 6**)
3. Effects of NMs on the gut microbiome of the earthworm *Eisenia fetida* occur at similar concentrations as effects on associated soil bacterial communities (**Chapter 4**).
4. Due to the known higher biocidal toxicity of silver compared to copper oxide, silver NMs have greater effects on bacterial communities than copper oxide NMs (**Chapter 4**)
5. *E. fetida* that have their microbiome changed through an exposure to NMs are more susceptible to a bacterial infection (**Chapter 5**).
6. Exposure to NMs affects the earthworm innate immune response towards a bacterial infection (**Chapter 5**)

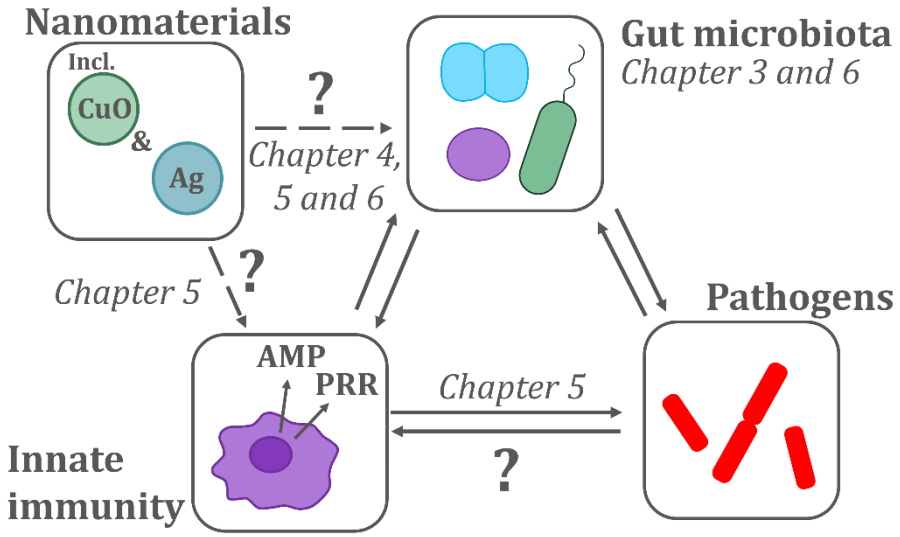


Figure 1.3: *schematic overview of the main lines of research conducted as part of this thesis. The scheme shows the interplay between nanomaterials, microbiota, the immune system and pathogens. Solid arrows indicate interactions well-described in literature. Question marks and dashed arrows indicate interactions not well studied but investigated in this thesis.*

2

Methods

2.1 Earthworm tissue sampling

Chapter 3 details a method to physically remove residual soil from the earthworm gut through repeated rinsing. Based on that method, all collected earthworm gut tissue samples in the chapters that follow were processed according to the below procedure.

2.1.1 Dissection and sampling of earthworm gut tissue

After removal from soils earthworms were depurated (i.e. starved) for two days on wetted filter paper to allow egestion of gut contents. The filter paper was replaces by a new filter paper after one day to prevent ingestion of depurated gut contents by the animals. After depuration, earthworms were euthanized in 100% ethanol and the midgut (spanning 20 segments posterior to the clitellum) was dissected using sterile equipment. Previous studies have shown that rinsing of the dissected gut of earthworms can lead to removal of soil particles and allow investigation of the resident gut wall associated bacteria (Singleton et al., 2003; Thakuria et al., 2010). Incisions along the length of the dissected midgut were made to facilitate the removal of any remaining soil particles during the following rinsing procedure. The dissected midgut was then rinsed by placing the sample in a microcentrifuge tube containing 1 ml of sterile phosphate buffered saline (PBS) and subsequently vortexed for 1 minute. The rinsed midgut was subsequently placed in a new tube containing PBS and rinsed through vortexing for an additional minute. The rinsed midgut was subsequently placed in a bead beating tube containing 900 µl RNA/DNA shield (Zymo Research, Irvine, CA, USA) and homogenised for 30 seconds at 4.5 m/s. Homogenized samples were placed at 4°C overnight and subsequently stored at -20°C until DNA extraction.

2.1.2 Sampling of soil samples for microbiome analysis

For the microbiome analysis of soils, soil samples (50 mg wet weight soil) were transferred using sterile equipment to a bead beating tube containing 500 µl of RNA/DNA shield. The sample was then bead beaten for 30 seconds at 4.5 m/s. All samples were subsequently incubated overnight at 4 °C and then stored at -20 °C until DNA extraction.

2.2 Metabarcoding

In each research chapter, the bacterial community of soil and midgut samples were measured through a metabarcoding approach. All metabarcoding in this thesis is done according the below procedure except where stated otherwise.

2.2.1 DNA extraction from earthworm tissue and soil samples

Total genomic DNA was extracted from the dissected guts and soil samples using a Soil/Fecal microbe miniprep kit (Zymo Research, Irvine, CA, USA) according to manufacturer's instructions.

DNA quantity and purity was measured by Nanodrop (ThermoFisher Scientific, Waltham, MA, USA). Visual inspection through agarose gel electrophoresis under denaturing conditions was used to verify that the total genomic DNA in the samples was not degraded.

2.2.2 Amplification and sequencing of the bacterial 16S-rRNA gene

The prokaryotic community in genomic DNA extracted from soil and gut tissue was determined by PCR amplification and sequencing following the method outlined by Kozich et al. (2013). Briefly, a ~555 bp fragment spanning the V3–V4 region of the 16S small subunit rRNA gene (herein, '16-rRNA') was amplified using the forward primer 5'-CCTACGGGAGGCAGCAG-3' and reverse primer 5'-GGACTACHVGGGTWTCTAAT-3', each modified with the addition of a sequencing primer, an indexing region, and an Illumina flow-cell adaptor such that each sample was uniquely barcoded. This specific region was chosen because the V4 region of the 16-rRNA gene is currently the most commonly used region in metabarcoding studies and for example used as standard in the Earth Microbiome Project (www.earthmicrobiome.org). PCR amplification was done using Q5® High-Fidelity DNA Polymerase and reaction buffer (New England Biolabs, Ipswich, MA, USA) using the following programme: initial denaturing at 95 °C for 2 min, followed by 30 cycles of (1) denaturing at 95 °C for 30 s, (2) annealing at 55 °C for 15 s, and (3) extension at 72 °C for 40 s, followed by a final extension step at 72 °C for 10 min. Gel electrophoresis was used to verify amplification of a single product. The PCR product was normalized using SequalPrep™ Normalization Plate Kit (ThemoFisher Scientific) and samples from each normalization plate were pooled. The pooled samples were each purified using QIAquick Gel Extraction Kit (QIAGEN, Venlo, the Netherlands). Gel extracted libraries were quantified using Qubit dsDNA HS Assay Kit (ThemoFisher Scientifc) and equimolary pooled and diluted to 7 pM. The pooled library was sequenced with 10% PhiX on a MiSeq using MiSeq Reagent Kit v3—600 cycles (Illumina, Inc., San Diego, CA, USA).

2.2.3 Bioinformatics

The Illumina demultiplexed sequences were processes using the DADA2 bioinformatics pipeline (Callahan et al., 2016) to generate an amplicon sequence variant (ASV) table. DADA2 was used as bioinformatics pipeline as this platform fully operates in R (www.R-project.org) and thereby has a wider accessibility in comparison to other platforms. Further, at the time of the start of this project, DADA2 was one of the few platforms that had a fully developed bioinformatics pipeline that used the ASV concept of taxa instead of the Observed Taxonomical Unit clustering approach, and thereby provided more precise and reproducible results. The quality of the reverse reads was too low to successfully merge with the forwards reads and therefore only the forward reads were used for the ASV table generation. DADA2 settings were maxEE(2), maxN (0), and truncQ(2). Sequences were

trimmed to 290 bases. Sequences were dereplicated and the DADA2 core sequence variant inference algorithm was applied. Chimeric sequences were removed using removeBimeraDenovo default settings. ASVs were subjected to taxonomic assignment using assignTaxonomy at default settings and using the Silva database (Callahan, 2018). ASVs assigned to mitochondria, chloroplasts, Archaea, Eukaryotes, and ASVs with unknown kingdom or phylum were removed from the dataset.

3

The midgut of the earthworm *Eisenia fetida* harbours a resident bacterial community independent from soil

3.1 Introduction

Host associated microbes (collectively known as the microbiome) of invertebrate animals are known to play an essential role in the host functioning (Chaston & Goodrich-Blair, 2010; Dillon & Dillon, 2004; Engel & Moran, 2013). In invertebrates, microbes can contribute to digestion and nutrition by enabling the uptake or provision of otherwise inaccessible or unavailable nutrients (Akman Gündüz & Douglas, 2012; Hosokawa et al., 2010; Peterson et al., 2015). Moreover, microbiota colonizing the surfaces of invertebrate animals can provide a protective function by increasing resistance to invading pathogens as well as directly interact with the host immune functioning preventing bacterial infection (Dillon et al., 2005; Eleftherianos et al., 2013; J.K. Kim et al., 2015; B. L. Weiss et al., 2012). Specialized symbiosis is often found among animals feeding on recalcitrant plant fibres. Termites, for example, rely on the degradation of lignocellulose from wood as an energy source. The degradation of lignocellulose in the intestinal tract of these detritivorous animals is largely mediated by various microbial communities inhabiting the termite hindgut. These microbial communities allow degradation of wood particles, ultimately leading to the release of host accessible fatty acids (Brune & Dietrich, 2015; Köhler et al., 2012). Disruption by antibiotics of the gut microbiome in termites, can lead to reduced host longevity and fecundity, likely due to the nutritional interaction between host and microbes being compromised (Rosengaus et al., 2011).

Earthworms are a group of mainly detritivorous invertebrates that play an essential role in soil ecosystems by fragmenting biomass, facilitating nutrient cycling and aerating the soil though bioturbation and burrow formation (Edwards & Bohlen, 1996). In earthworms two major host associated microbial communities have so far been recognized: the nephridial and gut communities. Nephridial communities are dominated by a consortium of bacteria that are vertically transmitted from parents to offspring (Davidson & Stahl, 2006, 2008; Lund, Davidson, et al., 2010; Møller et al., 2015). Loss of nephridial symbionts can lead to reduced cocoon production and juvenile development, however the exact interaction between the host organism and these symbionts is not fully understood (Lund, Holmstrup, et al., 2010; Viana et al., 2018).

The largest microbial community in earthworms can be found in the intestinal tract. By far the greatest part of this community is a transient community that is associated with the ingested food or soil. This component passes through the gut during which time changes in community composition can occur (Fischer et al., 1997; Pedersen & Hendriksen, 1993; Zeibich et al., 2019b). In addition to this transient community, there is also evidence for a resident gut microbial community (Furlong et al., 2002; Jolly et al., 1993; Toyota & Kimura, 2000). The non-transient component of the intestinal microbiome community would remain in the gut after depuration of the gut contents.

Additionally, the expected composition of this resident gut component would be different to that of the outside environment. Because the dominant resident gut taxa are also shown to be present in the soil, the presence of a stable resident community is often questioned (Egert et al., 2004; Karsten & Drake, 1995; Knapp et al., 2008; Nakamura & Sakai, 2011). More recent next-generation sequencing studies have indicated evidence for the existence of host specific bacterial gut community in *Lumbricus rubellus* (Pass et al., 2015). However, it is unknown whether the presence of a gut specific community is common among other earthworm species and to what extend this community is environment dependent.

Demonstrating the resident earthworm gut bacterial community, without contamination from transient environmental members poses a challenge. Unlike other organs containing bacteria (like the nephridia) that are relatively closed off from the outside environment, the gut of earthworms is constantly in contact with ingested food and soil particles and its associated bacteria. It is therefore difficult to distinguish truly gut associated taxa from bacteria associated with ingested food. Thorough rinsing has been shown to improve extraction methods targeting the gut specific bacterial community (Singleton et al., 2003). However the efficacy of this method has yet to be tested with the latest next-generation sequencing methods. As the sample preparation can be crucial for microbial community analyses (Ainsworth et al., 2015), this paper aims to study the effect of different sample treatments on the bacterial 16S rRNA gene sequence profile of the midgut of the earthworm *Eisenia fetida* to allow the isolation and identification of the resident gut community. Combinations of depuration to allow soil egestion and repeated rinsing of the midgut in a saline solution are used in order to identify an efficient approach for isolation of the host associated community, as distinct from that of the bulk soil. The structure of this resident gut community is then investigated in different soils by means of a transfer experiment. Through the application of these improved sampling and molecular techniques, we sought to establish the presence of a truly environmentally independent, host associated microbiome in *E. fetida* that is distinct and independent from the transient environmentally derived microbiome.

3.2 Methods

3.2.1 Culturing of Eisenia fetida

Eisenia fetida were reared in a culture medium soil consisting of sandy loam soil (Topsoil Grade A, Country Supplies, High Wycombe, UK), composted bark and garden peat substitute compost, mixed in a 1:1:1 volume ratio. Animals were kept at 20 °C and fed with horse manure from animals grazing on uncontaminated pasture and that have not been subjected to recent medication. All *E. fetida* used in this study were adults with fully developed clitella within a weight range of 300-600 mg.

3.2.2 Sampling procedure of the midgut, cast and soil

Five different sampling methods of the *E. fetida* midgut were tested, each comprised a different combination of depuration and repeated rinsing steps for the *E. fetida* midgut samples (n=8 for all sample groups). All animals were initially rinsed in purified laboratory grade water (Barnstead™ Nanopure™, ThermoFisher Scientific, Waltham, MA, USA) upon collection from the soil and subsequently either immediately dissected ('non-depurated midgut', herein) or depurated (e.g. starved to clear gut contents). All midgut samples, spanning a twenty segment region posterior to the clitellum, were dissected using single-use sterile scalpels. All *E. fetida* were euthanized in 100% molecular grade ethanol prior to dissection. Depurations of *E. fetida* to allow them to egest their gut contents was done by placing the animals in a sterile Petri dish containing wetted filter paper for 48 hours prior to dissection. Dissected midguts from depurated animals were either collected directly ('depurated midgut') or rinsed once ('gut rinsed 1x'), twice ('gut rinsed 2x') or four times ('gut rinsed 4x') in sterile phosphate buffered saline (PBS). For rinsing of the midgut, incisions along the length of the gut were made and subsequently the midgut was placed in a sterile 1.5 ml micro centrifuge tube containing PBS and vortexed for one minute. When multiple rinsing steps were performed, the midgut was removed from the tube and placed in another tube containing PBS and rinsed a further minute. After sampling and the relevant clean-up steps, each midgut sample was placed in 900 µl DNA/RNA Shield™ (Zymo Research, Irvine, CA, USA). All soil samples were sampled on the day *E. fetida* were collected. For each sample, 100 mg of soil was mixed with 900 µl of DNA/RNA Shield™. Cast samples were collected from the Petri dishes after two days of depuration. Up to 30 mg of cast was mixed with 900 µl of DNA/RNA Shield™. All gut, cast and soil samples were homogenized immediately after sampling using a FastPrep®-24 (MP Biomedicals, Irvine, CA, USA) at 4.5 m/s for 30 seconds and stored at 4 °C overnight and subsequently at -20 °C until DNA extraction.

3.2.3 Soil transfer experiment 1

In order to test the stability of the resident gut microbiome upon a transfer to a different soil, a soil transfer experiment was conducted in which *E. fetida* were transferred from the culture soil ('pre-transfer') to a soil with a different community composition (Lufa) (LUFA 2.2, LUFA-Spreyer, Germany) for 35 days. For this purpose, air dried Lufa soil was wetted to 50% of the water holding capacity (WHC). *E. fetida* were then collected from the culture soil, depurated for two days and subsequently transferred to the wetted Lufa soil. *E. fetida* midgut samples were then taken every week following the procedure described in **2.1.1**.

3.2.4 Soil transfer experiment 2

In order to test whether the bacterial midgut community is dependent on the soil type, a soil transfer experiment was conducted in which worms that were reared in culture medium were either transferred to four different natural soils or to back-transferred to a freshly prepared culture soil. Three of the four natural soils were collected directly from an identified field site under agricultural (Woburn), pasture (North Wales) and a mixed woodland land-use (Chiltern). In addition, an agricultural soil frequently used as a test soil in invertebrate toxicological studies (Lufa 2.2) supplied by LUFA Spreyer (Germany) was also used. On receipt, all soils were air dried to constant weight. Soil texture characterization and determination of WHC of Chiltern, Woburn, North Wales and Lufa 2.2 soils were performed by Department of Soil and Environment at the Swedish University of Agricultural Sciences. WHC of culture medium was determined by adding of 25 ml of ultrapure water on top of 25 g d.w. soil placed in a funnel. Funnel tube was blocked using wetted glass wool to prevent soil from sliding down. WHC was determined by measuring the volume of flow-through one hour after addition of water. Each soil was wetted to either 47.5% (Woburn), 50% (Lufa, Chiltern, N. Wales) or 52.5% (Culture) of the respective WHC. Water levels were determined by looking at the suitability of the soil structure for earthworm culturing based on expert opinion. Wetted soils were incubated for two weeks at 20 °C room before the start of the soil transfer experiment to allow activation of indigenous soil bacteria. Eight replicates were prepared for each soil type. Each replicate consisted out of a plastic tub containing a w.w. equivalent of 75 g d.w. soil. At the end of the experiment pH of all soils were measured following the description given in ISO no. 10390 (ISO, 2005). For this purpose, five grams of d.w. soil was mixed with 25 ml of 0.01M $CaCl_2$ in a 50 ml centrifuge tube and shaken for five minutes. Tubes containing the soil suspension were subsequently left to settle for two hours before the supernatant of the suspension was measured using a calibrated electrode. Loss of ignition of each soil type was determined by four hour long ignition of two grams of oven dried soil using a muffle furnace set at 500°C.

Prior to the start of the soil transfer experiment, all *E. fetida* were weighed and were depurated for two days on wetted filter paper to limit inoculation of soils with bacteria from *E. fetida* casts arising from the culture soil. Eight depurated *E. fetida* were dissected and the midgut was collected following the procedure described above using two rinsing steps to provide a baseline sample to assess the impact of habitat transfer on the resident gut microbiome. The remaining depurated *E. fetida* were subsequently transferred to the experimental replicates. Each replicate contained one animal, with four grams of autoclaved wetted horse manure added to the soil surface as a source of food. The incubation period used for the transfer experiment was two weeks. This duration was

selected based on the results from 'Soil transfer experiment 1' (**3.2.2**). At the end of the incubation period, the individual earthworms were removed from the soil, weighed and rinsed in deionised water and subsequently depurated for two days on wetted filter paper. Depurated animals were subsequently dissected according to the procedure described in **2.2.1** and stored in RNA/DNA shield until DNA extraction.

3.2.5 DNA extraction, 16S-rRNA gene amplification and sequencing

Soil and gut samples were incubated with 2 µl of proteinase K (New England Biolabs, US) for 30 minutes at room temperature, after which total genomic DNA was extracted according to the procedure described in **2.2.1**. The bacterial community was subsequently measured following **2.2.2**.

3.2.6 Bioinformatics and data analysis

Amplicon Sequence Variant (ASV) tables were generated following the procedure described in **2.2.3**. All data analysis was conducted in R (www.r-project.org). For all microbiome data analysis, sequencing data was subjected to rarefaction using the R package 'vegan' (Oksanen et al., 2017). All samples (including soil, cast and *E. fetida* midgut samples) derived from the experiment testing different sample treatments were rarefied to 15,192 reads. Rarefication curves were calculated for the 'depurated' and the '2x rinsed' samples using the 'rarefy_even_depth' function from the 'Phyloseq' package (McMurdie & Holmes, 2013). Samples from the 'soil transfer experiment 1' were rarefied to 2199 reads per sample, with removal of samples with a total read counts below the rarefaction threshold. Samples from the 'soil transfer experiment 2' were rarefied to 5,980 and 10,833 reads per sample for midgut and soil samples respectively, with removal of samples with a total read counts below the rarefaction threshold. Non-metric dimensional scaling (NMDS) (using Bray-Curtis distance matrix), Permanova and calculation of diversity indices was performed using the R package 'vegan'. Statistical difference in diversity indices were tested using two-way ANOVA (2 way-ANOVA) and Tukey's post hoc test. The core midgut taxa were determined through using the 'core' function in R package 'microbiome' (Lahti et al., 2017) and defined as any ASV present in more than 50% of the midgut samples with no minimal detection threshold set (e.g. detection = 0, prevalence = 50/100). Venn diagram was produced using the R package 'VennDiagram' (H. Chen, 2017). Correlation between abundance of 16S-RNA gene reads in soils and midgut samples was determined by calculation of Pearson's correlation coefficient. Due to low effective number of parties in *E. fetida* midgut dataset (average inverse Simpson = 4.3), effects of compositionality of microbiome sequence data is high (S. Weiss et al., 2016). Accordingly, network analysis was done using the R package 'SpiecEasi' (Kurtz et al., 2015) which takes statistical issues relating to compositionality into account (Gloor et al., 2017). 'SpiecEasi' model was run using midgut ASVs

present in at least ⅓ of all samples applying a neighbourhood joining method and using 'StaRS' procedure for model parameter selection. Nucleotide sequence data have been submitted to NCBI and are available under submission number SUB7077245 as part of BioProject number PRJNA610159.

3.3 Results

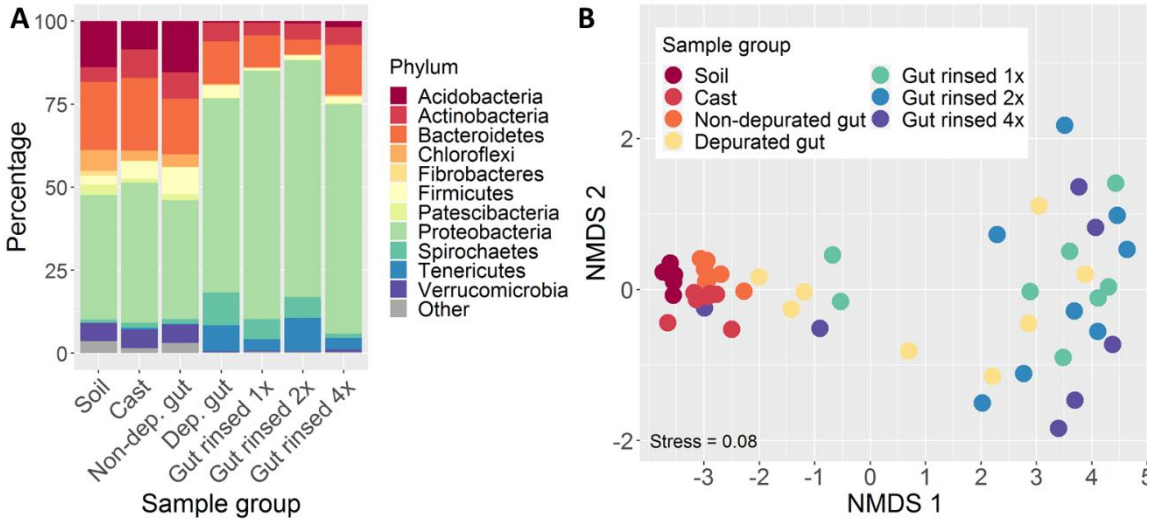


Figure 3.1: The relationship between sequence profiles of the total bacterial community for the different sample groups. (**A**) Bar chart showing the bacterial community composition at phylum level for the different sample groups (right). Bars are based on sample group averages (N = 7-8). Phyla with an abundance <1% of the total community are grouped under 'Other'. 'Non-dep. gut' and 'Dep. gut' stand for non-depurated and depurated gut, respectively. *Tenericutes* in *E. fetida* gut samples are for >90% attributed to '*Candidatus* Lumbricincola'. (**B**) NMDS plot showing ordination of samples. Different colours indicate different sample groups.

3.3.1 The effect of rinsing of the midgut on the bacterial community signal

In this study 16S rRNA gene sequencing was used to assess the bacterial community composition of the *E. fetida* midgut in addition to egested cast and soil samples. Combinations of *E. fetida* depuration (i.e. starvation to allow egestion of gut contents) and rinsing of the dissected midgut were used to optimise a method allowing efficient identification of the host associated midgut community. Sequencing generated 5,335,540 reads that could be assigned to 16,487 different bacterial amplicon sequence variants (ASVs). After rarefaction, 15,192 reads per samples remained. The community composition at phylum level in the soil, cast and non-depurated midgut samples were very similar to each other and dominated by *Acidobacteria* (between 8.6-15.4%, on average), *Actinobacteria* (4.5-8.5%), *Bacteroidetes* (16.8-21.9%), *Chloroflexi* (2.9-6.3%), *Firmicutes* (2.6-8.1%), *Proteobacteria* (35.8-42.1%) and *Verrucomicrobia* (5.5-5.6%) (**Figure 3.1A**). In depurated and rinsed midgut samples, the community composition at phylum level was dominated by *Actinobacteria* (3.7-5.6), *Bacteroidetes* (4.5-15.0%), *Firmicutes* (1.4-8.0%), *Proteobacteria* (58.4-74.8%),

Spirochaetes (1.3-9.9%) and *Tenericutes* (3.4-10.1%). The abundance of *Proteobacteria* in the depurated and rinsed *E. fetida* midguts was largely attributed to the *Verminephrobacter* earthworm symbiont which comprised on average 58% of all *Proteobacteria* in these sample groups.

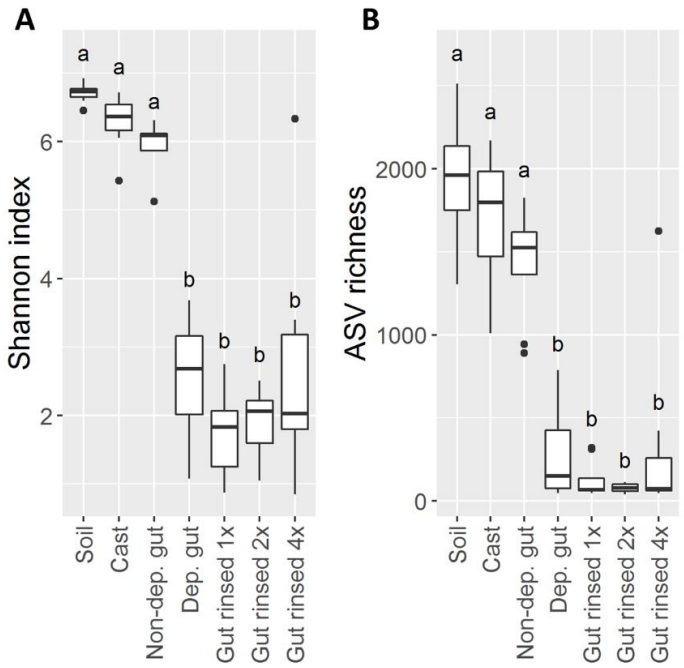


Figure 3.2. Shannon index (**A**) and ASV richness (**B**) per sample group. Letters indicate groups of statistical significance at α=0.05.

The ASV richness in the soil (mean: 1945 ±SD 370), cast (1698 ±408) and non-depurated (1439 ±341), samples was significantly higher (2 way-ANOVA: p<0.001) than the ASV richness in the depurated (275 ±269), and once (117 ±125), twice (80 ±27) and four times (351 ±582) rinsed midgut (**Figure 3.2**). The Shannon diversity index in soil (mean: 6.7±SD 0.1), cast (6.23±0.4 and non-depurated (5.9±0.4) samples was also significantly higher than the diversity in the depurated (2.5 ±0.9), and the once (1.8 ±0.6), twice (1.9 ±0.5) and four times (2.7 ±1.8) rinsed midguts (2 way-ANOVA: p<0.001) (**Figure 3.2**). NMDS and Permanova analysis indicated that different sample groups showed distinct clustering with sample group explaining 53% of the variance (Permanova: $F(6,53)=8.723$, $R^2=0.527$, $p<0.001$) (**Figure 3.1B**). Soil (dark red), cast (red) and non-depurated midgut (dark orange) samples clustered closely together, whereas the rinsed midgut samples (green, blue and purple) formed a separate and also more widely divergent cluster. Depurated midgut samples (yellow) showed an intermediate pattern, with some samples clustering closer the soil and the non-depurated midgut samples and others being closer to the rinsed midgut treatments.

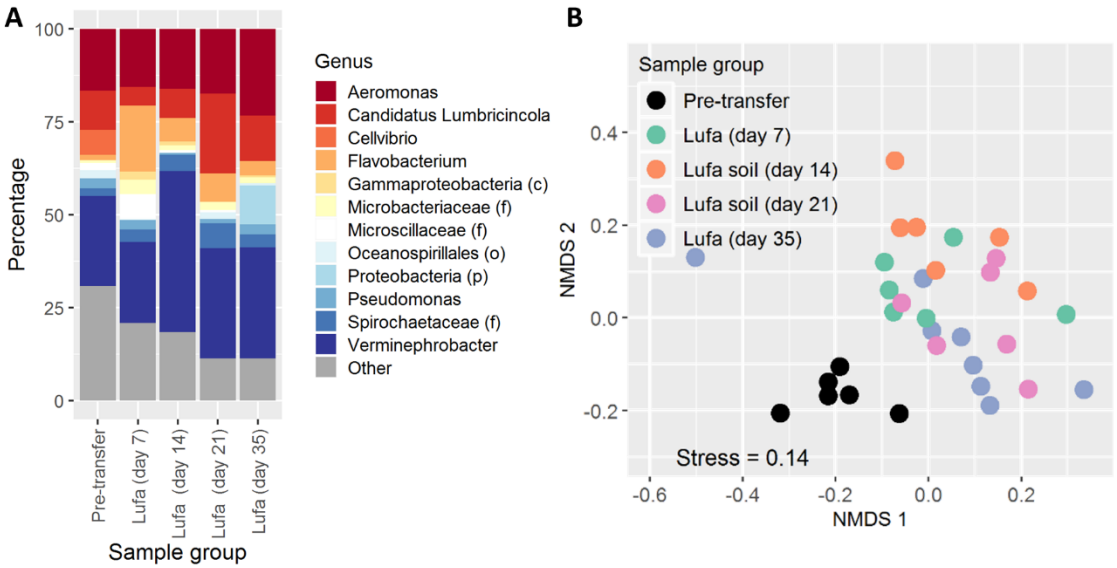


Figure 3.3: Effects of soil transfer experiment 1 on the *E. fetida* resident gut microbiome composition and structure. (**A** The average relative abundance for each genus per sample group. (**B**) NMDS plot. ASV with unknown genus are annotated by their next taxonomical level: 'f' (family), 'o' (order) and 'p' (phylum). All taxa with a relative abundance <2% are grouped under 'Other'.

3.3.2 Soil transfer experiment 1

Transfer from the pre-transfer soil to $^{5.6}Lufa_4$ soil resulted in a shift in the bacterial community structure after one week (Permanova: $F(1,10)=2.264$, $R^2=0.175$, $p<0.05$) (**Figure 3.3B**). The community structure of *E. fetida* samples after one week in $^{5.6}Lufa_4$ soil was not significantly different to the that of *E. fetida* samples during the following weeks (Permanova: $p>0.05$ for all comparisons). At all tested sampling points, the bacterial community was dominated by a similar consortium of bacteria (**Figure 3.3A**).

3.3.3 Characterization of soils and mediums used in soil transfer experiment 2

In order to determine whether the resident midgut bacterial community was independent from the bacterial community in the surrounding bulk soil, a second soil transfer experiment was conducted in which *E. fetida* were transferred from culture medium to four different natural soils or back transferred to the culture medium (**Table 3.1**). The four different natural soils ranged in pH (between 5.5 and 7.2) and organic matter content (loss of ignition between 2.7% and 19.6%). The four natural soils also had different soil texture compositions (Sand: 29.5–75.6%, Clay: 11.8–46.8% and Silt: 12.6–23.7%) (**Table 3.1**). Soil pH, soil texture and organic matter content are among the most important factors driving the bacterial community composition in natural soils (Rousk *et al.*, 2010; Griffiths *et al.*, 2011; Plassart *et al.*, 2019) and therefore these four soils were likely to have diverging bacterial community compositions making them ideal to test our hypothesis that the resident midgut bacterial community is independent from the surrounding bacterial community. In the remainder of the paper all soils and mediums used in the transfer experiment will be addressed

by their name and soil properties as ^{pH}Soil name$_{\%\ Organic\ matter}$ (i.e. $^{6.5}$Culture$_{20}$; $^{5.6}$Lufa$_4$; $^{5.6}$Woburn$_3$; $^{5.5}$Chiltern$_{19}$; $^{7.2}$N. Wales$_{18}$). Culture medium that was used for *E. fetida* rearing prior to the soil transfer experiment will be addressed as '$^{6.0}$Pre-transfer$_{33}$'.

Table 3.1: Soil properties of the tested soils used in the soil transfer experiment. pH, loss of ignition (LOI), and bacterial community diversity values are means (±SD) of measured values.

Soil/medium type	Soil texture (%)			WHC ¥ (%)	pH	LOI ǂ	Bacterial diversity	
	Sand	Clay	Silt				Shannon diversity	Species richness
$^{6.0}$Pre-transfer$_{33}$ ‡	na	na	na	77.3	6.0 (±0.02)	33.2 (±2.0)	5.6 (±0.2) ^{ab}	929 (±208) ^{a}
$^{6.5}$Culture$_{20}$ ‡	na	na	na	73.0	6.5 (±0.07)	19.6 (±0.4)	5.5 (±0.1) ^{a}	790 (±116) ^{ab}
$^{5.6}$Lufa$_4$	67.3	17.5	15.2	41.8	5.6 (± 0.04)	4.2 (±0.2)	5.8 (±0.2) ^{b}	914 (±153) ^{a}
$^{5.6}$Woburn$_3$	75.6	11.8	12.6	32.0	5.6 (± 0.04)	2.7 (±0.1)	5.7 (±0.1) ^{ab}	1035 (±97) ^{a}
$^{7.2}$N. Wales$_{18}$	57.7	29.7	12.6	77.6	5.5 (± 0.06)	18.5 (±0.5)	4.8 (±0.2) ^{ab}	601 (±65) ^{a}
$^{5.5}$Chiltern$_{19}$	29.5	46.8	23.7	96.0	7.2 (± 0.04)	17.8 (±0.1)	5.6 (±0.1) ^{c}	860 (±116) ^{b}

$^{a\ b\ c}$ Letters indicate statistical significance groups at $\alpha = 0.05$; ‡ Soil texture values for culture mediums were not determined as mediums were composed out of mostly compost and so soil texture values do not apply; ¥ WHC, water holding capacity; ǂ LOI, loss of ignition.

Sequencing of soil and *E. fetida* midgut samples derived from the soil transfer experiment, generated 3,703,220 reads that could be assigned to 23,604 different bacterial amplicon sequence variants (ASVs). The test soils had different bacterial phyla compositions (**Figure 3.4**). $^{6.5}$Culture$_{20}$ medium, for example, was characterized by a high relative abundance of *Patescibacteria* (10.9% vs. 0.0-0.3% in other soils) and the absence of *Acidobacteria* (0.0% vs. 2.0-4.7%). The $^{7.2}$N. Wales$_{18}$ was most distinct in terms of relative abundance of bacterial phyla. The bacterial community in $^{7.2}$N. Wales$_{18}$ was characterized by a relative high abundance of *Firmicutes* (56.6% vs. 23.0-33.6%) and the absence of *Verrucomicrobia* and *Gemmatimodades* (1.0-3.6% in other soils). *Actinobacteria* were relatively scarce in $^{7.2}$N. Wales$_{18}$ (14%) and $^{5.6}$Woburn$_3$ (19%) compared to the other soils (25.4-37.4%). Both $^{5.6}$Lufa$_4$ and $^{7.2}$N. Wales$_{18}$ had fewer *Bacteroidetes* (1.1% and 1.4%, respectively) than other soils (4.1-8.3%). Mean bacterial ASV richness was the lowest in $^{7.2}$N. Wales$_{18}$ (601) and was significantly lower than all other soils (between 860 and 1035) except for the $^{6.5}$Culture$_{20}$ medium (790) (2 way-ANOVA: $p<0.05$) (**Table 3.1**). Mean Shannon diversity in the soils was between 5.5 and 5.8 except for $^{7.2}$N. Wales$_{18}$ which at 4.8 was also significantly different from the other soils (2 way-ANOVA: $p<0.05$). (**Table 3.1**). NMDS at ASV level showed that soil samples clustered by soil type (**Figure 3.5A**). Permanova indicated that soil type significantly affected soil bacterial community composition ($F(5,35)=30.1$, $p<0.0001$). Soil type explained 82% of the model variance. Both soil pH and LOI significantly affected community composition with these two factors explaining 24% and 17% of the total variance, respectively (Permanova: $F(1,40)=11.1$, $p<0.0001$ for pH; $F(1,40)=15.2$,

p<0.0001 for LOI). Out of the 4660 ASVs that had a relative abundance >0.1% of the total community in any of the soil samples, only 17 ASVs were detected in all soil types (**Figure S3.1**).

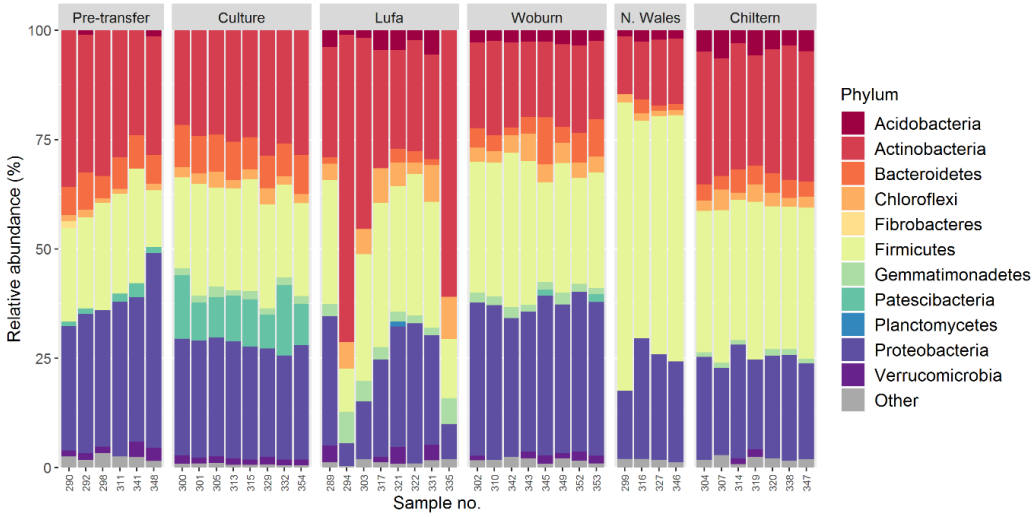


Figure 3.4: Relative abundance of soil bacterial phyla per sample and soil type. All phyla with a relative abundance <1% are grouped in 'Other'.

3.3.4 The impact of soil transfer on the resident E. fetida midgut bacterial community

In all test soils, the resident midgut bacterial community was dominated by *Acidobacteria* (2.9-14.2%), *Bacteroidetes* (1.3-11.1%) and *Proteobacteria* (46.6-93.3%) (**Figure 3.5C**). *Spirochaetes* were absent in $^{5.6}$Woburn$_{3}$, but abundant in $^{5.5}$Chiltern$_{19}$ (31.7%). *E. fetida* midgut *Tenericutes* were only present in $^{5.5}$Chiltern$_{19}$ and *E. fetida* from $^{6.0}$Pre-transfer$_{33}$. The relative abundance of *Firmicutes* was low in in $^{7.2}$N. Wales$_{18}$ (0.3%) in comparison to *E. fetida* reared in all other soils (1.2-6.1%). No significant differences in species ASV richness was found with the mean ASV richness per soil type ranging between 43−84 (2 way-ANOVA: $F(5,33)=0.47$, $p>0.05$) (**Table 3.2**). Shannon diversity of the midgut microbiome was between 1.4 and 2.3 in all soils, except $^{7.2}$N. Wales$_{18}$ in which it was lower (0.9) and significantly different to $^{6.5}$Culture$_{20}$ (2 way-ANOVA: $F(5,33)=2.52$, $p<0.05$; Tukey's test: p<0.05) (**Table 3.2**). NMDS indicated high sample and soil type variability in the bacterial community composition (**Figure 3.5B**). Permanova indicated a significant impact of soil type on composition ($F(4,34)=2.0$, $p<0.05$) which explained 19% of the variance. Soil pH significantly explained 11.7% of the model variance (Permanova: $F(1,38)=4.9$, $p<0.001$). LOI had no significant effect on the midgut community composition (Permanova: $F(1,38)=4.9$, $R^2 = 0.02$ $p=0.453$). In total thirteen ASVs were present in at least 50% of all samples across all soil types ('core midgut ASVs' hereafter) (**Table 3.3**). Among these thirteen ASVs, the abundance of *Pseudomonas* (ASV 7) and *Aeromonas* (ASV 11) in *E. fetida* midguts were significantly correlated ($p<.0.05$) to the abundance of these specific taxa in soils (ASV 7: $r=0.51$; ASV 11: 0.36). The earthworm symbiont *'Candidatus* Lumbricincola' (*Tenericutes*) was only found in three samples at a relative abundance of >1% of the total community. Network

analysis on taxa present in more than ⅓ of all midgut samples, suggested four separate ASV clusters (network stability = 0.046) (**Figure 3.5D**). The three largest clusters were each composed out of four to six ASVs. The largest of these networks was composed of the *Verminephrobacter* symbiont (ASV 1), three *Aeromonas* ASVs (ASV 11, 13 and 48), *Microbacteriaceae* (ASV 9) and *Microscillaceae* (ASV 17). A further cluster was composed of *Bradyrhizobium* (ASV 64), *Deftia* (ASV 438), *Pseudomonas* (ASV 350) and an *Enterobacteriaceae* (ASV 400). The third largest cluster was composed out of *Cutibacterium* (ASV 224), *Aeromonas rivuli* (ASV 4) and two other *Aeromonas* (ASV 2 and 10).

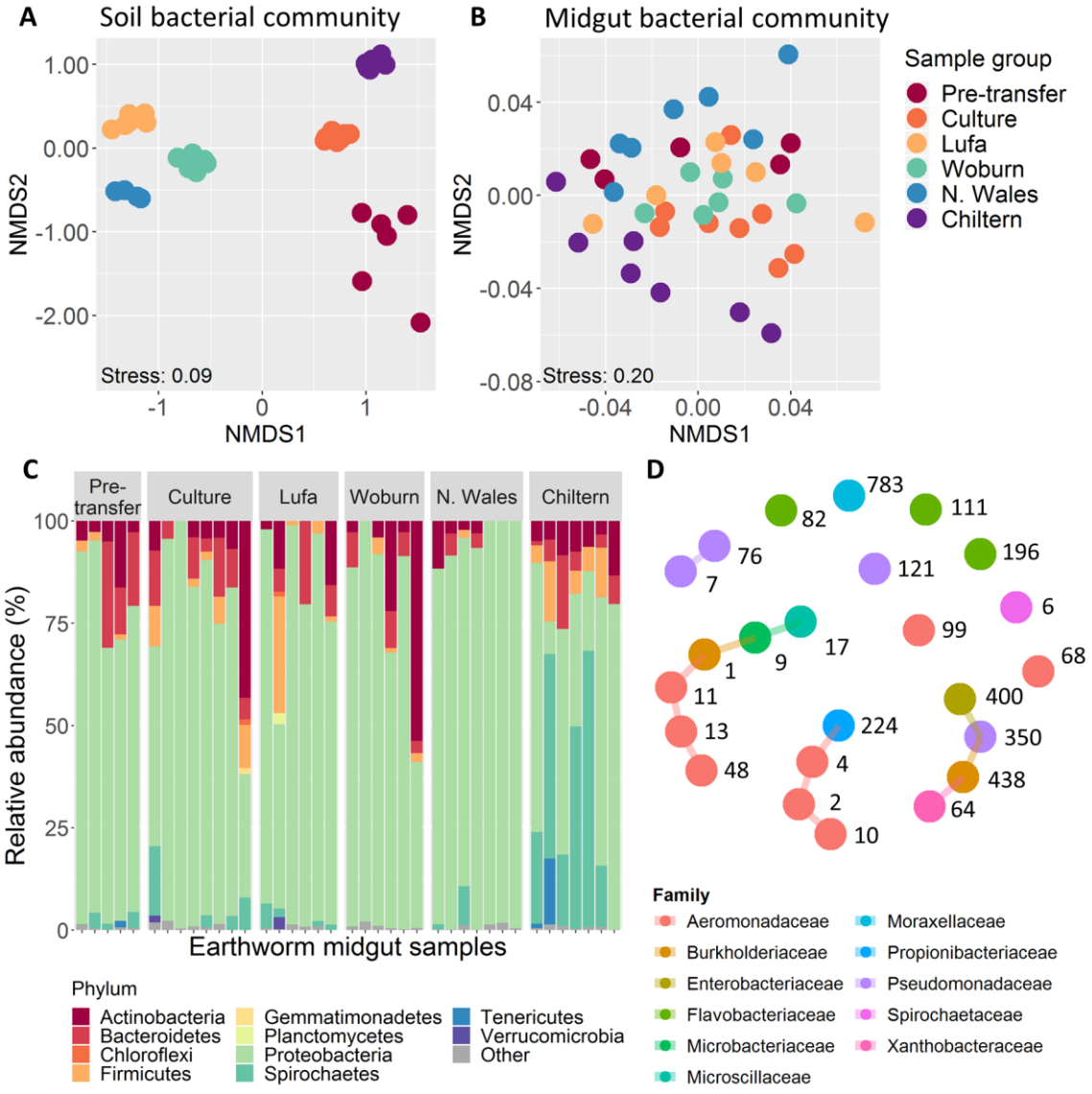


Figure 3.5: (A-B) NMDS plots showing ordination of soil samples and *E. fetida* midgut samples. Different colours indicate different soil types. **(C)** Relative abundance of *E. fetida* midgut bacterial phyla per sample and soil type. All phyla with a relative abundance <1% are grouped in 'Other'. *Tenericutes* is fully attributed to a single ASV ('*Candidatus* Lumbricincola'). **(D)** Outcome of network analysis using 'SpiecEasi' model applying a neighbourhood selection method and using 'StaRS' as parameter selection procedure. Network analysis was done using *E. fetida* midgut ASVs present in at least ⅓ of all midgut samples. Each node indicates a unique ASV. Numbers indicate ASV identifier number. Colours indicate taxonomical assignment at family level. Connecting lines indicate significant connectivity between respective ASVs.

Table 3.2: Mean ASV richness (±SD) and Shannon index (±SD) in *E. fetida* midguts per soil/medium.

Soil/medium	Bacterial richness	Shannon index
$^{6.0}$Pre-transfer$_{33}$ ‡	56±28	1.45±0.4 ^{ab}
$^{6.5}$Culture$_{20}$ ‡	82±55	2.27±0.8 ^{a}
$^{5.6}$Lufa$_{4}$	84±111	1.96±1.3 ^{ab}
$^{5.6}$Woburn$_{3}$	63±27	1.72±0.6 ^{ab}
$^{7.2}$N. Wales$_{18}$	43±37	0.95±0.6 ^{ab}
$^{5.5}$Chiltern$_{19}$	73±60	1.62±0.6 ^{b}

$^{a\,b\,c}$Letters indicate statistical significance groups at $\alpha = 0.05$

Table 3.3: Mean (±SD) relative abundance (%) of the core midgut ASVs in the resident gut microbiome of *E. fetida* reared in five different soils. Limit of detection in relative abundance is 0.016%.

ASV id	Phylum	Genus/species	Mean (±SD) relative abundance (%)					
			$^{6.0}$Pre-transfer$_{33}$	$^{6.5}$Culture $_{20}$	$^{5.6}$Lufa $_{4}$	$^{5.6}$Woburn $_{3}$	$^{5.5}$Chiltern $_{19}$	$^{7.2}$N. Wales $_{18}$
1	*Proteobacteria*	*Verminephrobacter*	37.5 ±33.2	21.0 ±25.4	32.2 ±37.1	17.2 ±33.9	40.4 ±30.1	47.9 ±40.3
2	*Proteobacteria*	*Aeromonas*	28.6 ±31.0	15.8 ±24.7	20.4 ±20.2	30.6 ±23.2	0.3 ±0.4	32.4 ±33.5
4	*Proteobacteria*	*Aeromonas rivuli*	7.6 ±7.8	2.8 ±4.7	8.1 ±8.6	4.1 ±3.9	<0.1 ±0.1	4.1 ±5.3
6	*Spirochaetes*		1.9 ±1.7	4.1 ±5.9	1.5 ±2.2	n.d.	31.7 ±24.2	1.6 ±3.4
7	*Proteobacteria*	*Pseudomonas*	<0.1 ±0.0	11.1 ±10.7	3.3 ±4.6	7.9 ±15.8	0.2 ±0.3	<0.1 ±0.0
9 ǂ	*Actinobacteria*		4.8 ±5.9	5.8 ±12.3	2.8 ±5.9	13.4 ±20.3	4.4 ±4.9	2.8 ±4.0
11	*Proteobacteria*	*Aeromonas*	1.2 ±1.4	5.4 ±4.8	2.9 ±3.0	4.6 ±5.2	0.8 ±0.8	1.9 ±4.7
13	*Proteobacteria*	*Aeromonas*	0.8 ±01.0	4.9 ±4.4	2.6 ±2.8	3.0 ±3.7	0.6 ±1.3	1.7 ±4.6
17 ‖	*Bacteroidetes*		4.4 ±6.7	1.7 ±3.3	1.0 ±2.5	3.2 ±3.5	3.7 ±6.0	1.3 ±2.2
48	*Proteobacteria*	*Aeromonas*	0.3 ±0.4	1.7 ±1.6	0.7 ±0.7	1.0 ±1.3	0.2 ±0.4	0.4 ±1.1
64	*Proteobacteria*	*Bradyrhizobium*	<0.1 ±0.0	0.3 ±0.2	0.2 ±0.2	0.7 ±1.0	0.8 ±1.1	<0.1 ±0.0
224	*Actinobacteria*	*Cutibacterium*	<0.1 ±0.0	0.7 ±1.2	0.1 ±0.2	<0.1 ±0.1	0.4 ±0.6	<0.1 ±0.0
438	*Proteobacteria*	*Delftia*	0.1 ±0.1	0.2 ±0.2	0.1 ±0.1	0.1 ±0.3	0.2 ±0.3	<0.1 ±0.0

n.d. indicates 'not detected'; ǂ NCBI blastn showed that the 16S rRNA gene sequence of *Microbacteriaceae* (ASV 9) aligned with 100% identity to a Agromyces-like symbionts which has the proposed name '*Candidatus* Lumbricidophila' (NCBI accession KX078350.1; Lund et al., 2018); ‖ NCBI blastn showed 16S rRNA gene sequence of *Microscillaceae* (ASV 17) aligned with 99.66% identity to '*Candidatus* Nephrothrix' (NCBI accession KP420702.1; Møller et al., 2015).

The relative abundance of the core midgut ASVs were between one and three orders of magnitude lower in soils than in midguts, with exception of *Bradyrhizobium* (ASV 64) (**Table S3.1**). The relative abundance of *Bradyrhizobium* (ASV 64) in midguts was comparable to soils except for $^{5.6}$Woburn$_{3}$ where this bacterium was below detectable levels in soil but on composed on average 0.67% of the total community in midguts (**Table 3.3 and Table S3.1**). NMDS and Permanova on midgut and soil

samples together, indicated separation of samples by soil type (Permanova: $F(5,85)=4.61$, $p<0.001$) and sample type (Permanova: $F(5,85)=20.8$, $p<0.001$) (**Figure 3.6A**), with these factors explaining 21% and 19% of the model variance, respectively. The 'core midgut ASVs' were subsequently removed from the dataset in order to test whether these remaining non-core ASVs were a reflection of the outer environment. After removal of the thirteen core midgut ASVs, samples clustered more by soil type (Permanova: $F(5,66)=7.08$, $p<0.001$) then by sample type (Permanova: $F(1,70)=7.43$, $p<0.001$) (**Figure 3.6B**), with these two factors explaining 35% and 10% of the model variance, respectively.

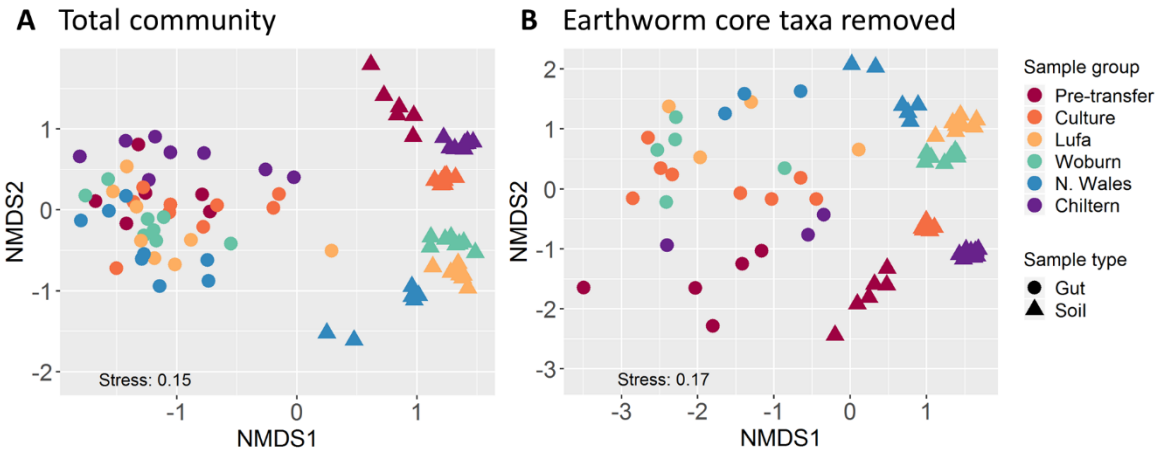


Figure 3.6: Plots of NMDS analysis of **(A)** total bacterial community and **(B)** bacterial community after removal of core midgut taxa. Different colours indicate treatment, different shapes indicate sample type.

3.4 Discussion

Studying host specific bacterial communities poses a challenge for biota that are surrounded or interact with complex media (Ainsworth et al., 2015; Kooperman et al., 2007). The earthworm gut is continuously in contact with ingested soil making it difficult to separate the bacterial community that is strongly associated with the earthworm tissues from the transient bacteria that pass though associated with the ingested gut content. Given the critical role that earthworms play in the provision of soil ecosystem services, knowledge of their true host associated microbiome is needed to understand the contributions that symbiotic relationships make to these functions. Identification of host specific microbes can be achieved by looking at which microbe is present in the host but not in the environment. This approach, however, could lead to exclusion of a true symbiont when hosts are highly interactive with their environment and symbionts frequently detached into the environment. This study aimed to physically remove the ingested soil from the midguts of *E. fetida* through repeated rinsing with a saline solution. The data presented here suggest, in line with previous research (Singleton et al., 2003; Thakuria et al., 2010), that rinsing of the *E. fetida* midgut

leads to the removal of remaining soil particles (**Figure 3.1**) allowing for clear identification of the resident *E. fetida* gut bacterial community.

Removal of the gut content from the gut tissue sample is an obvious way by which to separate the true gut associated from transient microbial communities. However, it is important to consider that rinsing of the gut may not lead to complete removal of all residual soil particles. Therefore, the comparison of bacterial communities of hosts from different habitats, can help to further elucidate members of the truly host specific bacterial community (Shade & Handelsman, 2012). With this aim, *E. fetida* midgut bacterial communities were further investigated in a soil transfer experiment using five different test soils/mediums with highly divergent bacterial community compositions (**Figure 3.4 and 3.5A**), to identify which gut bacterial taxa are present independent from the soil community.

Through this approach we identified, thirteen bacterial ASVs that were consistently present associated to *E. fetida* midguts irrespective of the larger soil community (**Table 3.3**). These core midgut ASVs comprised on average 83% (±14%) of the total midgut community. In contrast to other soil dwelling organisms (Johnke et al., 2020), the core midgut microbiome of *E. fetida* is thus largely soil independent. Analysis of the total community following removal of the core set of midgut bacteria taxa showed a closer relationship between soil and remaining midgut community (e.g. soil type explained 35% of variance after removal of core bacteria, in contrast to 21% when all taxa were included) (**Figure 3.6**), indicating that these remaining taxa bacteria are not consistently associated with *E. fetida* midgut, but are instead predominantly environmentally derived. The total bacterial community closely associated with the *E. fetida* gut, thus, likely contains both transient and resident bacteria even after repeated rinsing.

Thakuria and colleagues found that the bacterial communities associated with rinsed guts of the earthworms *Aporrectodea longa* and *Aporrectodea caliginosa* were largely driven by soil and to a lesser extent by species (Thakuria et al., 2010). This contracts with the data presented here, which indicates that although soil is a driver of the *E. fetida* midgut bacterial community, most of this host associated microbiome comprises a small consortium of bacteria that is independent from soil. *E. fetida* is, however, an epigeic earthworm that feeds on decomposed organic matter or manure. In contrast, *A. longa* and *A. caliginosa* are endogeics that feed on top soil (Eisenhauer et al., 2008). In the same study, Thakuria and colleagues also showed that ecological group is a major driver of the bacterial community composition of the earthworm gut (Thakuria et al., 2010). Differences between different earthworm species in the role of habitat on the resident gut microbiome may therefore relate to ecological feeding group.

Notably, four of the soil independent ASVs (e.g. *Verminephrobacter* – ASV 1; *Spirochaetaceae* – ASV 6; *Microscillaceae* – ASV 17 and *Microbacteriaceae* – ASV 9), are known nephridial symbionts widespread within the earthworm clade (Davidson et al., 2013; Lund, Davidson, et al., 2010). Although mostly described as a nephridial symbiont, the presence of the *Verminephrobacter* in the gut of developing earthworms has been confirmed through FISH staining (Davidson & Stahl, 2008). Recent next-generation sequencing based research has also indicated the presence of *Verminephrobacter*, as well as *Spirochaetaceae* and *Microbacteriaceae* in the intestinal tract of adult animals (Pass et al., 2015; Procházková et al., 2018; Tang et al., 2019). These findings together with the results presented here, point to the presence of these symbionts associated the intestinal tract in the adult earthworm. The coelomic cavity of earthworms is typically aseptic. The approach applied in this study, does not allow differentiation between bacteria that are present inside the gut and bacteria associated to outside of the intestinal tract. Confirmation of the presence of these symbionts inside the gut of adult animals using fluorescent labelling is therefore needed to fully confirm the presence of these bacteria inside the intestinal tract. The potential presence of these established nephridia associated taxa in the gut microbiome sample, suggests that there is a degree of cross association of the intestinal bacterial and nephridial bacterial communities, possibly indicating translocation between the two tissues. Several possible routes of migration of these nephridial bacteria into the gut are possible. Migration to the gut could occur during development of the juvenile in the cocoon through consumption of the egg albumin substance, which is known to that contain a consortium of bacteria (Davidson & Stahl, 2006). In this study, some of the nephridial associated bacteria were sporadically recorded in the soil. It is, therefore, also possible that bacteria excreted into the soil by nephridia, are able to colonize the intestinal tract of *E. fetida*. Given the specialized recruitment into the nephridia during development (Davidson & Stahl, 2008), it can be expected that the primary interaction between host and symbionts takes place in the nephridia. Presence of the same taxa in the midgut may be circumstantial and presence does not necessarily mean an interactive host association.

The second most abundant core taxonomical group, comprised five ASVs belonging to the *Aeromonas* genus. *Aeromonas* is a widespread group of Gram-negative bacteria well known as an opportunistic pathogen in humans and fish (Janda & Abbott, 2010; Reith et al., 2008; Teunis & Figueras, 2016). However, *Aeromonas* is also found in various invertebrate animals in non-pathogenic interactions (Harris, 1993). *Aeromonas* (or *Aeromonadaceae*) has also been frequently observed in the gut and the cast of various earthworm species (Byzov et al., 2009; Furlong et al., 2002; Rieder et al., 2013; Tang et al., 2019; Toyota & Kimura, 2000; Yausheva et al., 2016; Zeibich et al., 2019a). In this study, most of the core midgut *Aeromonas* ASVs were also found in low

abundances in soil. Given the presence of these taxa in soils and the correlation between soil abundance and midgut abundance of one of the *Aeromonas* ASVs, it seems likely that *Aeromonas* is taken up from its environment and not vertically transmitted like other earthworm symbionts such as *Verminephrobacter*. The nature of interaction between *Aeromonas* and earthworm host is unknown. However, it has recently been shown that in earthworm cast containing bio-reactors supplementation of various polysaccharides strongly stimulates fermentation and increases the abundance of earthworm gut derived *Aeromonadaceae* (Zeibich et al., 2019a). This finding suggests a possible role of this bacterial family in the degradation of polysaccharides in the earthworm gut.

The remaining core midgut taxa that were identified include *Pseudomonas* (ASV 7), *Bradyrhizobium* (ASV 64), *Cutibacterium* (ASV 224) and *Delftia* (ASV 438). Previous studies have shown the association of *Pseudomonas* (or *Pseudomonadaceae*) with earthworm gut or cast (Byzov et al., 2009; Furlong et al., 2002; Knapp et al., 2009; Yausheva et al., 2016). Relative abundances of *Pseudomonas* (ASV 7) in soil and midgut were correlated, but, *Pseudomonas* was much more abundant in midguts compared to soils (0.02-15.5% in midgut vs. 0.003-1.430% in soils) suggesting a colonization of gut tissue by *Pseudomonas*. *Bradyrhizobium* is well studied soil-dwelling genus capable of nitrogen fixation and is able to form symbiotic nodules with legumes plants. Association of *Bradyrhizobium* with earthworm gut tissue has previously been reported (Thakuria et al., 2010). Free-living *Bradyrhizobium*, however, do not always possess genes involved in nitrogen fixation (VanInsberghe et al., 2015). Previous work has shown that higher rates of nitrogen fixation in earthworm guts and cast compared to soils are associated with a presence of nitrogen fixing bacteria in the earthworm gut (Citernesi et al., 1977; Tomati & Galli, 1995; Umarov et al., 2008). This suggests that the earthworm gut may be a site of enhanced nitrogen fixation resulting from the simulation of ingested nitrogen fixating bacteria by the specific gut conditions, as has been demonstrated for earthworm intestinal denitrification (Ihssen et al., 2003; Karsten & Drake, 1997). In this study, relative midgut abundance *Bradyrhizobium* was comparable that of soils (0.02-0.93% in midguts vs. 0.2 and 0.51% in soils), suggesting that *Bradyrhizobium* may be a transient gut bacterium. *Bradyrhizobium* was however not detected in $^{5.6}Woburn_3$ soils but had a relative abundance of 0.78% in midguts of *E. fetida* reared in $^{5.6}Woburn_3$ soils, indicating this bacterium may sustain presence in the midgut without continued soil ingestion. The consistent association of among others *Bradyrhizobium* and *Pseudomonas* with the midgut of *E. fetida* as reported here, warrants further investigation into possible roles of these bacteria in *E. fetida* gut system.

'*Candidatus* Lumbricincola' was consistently found in *E. fetida* midguts during the pilot transfer experiment (**Figure 3.3**) and the experiment testing different midgut sampling methods (**Figure 3.1).** In samples from the soil transfer experiment, however, this taxa was below detectable levels

in most midgut samples (**Figure 3.5**), indicating a possible loss of symbiont event in culturing conditions. '*Candidatus* Lumbricincola' is novel lineage of *Mollicutes* which has been detected in various earthworm tissues and casts of different earthworm species (Nechitaylo et al., 2009; Rieder et al., 2013; Singleton et al., 2003; Zeibich et al., 2019a). Recent studies on the impact of environmental pollutants on the earthworm microbiome indicate that this taxa is particularly sensitive to exposure to metal pollutants (Šrut et al., 2019). '*Candidatus* Lumbricincola' is phylogenetically related to '*Candidatus* Hepatoplasma crinochetorum' (Nechitaylo et al., 2009), a bacterium that can infect the hepatopanceas of isopods and is beneficial to the host in nutrient limited conditions (Collingro et al., 2015; Fraune & Zimmer, 2008; Wang et al., 2004). The nature of the interaction between earthworms and '*Candidatus* Lumbricincola' is still unknown. However, recent studies suggest that earthworm *Mollicutes* can be stimulated by supplementation of chitin and xylan (Zeibich et al., 2019a), suggesting a possible role of this taxa in the degradation of structural polysaccharides.

In this paper it was aimed to study the composition of the bacterial community closely associated with the midgut of the earthworm *E. fetida*. It is this host associated community that could possibly have a strong interaction with its host. Host specificity is however not a condition for microbial-host interaction. Studying solely the host specific community independent from habitat will thus exclude taxa of which the interaction is of facultative or opportunistic nature. Animal associated microbiome can be highly variable between individuals and habitats (Falony et al., 2016; Salonen et al., 2012; Wong et al., 2013). Despite the consistent presence of the some core taxa, this papers show that the bacterial midgut community of *E. fetida* is highly variable between individuals. This paper only discusses the community composition of the midgut of *E. fetida*. In order to understand the intrinsic nature of the host-microbiome interactions, it is crucial to study the functionality of the microbiome. Defining the microbiome structure, as reported here, can form the basis for such work. Previous research has also shown that anthropogenic pollution can alter the total bacterial community composition (i.e. transient gut, resident gut and nephridial communities together) in some earthworm species (Pass et al., 2015). Impact on environmental pollutants on the resident earthworm gut microbiome and subsequent effects on host-microbe interactions are, however, not well described. As earthworms play a crucial role in soil functioning, and earthworm symbionts are known to contribute to host health (Lund, Holmstrup, et al., 2010; Viana et al., 2018), the effects of environmental conditions including soil properties, food quality and pollutant exposures on the earthworm microbiome should be further investigated.

4

The earthworm microbiome is resilient to exposure to biocidal metal nanoparticles

4.1 Introduction

The microbiome of invertebrates can play a crucial role in host health by aiding in digestion and nutrition (Brune, 2014; Hosokawa et al., 2010), providing resistance to invading pathogens (Dillon et al., 2005; Eleftherianos et al., 2013; B.L. Weiss et al., 2012) and providing aid in dealing with abiotic factors (Cheng et al., 2017; De Almeida et al., 2017; Kikuchi et al., 2012). Earthworms play a vital role in the functioning of the soil through nutrient cycling, organic matter degradation and the aeration of the soil (Edwards & Bohlen, 1996). The earthworm microbiome has been relatively well described (Drake & Horn, 2007; Parle, 1963; Pass et al., 2015; Thakuria et al., 2010) with two microbial communities so far being recognized: the nephridial and the gut communities. Earthworm nephridia (the excretory organs of these animals) house an earthworm specific bacterial community of which some bacterial symbionts are vertically transmitted from parent to offspring (Davidson et al., 2006; Møller et al., 2015; Schramm et al., 2003). The bacterial community in the gut of earthworms is composed of both transient microbes and resident microbes, the latter being more tightly associated to the gut wall of earthworms (Singleton et al., 2003; Thakuria et al., 2010). Roles of the earthworm microbiomes are not fully understood (Møller et al., 2015) but some studies have highlighted the role of gut bacterial communities in anaerobic fermentation of dietary polysaccharides suggesting that gut microbes play a role in digestion (Zeibich et al., 2019a, 2019b). The loss of some earthworm symbionts has been shown to negatively impact earthworm development and reproductive output (Lund, Holmstrup, et al., 2010; Viana et al., 2018). The dependence of many animals on their symbiotic microorganisms, as with earthworms, makes it important to understand how environmental pollutants affect the microbiome, especially for those chemicals that are designed to target microbes.

Previous work has established that exposure to antimicrobial chemicals can influence gut microbiome structure in rodents, fish and invertebrates (Ding et al., 2019; Gaulke et al., 2016; Han et al., 2014; Williams et al., 2015). Also in earthworms, exposure to pollutants (e.g. antibacterial agents and heavy metals) can alter the structure of the gut microbiome (Liang et al., 2009; J. Ma et al., 2020; L. Ma et al., 2017; Rieder et al., 2013; H.T. Wang et al., 2019). Field-based studies have further shown that metal pollution can lead to the loss of core symbionts important to earthworm health (Pass et al., 2015). Through these exposure related changes and the dependence of animals on their microbiome, pollutants can negatively affect organisms, even in the absence of direct host toxicity (Blot et al., 2019; Motta et al., 2018). In a recent study, Motta and colleagues showed that the herbicide glyphosate can negatively affect some core symbionts in the honey bee gut (Motta et al., 2018). Under normal conditions, glyphosate has no negative impact on host survival. However, honey bees that have their microbiome changed by glyphosate exposure are much more

susceptible to the bee pathogen *Serratia* leading to high mortality rates when infected (Motta et al., 2018). Hence, pollutant induced changes to the gut microbiome can have important implications for exposed individuals and potentially also for populations.

Over recent decades, there has been a major increase in the production and usage of nanoparticles (NPs), including as biocidal agents (Khot et al., 2012; Prabhu & Poulose, 2012). Two of the most commonly applied biocidal NPs are copper and silver. The soil ecosystem can be exposed to biocidal NPs through the application of fungicides (Cu-based NPs) or the application of sewage sludge onto soils (Ag-NPs) (Keller et al., 2013). Current predicted environmental concentrations (PEC) for Cu-based NPs have been estimated to be in the ppb range, whilst the current PEC for Ag-NPs may range between 7 and 5000 ng/kg soil (Garner et al., 2017; Giese et al., 2018). These concentrations are based on total NP production values and the predicted fate of NPs in the environment. However, these predictions do not take into account point source releases such as direct application of NP-based pesticides, which could produce locally higher concentrations. The release of these biocidal NPs into the soil ecosystem may lead to exposure of non-target soil microbes to these chemicals with possible effects on the soil bacterial community structure and function (Collins et al., 2012; Colman et al., 2013; J. Liu et al., 2019; Read et al., 2016; Samarajeewa et al., 2017; Simonin & Richaume, 2015). Colman and colleagues, for example, showed that even low concentrations of Ag-NPs (e.g. 0.14 mg/kg soil), significantly alter the community composition of soils and reduce soil enzymatic activity (Colman et al., 2013). Recent research indicates that biocidal NPs can also disturb the gut microbiome of the soil invertebrate *Folsomia candida* (Zhu et al., 2018b). Less is known, however, about the impact of biocidal NPs on the earthworm microbiome, which may play a key role in soil functions like nitrogen cycling and degradation of organic matter (Horn et al., 2003; Zeibich et al., 2019a).

The aim of this paper was to study how exposure to two metal biocides (copper and silver) in nanomaterial forms (e.g. as uncoated CuO-NPs and acrylic co-polymer coated Ag-NPs) affect the structure of the soil microbial community and the resident gut microbiome of the earthworm *Eisenia fetida.* The resident gut microbiome is here defined as the microbial community that remains in the gut after all gut contents (i.e. food and soil particles) have been physically removed. This closely associated soil independent bacterial community may play a beneficial role in host health (Thakuria et al., 2010) and it is therefore important to understand how it responds to environmental pollutants. Effects of the two NPs were studied in two separate 28-day long experiments which were conducted following the basis of OECD test no. 222 (OECD, 2016) in order to compare conventional endpoints (i.e. earthworm reproduction) to microbiome endpoints (i.e. community structure and bacterial taxa abundance). Effects induced by the two biocidal NPs were

furthermore compared to the effects of non-biocidal NPs (TiO_2) (Chen et al., 2017; Dudefoi et al., 2017) and also to the ionic form of both metals (e.g. Cu^{2+}, Ag^{+}) to test whether any observed effects were attributed to particle or ionic toxicity. Our hypothesis was that exposure to CuO-NPs and Ag-NPs and their ionic counterparts would have effects on the structure of the soil microbial community and *E. fetida* gut microbiome at similar concentrations. Greater impacts of silver forms than copper forms were expected due to the known higher biocidal toxicity of silver compared to copper (Bondarenko et al., 2013; Suppi et al., 2015). Finally, exposure to ionic metals was hypothesised to have a greater impact than their NP counterparts (Notter et al., 2014).

4.2 Methods

4.2.1 Test compounds

Ionic exposures used $CuCl_2$ and $AgNO_3$ salts (Sigma-Aldrich Ltd, Poole, UK). Uncoated rod shaped CuO-NPs and acrylic co-polymer coated spherical Ag-NPs were obtained from Promethean Particle Ltd (Nottingham, UK). These materials were chosen to reflect a range or properties (size, shape and coatings) and were used as generic models for Cu-based-NPs and Ag-NPs. Both materials were dispersed in water. The stated mean width and length of the rod-shaped CuO-NPs were 20 and 50 nm, respectively. The stated mean diameter for the spherical Ag-NPs was 50 nm. Size distributions were measured using nanoparticle tracking analysis (NTA) using a Nanosight NS500 instrument (Malvern Instruments, Salisbury, UK). The mean measured hydrodynamic diameter for CuO-NPs was 183 nm (±SE) nm and 177 nm (±20) for Ag-NPs (**Figure 4.1**) (n.b. size estimations using NTA for rod-shaped particles are not as accurate as for spherical particles, therefore the true size distributions may be different from the values reported here). Zeta potential of the CuO-NPs and the Ag-NPs were determined using phase analysis light scattering using a Zetasizer Nano ZS (Malvern Instruments). The zeta potentials were 33 mV (±SD 0.3) for CuO-NPs and -34 mV (±0.7) for Ag-NPs. To ensure that any responses were due to the particles themselves and not to any impurities in the stock dispersion, an exposure was undertaken using the NP dispersion carrier. The carrier control medium was collected after centrifugation of the dispersions at 41000 rpm for one hour at 4°C using a OptimaTM L-100 XP ultracentrifuge (Beckman Coulter, Brea, CA, USA). The supernatant was subsequently filtered using an Amicon Ultra -15 Ultracel -10K filter (Merck, Darmstadt, Germany) and centrifuged at 5000 rpm at 4°C using an Eppendorf centrifuge 5810R (Eppendorf, Hamburg, Germany). The flow-through (i.e. the carrier control medium) was collected and stored at 4 °C until soil spiking. The TiO_2-NPs used were uncoated spherical 25 nm anatase NPs supplied by Sigma-Aldrich Ltd.

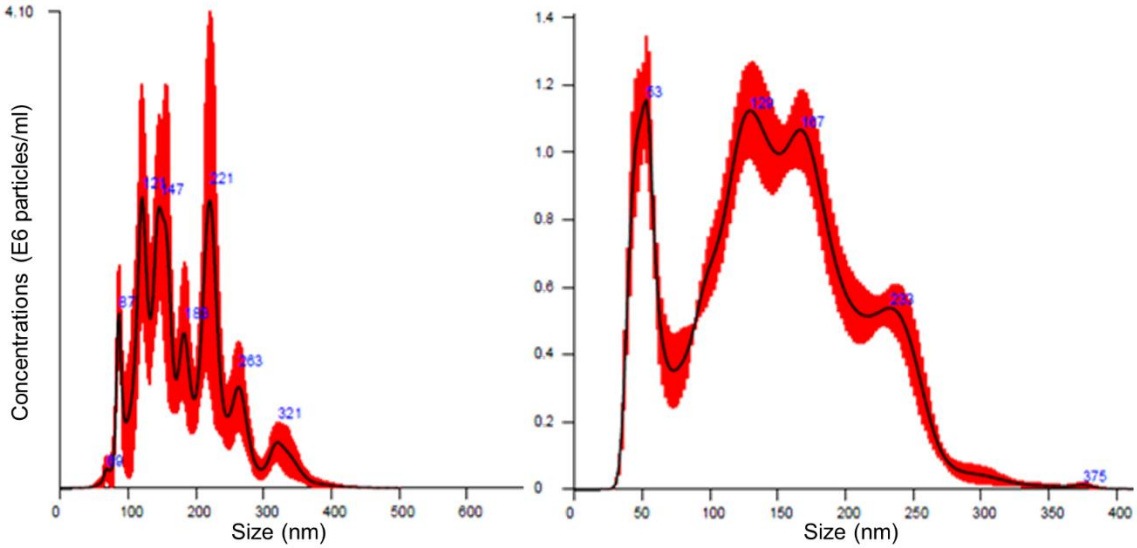


Figure 4.1: Size distributions of CuO-NPs (left) and Ag-NPs (right) derived through nanoparticle tracking analysis. X-axis indicates size in nm. Solid lines indicates mean. Red ribbons indicate ± standard error of the mean. Estimates of the size distributions were derived by 5 captures of each 60 seconds.

4.2.2 Eisenia fetida toxicity testing

Eisenia fetida were cultured in a 1:1:1 medium of loamy soil, composted bark and garden compost at 20 °C and fed with uncontaminated horse manure. All *E. fetida* used in the experiments were adults with a weight between 300 and 600 mg. Exposures were in LUFA 2.2 soil (LUFA-Speyer, Germany). The protocol used for the *E. fetida* bioassay followed the basis of the OECD test no. 222 (OECD, 2016) with modifications following Heggelund et al. (2014). Two separate experiments were conducted. In the first experiment, *E. fetida* were exposed to a concentration series of CuO-NPs, a single ionic control ($CuCl_2$) and a single NP dispersion carrier control. In the second experiment, *E. fetida* were exposed to a concentration series of Ag-NPs, a single ionic control ($AgNO_3$), a single NP dispersion carrier control and an additional particle control, which used TiO_2-NP.

The concentration series of the NPs were designed so that effects on *E. fetida*'s reproduction were only expected at the highest concentration, based on previous research (Schlich et al., 2013; Shoults-Wilson et al., 2011b, 2011a). We expected that the microbial communities would be particularly sensitive to CuO-NP and Ag-NP (Colman et al., 2013). Therefore, we used a large concentration range similar to previous studies (Doolette et al., 2016; Yang et al., 2018), with different treatments separated in concentration by a factor of 2.5. Test concentrations for CuO-NPs and Ag-NPs were 0, 10, 26, 64, 160 and 400 mg Cu or Ag per kg dry weight soil. Ionic controls ($CuCl_2$ and $AgNO_3$) were tested at 160 mg Cu or Ag per kg dry weight soil. Ionic metals are typically more toxic than their NP counterparts. To prevent lethality of *E. fetida* in the ionic control, we therefore chose to compare ionic metals and NPs not at the highest NP concentration, but rather at the

second highest NP concentration (i.e. 160 mg/kg). At these test concentrations, it is unlikely that the anions of the metals salts will have an effect on the earthworm endpoints (Owojori & Reinecke, 2014). TiO_2-NPs were tested at 308 mg TiO_2 per kg dry weight soil. The TiO_2-NP treatment was used as a control to test whether any effects of CuO-NPs and Ag-NPs could be related to the physical interaction of the particles with organisms. Based on the stated dimensions of the CuO-NPs and the Ag-NPs and the density of the materials we calculated the theoretical number of particles that were added in the highest NP treatment replicates. The theoretical number of particles in the TiO_2-NP controls was calculated to be equivalent to that in the highest CuO-NP treatment.

Nine replicates were used for all copper and silver treatments (i.e. for CuO-NP, Ag-NP, $AgNO_3$ and $CuCl_2$ treatments) and six for the TiO_2-NP control and the carrier controls. Carrier controls were spiked at a level equivalent to the amount of dispersion carrier present in the highest tested concentration of the respective NP exposure. $CuCl_2$ and $AgNO_3$ were treated one week before the initiation of exposure to allow the metal speciation of the soil to reach an initial quasi-equilibrium (Smit & Van Gestel, 1998). In order to expose the *E. fetida* and the bacterial communities to pristine NPs (Diez-Ortiz et al 2015a), the NPs (i.e. CuO-NP, Ag-NP and TiO_2-NP) and carrier control medium were spiked one day prior to the initiation of the exposure. For the CuO-NP, Ag-NP, $AgNO_3$, $CuCl_2$ and the carrier control exposures, mixing of soils with chemicals was done per replicate. For this purpose, a stock solution was prepared per treatment. 15 ml of the stock solution was then added to 300 g dry soil and mixed thoroughly using a spoon until all clumps were gone and the mixture was homogenous in colour. After mixing, soils were wetted to 55% of the water holding capacity (WHC) and then mixed further. TiO_2-NPs were added as a dry powder following Waalewijn-Kool et al. (2012), initially using 30 g of dry soil. This mixture was then thoroughly mixed with 270 g of dry soil. For each replicate, an aliquot of 50 g of this mixture was added to an exposure container and thoroughly mixed with the remaining soil (250 g). After mixing, de-ionised water was added to a moisture content of 55% of the WHC.

To initiate the exposure, six *E. fetida* that had been acclimatised in LUFA 2.2 for one week were added to each test replicate. Three grams of spiked manure was added to the soil surface on a weekly basis. Exposures were conducted at 20 °C for 28 days. Mortality was measured at 28 days when *E. fetida* were removed and a soil sample was taken. *E. fetida* samples and soil samples were processed according to the description below. After removal of the *E. fetida*, soils were incubated for a further 28 days to allow the laid cocoons to hatch. After the second exposure period, juveniles were extracted from the soil by placing the containers in a water bath set at 60 °C for approximately ten to fifteen minutes, following (OECD, 2016). Collected juveniles were counted and the total juvenile biomass per replicate was recorded.

Concentration response models and half maximal effective concentration (EC_{50}) values derived from the *E. fetida* reproduction and biomass data were calculated using the 'drc' package (Ritz & Streibig, 2005) in R (www.r-project.org). Chronic effect levels on reproductive output and juvenile biomass were determined using log-logistic models. One-way analysis of variance (ANOVA) was used to test the significance of differences between the negative controls and the carrier controls, and the differences between the ionic treatment and equimolar NP treatment. Significance of differences between treatments in adult weight was calculated using two-way ANOVA and Tukey's test.

4.2.3 Soil pH measurements and pore water extractions

The pH of the collected soil samples was measured in a 0.01 M $CaCl_2.2H_2O$ slurry (ISO, 2005). Soil pore water samples were extracted from three replicates per treatment for all CuO-NP, Ag-NP, $CuCl_2$ and $AgNO_3$ treatments following the approach of Heggelund et al. (2014). To extract the pore water, 15 grams wet weight soil was collected at the end of the 28-day exposure in a 50 ml centrifuge tube. Ultrapure water was added to raise the soil to 100% WHC and the samples were then left overnight. Subsequently, the soil mixture was added to a Thermo Scientifc™ 25 ml Centrifugal Filters tube containing 0.2 g of glass wool and centrifuged for 1h at 2000xg. For silver spiked soil samples, both the filters and glass wool were soaked in 0.1 M $CuSO_4.5H2O$ overnight and subsequently rinsed two times using ultrapure water prior to prevent the binding of the silver to both components by the occupation of binding sites with a high affinity metal ion. For copper spiked soil samples, filters and glass wool were soaked in ultrapure water. In addition, for the control, the two highest NP concentrations and the ionic control pore waters, a sample representing the ionic fraction of metals in soil pore water was collected from filtered extracts by ultracentrifugation. For each sample, three ml of the filtrate was mixed with seven ml ultrapure water and subsequently ultra-centrifuged at 41000 rpm for one hour using a Beckman Coulter Optima™ L-100 XP Ultracentrifuge. All pore water samples were acidified using HNO_3 for copper derived samples (1% HNO_3 final concentration) and aqua regia (33% HCl, 67% HNO_3) for silver derived samples (50% final concentration).

4.2.4 Pore water and soil metal analysis

To measure total copper and total silver concentrations in the soil samples, for each measurement 130 mg of d.w. soil was mixed with a mixture of nitric acid and hydrochloric acid (4:1 on a volume basis) and digested for seven hours at 150 °C. Metal concentrations of pore waters and the digested soil samples were determined by atomic absorbance spectrometry at the Vrije Universiteit Amsterdam (The Netherlands). The quality of metal analysis was tested using a certified reference

soil (ISE 989, International Soil-Analytical Exchange). Recovery of copper in the reference soil sample ISE 989 was 89%. Correlations between measured and nominal soil metal concentrations were tested by calculation of the Pearson's correlation coefficient. Differences between ionic controls and their equimolar NP treatment (160 mg/kg) and negative control and the carrier control were tested using one-way ANOVA.

4.2.5 Sampling of E. fetida midguts and soil samples

After removal from the soils at the end of the 28 day exposures, one *E. fetida* per replicate was depurated (i.e. starved) for two days on wetted filter paper to allow egestion of gut contents. After depuration, *E. fetida* were euthanized in 100% ethanol and the midgut portion (spanning 20 segments posterior to the clitellum) was dissected following **2.1.1**. Soil samples for microbiome analysis were collected at the end of the 28 day exposure following **2.1.2**.

Table 4.1: *Eisenia fetida* 18S-rRNA gene sequences used to design 18S-rRNA gene primers using Primer 3.

NCBI identifier	Authors	Sequence length
AB076887.1	Hiraishi A et al.	1818 bp
AB558505.1	Abe N et al.	1734 bp
EF534709.1	Wright ADG	2972 bp
KF823788.1	Zhao C and Li Y	668 bp
KX651309.1	Csuzdi C et al.	652 bp
X79872.1	Winnepenninckx B et al.	1818 bp

4.2.6 Measuring of the bacterial load in midguts

The bacterial load in midgut samples was determined by measuring the amount of bacterial 16S-rRNA gene relative to the amount of *E. fetida* 18S-rRNA gene using quantitative PCR. Total genomic DNA samples were diluted to 10 ng/µl. Two and a half millilitre of diluted DNA was used in qPCR reactions using GoTaq qPCR Master Mix (Promega, Madison, WI, USA), which uses BRYT Green® Dye for DNA quantification. To quantify the amount of bacterial 16S-rRNA gene, a 122 bp fragment was amplified using primers 5'-CGGTGAATACGTTCYCGG-3' (BACT1369F) as forward primer and 5'-GGWTACCTTGTTACGACTT-3' (BACT1492R) as reverse primer (Suzuki et al., 2000). To measure the quantity of the *E. fetida* 18S-rRNA we designed an 18S-rRNA primer pair based on five *E. fetida* 18S-rRNA DNA sequences retrieved from NCBI using Primer 3 in Geneious v9 (**Table 4.1**). The primer pair (forward: 5'-AACGGCTACCACATCCAAGG-3', reverse: 5'-CACCAGACTTGCCCTCCAAT-3') spanned a 165 bp region of the *E. fetida* 18S-rRNA gene. qPCR assays were performed using a Roche LightCycler® 480II using the following program: initial denaturing at 95 °C for three minutes followed by 40 cycles of 1. denaturing at 95 °C for 10 seconds and 2. annealing and extension at 60 °C for 30 seconds. Melt curve analysis and gel electrophoresis were conducted to verify the reaction

produced a single product. The efficiency of the used primers was tested through the amplification of serial diluted and cleaned qPCR products and was between 90% and 110% for each primer pair. All qPCR reactions were done in duplicates. Total bacterial load was assessed by employing the $\Delta\Delta C_t$ method using the 18S-rRNA C_t values as reference gene against which 16S-rRNA C_t values were normalized. Because of this normalisation, no standard curves were calculated per reaction. The differences in the bacterial load between the different treatments were tested using Kruskal-Wallis tests.

4.2.7 Metabarcoding and sequencing

Details of the method used for the characterisation of the soil bacterial community and *E. fetida* gut microbiome are described in **2.2.1-3**. Briefly, a ~550 bp fragment spanning the V3-V4 region of the bacterial 16S-rRNA gene was amplified through PCR following the metabarcoding pipeline developed by Kozich et al. (2013). Sequencing of 16S-rRNA gene amplicons was done using a MiSeq (Illumina, San Diego, USA). Bioinformatic analysis of Illumina reads was done using the DADA2 pipeline (Callahan et al., 2016) using the Silva database (Callahan, 2018) for taxonomical assignment.

Table 4.2: Average number of reads per sample (±SD) before and after rarefication per data set.

Dataset	Average number of reads per sample before rarefication (±SE)	Number of reads per samples after rarefication*
Cu –gut	40737 ± 5875	2447
Cu – soil	23200 ± 1324	13254
Ag – gut	37032 ± 4148	2722
Ag – soil	18741 ± 1354	7464
TiO_2 – gut	43583 ± 5565	3465
TiO_2 - soil	20354 ± 1353	8374

* Listed rarefied datasets were used for all microbiome analysis unless stated otherwise.

4.2.8 Microbiome data analysis

After ASV table generation and removal of ASVs belonging to Archaea, Eukaryotes, Archaea, mitochondria, chloroplast and ASVs with unknown kingdom or phylum (**2.2.3**), the remaining data was divided into six datasets for discrete analysis (i.e. Cu - gut, Cu - soil, Ag –gut, Ag – soil, TiO_2 – gut, TiO_2 – soil). All statistical testing on the microbiome data was done using R. Each separate dataset was subjected to rarefication, with the minimum number of reads per dataset listed in **Table 4.2.** The microbiome dataset were analysed as follow: **1)** The alpha-diversity (Shannon index and total richness) indices were calculated using the R package 'vegan' (Oksanen et al., 2017). Differences between treatments in Shannon index and species richness were tested using 2-way ANOVA. Linear models were used to test the relationship between soil metal concentration and the diversity indices. **2)** For each sample, the total and relative abundance of every bacterial phyla was

calculated. The relationship between the total phyla abundances and measured metal concentrations was then tested using linear modelling. Differences in the relative phyla abundance between control treatments from the two experiments were tested using the Mann-Witney-U test and applying Bonferroni correction **3)** Non-metric dimensional scaling (NMDS) was used to test for effects of treatments on community structure. NMDS analysis was done using the 'metaMDS' function in 'vegan and based on Bray-Curtis distance matrices. The relationship between the measured soil metal concentrations and microbial community structure was tested using distance-based redundancy analysis (Db-RDA) based on 999 permutations using the 'capscale' function in 'vegan'. Db-RDA models were run applying Bray-Curtis index as distance matrix and applying non-metric multidimensional scaling with stable solution from random starts, axis scaling and species scores. **4)** Only for midgut samples, the relationship between total read abundance (i.e. rarefied read abundance) of individual ASV and measured soil concentration was tested using linear modelling. **5)** In order to express the impact of treatments in a single number, for each treatment, the number of ASVs that was differentially abundant compared to their respective control was calculated using Mann-Witney U test. **6)** Using log-logistic modelling ASV specific EC_{50} values (i.e. the concentration of measured soil Cu or Ag at which the read abundance of an ASV is half of that in the negative control treatment) were calculated. For this purpose, datasets were rarefied to 2447 reads per sample prior to modelling to remove any library size biases between the datasets. Further, as it is not possible to run a log-logistic models on ASVs with too few data points, ASVs with a total read count below 50 were removed from the data. The EC_{50} values calculated from these ASVs were then used to calculate species sensitivity distributions (SSD) using the R package 'ssdtools' (Thorley & Schwarz, 2018). SSD models typically use EC_x or NOEC values derived from ecotoxicological tests using a single biological species. In this study, EC_{50} values were derived from the read abundance of ASVs. For clarity these models are herein referred to as SSD models, rather than ASV-sensitivity distributions models. For the calculation of the SSDs, only ASVs where included for which i) a log-logistic could be fitted, ii) there was a significant negative concentration dependent response (at $\alpha = 0.1$, calculated using the 'noEffect' function within the 'drc' package) and iii) the EC50 value of the log-logistic model was significant ($\alpha = 0.1$). The SSD models were then used to calculate the HC_5 values. The HC_5 values in this case refer to the concentration at which 5% of the total number of ASVs in the community is negatively affected in their read abundance by 50% or more compared to the negative control. For the calculation of the hazardous concentration for 5% of the taxa (HC_5) values were based on the total number of ASVs for which a log-logistic model was successfully fitted, irrespectively of whether there was a significant concentration-

response effect or whether the calculated EC_{50} value was higher than the highest test concentration.

4.3 Results

4.3.1 Test soil chemistry and exposure concentration validation

For the copper treated replicates, soil pH was not correlated to measured copper concentration with an average pH across treatments of 5.8 (±SD 0.08) (Pearson's *r*=-0.33, *p*=0.42). For the silver treated replicates, pH was significantly positively correlated to measured silver concentration in the soil (Pearson's *r*=0.78, *p*=0.02) and ranged between 5.6 in the controls and 6.2 and 6.3 in the highest NP treatment and the ionic control, respectively. Measured metal concentrations were on average 75% (±SD 15) and 77% (±7.0) of nominal concentrations for CuO-NPs and Ag-NPs, respectively. The measured concentrations in the ionic controls were the same as the equimolar NP replicates (Cu: $F(1,4)=0.648$, $p=0.466$); Ag: ($F(1,6)=2.944$, $p=0.137$). The measured copper concentration in the negative control replicates was not statistically different to the measured copper concentration in the CuO-NP carrier control replicates ($F(1,4)=0.08$, $p=0.792$). Silver concentration in the negative controls was below detectable levels but on average 0.58 mg/kg in the Ag-NP carrier controls. For all further data analysis, we used measured soil concentrations instead of the nominal concentrations. However, in this paper the nominal concentrations are used to describe treatments for clarity. Total and ionic pore water concentrations (**Table 4.3**) were correlated with nominal soil

Table 4.3: Mean (±SD) total and dissolved pore water concentrations in soils and ionic fraction per treatment.

Treatment	Nominal conc. (mg/kg)	Copper exposures			Silver exposures		
		Mean total pore water ±SD (mg/l)	Mean diss. pore water ±SD (mg/l)[a]	Avg. dissolved fraction ±SD	Mean total pore water ±SD (mg/l)	Mean diss. pore water ±SD (mg/l)[a]	Avg. dissolved fraction ±SD
Control	**0**	0.09 ±0.03	0.10 ±0.03	1.1 ± 0.06	0.06 ±0.00	0.00 ±0.01	n.a.
NP	**10**	0.17 ±0.03	n.d.	n.a.	0.06 ±0.03	n.d.	n.a.
NP	**26**	0.38 ±0.24	n.d.	n.a.	0.08 ±0.03	n.d.	n.a.
NP	**64**	0.74 ±0.10	n.d.	n.a.	0.28 ±0.13	n.d.	n.a.
NP	**160**	2.01 ±0.22	1.78 ±0.17	0.9 ±0.03	3.26 ±0.67	2.67 ±0.58	0.82 ±0.01
NP	**400**	4.47 ±0.57	3.35 ±0.44	0.7 ±0.01	4.74 ±0.63	3.64 ±0.50	0.77 ±0.01
Ionic ctrl.	**160**	1.58 ±0.29	1.41 ±0.23	0.9 ±0.02	3.16 ±0.54	2.53 ±0.42	0.80 ±0.01

[a] Mean diss. pore water, mean dissolved pore water. Dissolved pore water concentration were determined by measuring the concentrations in filtered and ultracentrifuged pore water, see 4.2.3

metal concentration for both copper and for silver (Pearson's *r:* 0.86-0.98, p<0.001). In both experiments, there were no statistically significant differences in the total pore water concentrations (Cu: $F(1,4)=2.926$, $p=0.162$; Ag: $F(1,4)=0.029$, $p=0.873$) and the ionic pore water concentrations (Cu: $F(1,4)=3.326$, $p=0.142$ and Ag: $F(1,4)=0.074$, $p=0.799$) between the metal salt controls (i.e. $CuCl_2$ and $AgNO_3$) and the equimolar NP treatments.

4.3.2 E. fetida adults and juvenile endpoints

There was no effect of the carrier control on adult biomass change (Cu: $F(1,13)=0.723$, $p=0.411$; Ag: $F(1,13)=1.406$, $p=0.267$), juvenile production (Cu: $F(1,15)=1.356$ $p=0.262$; Ag: $F(1,13)=2.895$, $p=0.113$) or total juvenile biomass (Cu: $F(1,5)=0.005$, $p=0.947$; Ag: $F(1,5)=2.045$, $p=0.212$) indicating no effect of the carrier control solution on *E. fetida*.

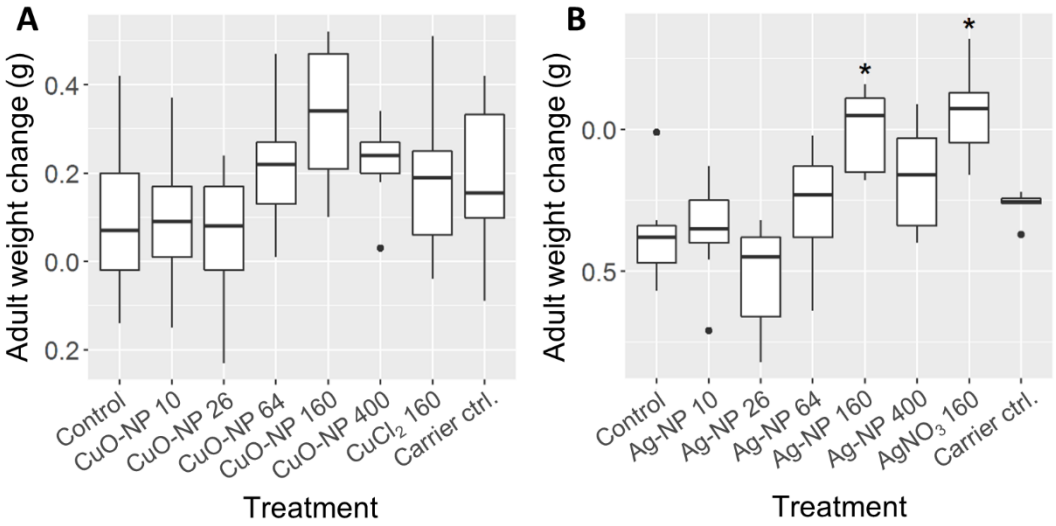


Figure 4.2: Adult weight change at day 28 (i.e. weight at day 28 – weight at day 0) in the copper experiment (**A**) and the silver experiment (**B**). Values are total weight of all earthworms per replicate. Asterisks indicate a statistical significant difference between treatment and the control.

In the copper experiment, there was 100% survival of animals in all treatments at day 28 of the exposure. A slight increase in adult biomass was observed in response to CuO-NP exposure ($F(1,43)=4.053$, $p=0.05$) (**Figure 4.2A**). Weight gain increased at higher exposure concentrations. CuO-NPs significantly negatively affected juvenile production ($X^2(2)=24.1$, $p<0.001$) with a 56d EC_{50} of 233 (CI: 163-304) mg/kg (**Figure 4.3A**). Total juvenile biomass after 56 days was significantly affected by CuO-NP exposure ($X^2(2)=28.0$, $p<0.001$), with an EC_{50} of 156 (CI: 96-215) mg/kg (**Figure 4.3C**).

Also in the silver experiment, there was 100% survival of animals in all treatments at day 28 of the exposure. Ag-NP exposure also had a positive effect on adult biomass ($F(1.66)=8.608$, $p<0.01$)(**Figure 4.2B**). Ag-NPs significantly negatively affected reproductive output in *E. fetida* ($X^2(2)=28.3$, $p<0.001$), with a 56d EC_{50} of 351 (CI: 258-444 mg/kg) (**Figure 4.3B**), although the effect

on juvenile production was <50% meaning the EC_{50} should be treated with some caution. Total juvenile biomass was also affected by the Ag-NP exposure ($X^2(2)=60.8$, $p<0.001$), with an EC_{50} of 166 (CI: 134-199) mg/kg (**Figure 4.3D**).

In both experiments, there were no statistically significant differences in the net adult biomass change (Cu: $F(1,16)=0.328$, $p=0.575$; Ag: $F(1,15)=0.553$, $p=0.469$), juvenile production (Cu: $F(1,13)=0.092$, $p=0.766$; Ag: $F(1,15)=0.420$, $p=0.527$) or total juvenile biomass (Cu: $F(1,6)=4.054$, $p=0.091$; Ag: $F(1,5)=0.032$, $p=0.864$) between the single ionic metal treatment and the equimolar NP treatment (**Figure 4.2 and 4.3**)

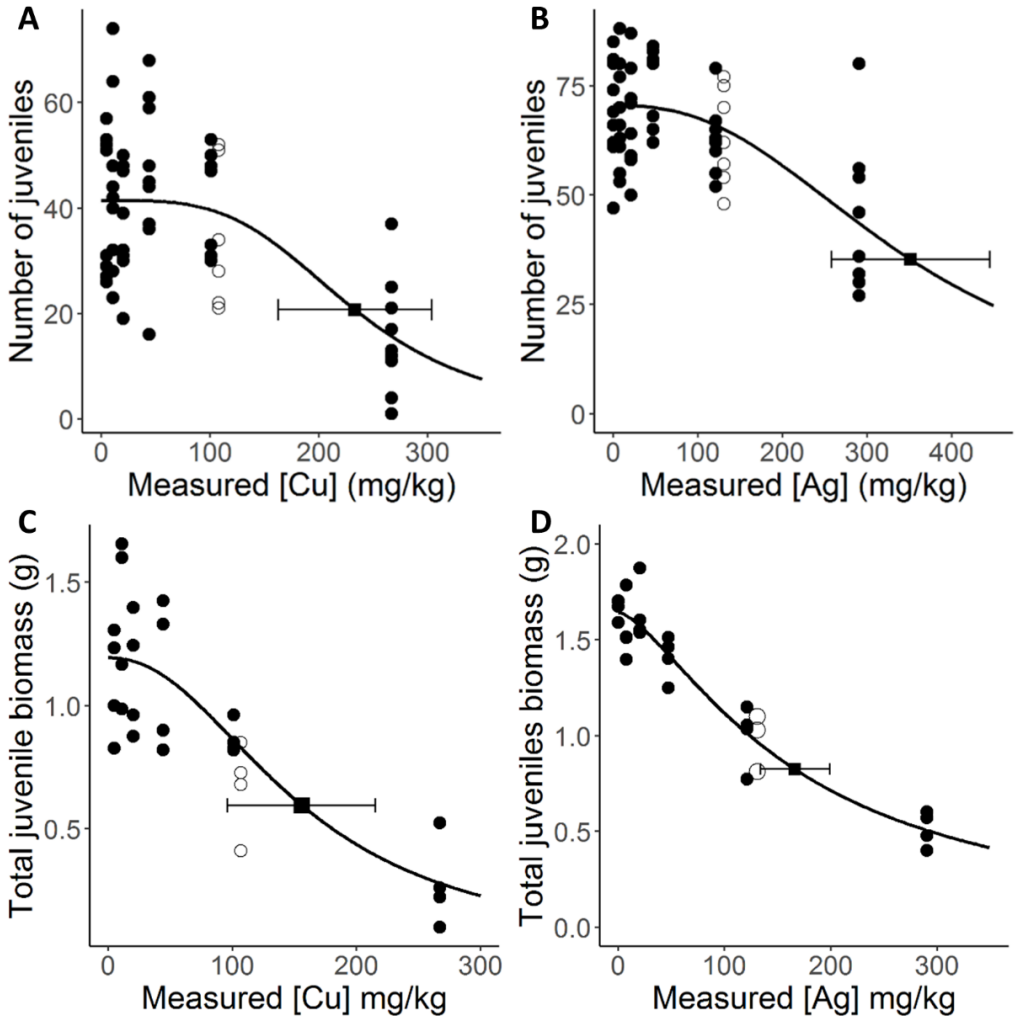


Figure 4.3: The number of hatched juveniles after a 56-day exposure to CuO-NPs and metal salt (**A**) and Ag-NPs and silver ions (**B**) and the total juvenile biomass after 56d exposure to CuO-NPs and copper ions forms (**C**) and Ag-NPs and silver ions (**D**). Black circles indicate NPs. Open circles indicate metal salt controls. Black square indicated the EC_{50} (±CI). Solid line indicates log-logistic model.

4.3.3 Illumina sequencing statistics

Illumina sequencing generated in total of 7,527,662 high quality forward reads with on average 27,983 (±8075) reads per sample. After sample interference and taxonomic assignment, the sequence data was split by metal (Cu, Ag and Ti) and by sample type (gut and soil) into six subset

(i.e. Cu–gut, Cu–soil, Ag–gut, Ag–soil, Ti–gut and Ti–soil). Subset specific sequencing and rarefication statistics are listed in **Table 4.2**. Each dataset was analysed separately and patterns in microbial community data are discussed separately below.

4.3.4 Effects of copper treatments on the soil bacterial community

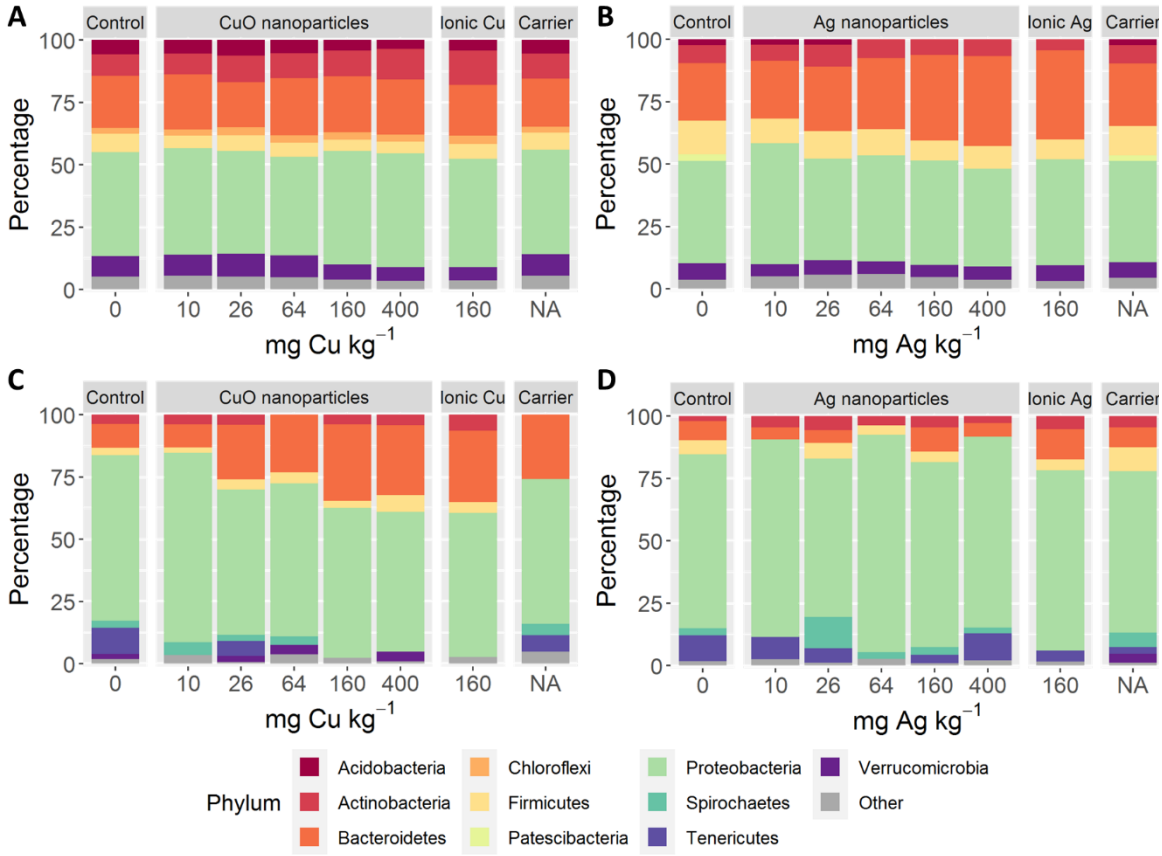


Figure 4.4: The average relative abundance of bacterial phyla per treatment in (**A**) soils from the copper experiment, (**B**) soils from the silver experiment, (**C**) the *E. fetida* midgut microbiome from the copper experiment and (**D**) *E. fetida* midgut microbiome from the silver experiment. Different colours indicate different phyla. All phyla with a relative abundance <2% are grouped under 'Other'.

Control soils in the copper experiment were dominated by *Proteobacteria* (41.8±SD3.1%), followed by *Bacteroidetes* (20.7±5.4%), *Actinobacteria* (8.7±2.4%), *Verrucomicrobia* (8.2±1.4%), *Firmicutes* (7.2±1.9%), *Acidobacteria* (5.7±2.1%) and *Patescibacteria* (1.3±0.6%) (**Figure 4.4A**). CuO-NP exposure concentration had a small but significant negative effect on the relative abundance of, among others, *Verrucomicrobia* ($F(1,46)=25.1$, $p<0.001$), *Firmicutes* ($F(1,46)=6.4$, $p<0.05$), *Acidobacteria* ($F(1,46)=10.3$, $p<0.001$) and *Patescibacteria* ($F(1,46)=16.9$, $p<0.001$) (**Table S4.1**) with the relative abundance of these taxa reduced to respectively 5.6% (±SD 1.5), 4.7% (±1.8), 3.6% (±SD 1.2) and 0.54% (±SD 0.7) at the highest NP treatment. CuO-NPs significantly increased relative abundance of *Proteobacteria* ($F(1,46)=6.54$, $p<0.05$) and *Actinobacteria* ($F(1,46)=6.71$, $p<0.05$) with the relative abundance of these taxa increased to 45.4% (±SD 3.5) and 12.2% (±4.2) at the highest NP concentration, respectively.

Table 4.4 Mean Shannon index and ASV richness (± SD) per treatment group of the soil and gut microbiomes at 28 day of the copper experiment.

Treatment	Nominal Cu conc. (mg/kg	Soil		Gut	
		Shannon	Richness	Shannon	Richness
Negative ctrl.	**0**	6.4 (± 0.1)	1117 (± 218)	2.6 (± 1.5)	138 (± 143)
NP	**10**	6.3 (± 0.2)	944 (± 209)	2.1 (± 0.9)	78 (± 51)
NP	**26**	6.3 (± 0.2)	993 (± 134)	3.2 (± 0.6)	147 (± 30)
NP	**64**	6.3 (± 0.3)	1008 (± 255)	3.0 (± 1.1)	158 (± 117
NP	**160**	6.4 (± 0.3)	1166 (± 497)	2.8 (± 1.0)	132 (± 81)
NP	**400**	6.2 (± 0.2)	1000 (± 101)	3.1 (± 1.2)	171 (± 127)
Ionic ctrl.	**160**	6.2 (± 0.2)	967 (± 116)	3.0 (± 0.9)	149 (± 67)
Carrier ctrl.	**NA**	6.6 (± 0.1)	1201 (± 186)	2.5 (± 1.1)	107 (± 44)

The Shannon index in control soils was 6.4 (±0.1). CuO-NPs had a significant negative effect on the Shannon index ($F(1,60)=7.097$, $p<0.01$) which was 0.22 lower at the highest concentration (**Table 4.4**). The species richness was on average 1045 (±247), with no significant differences between treatments. NMDS analysis showed that communities in the two highest NP concentrations and the 160 mg/kg ionic copper treatment were distinct from the controls. (**Figure 4.5A**). Db-RDA showed that soil copper concentration significantly changed the soil bacterial community composition at the ASV level (Db-RDA: $F(1,60)=4.03$, $p=0.001$).

The percentage of ASVs that was differentially abundant compared to control replicates was concentration related and amounted to 4.9% at the highest NP concentration (Pearson's $r=0.784$, $p<0.05$) (**Table 4.5**). A majority of the differentially abundant ASVs in the ionic control were also differentially abundant in the two highest NP treatments (**Figure S4.1**). Log-logistic models could successfully be fitted through 378 ASVs. Only 55 of those models showed a significant negative concentration response relation and allowed calculation of an ASV specific EC_{50}. The calculated HC_5 (the hazardous concentration for 5% of the taxa) amounted to 44.0 (±SE 7.5) mg Cu/kg (**Figure 4.6**). The most CuO-NP sensitive taxa are listed in **Appendix 2A**.

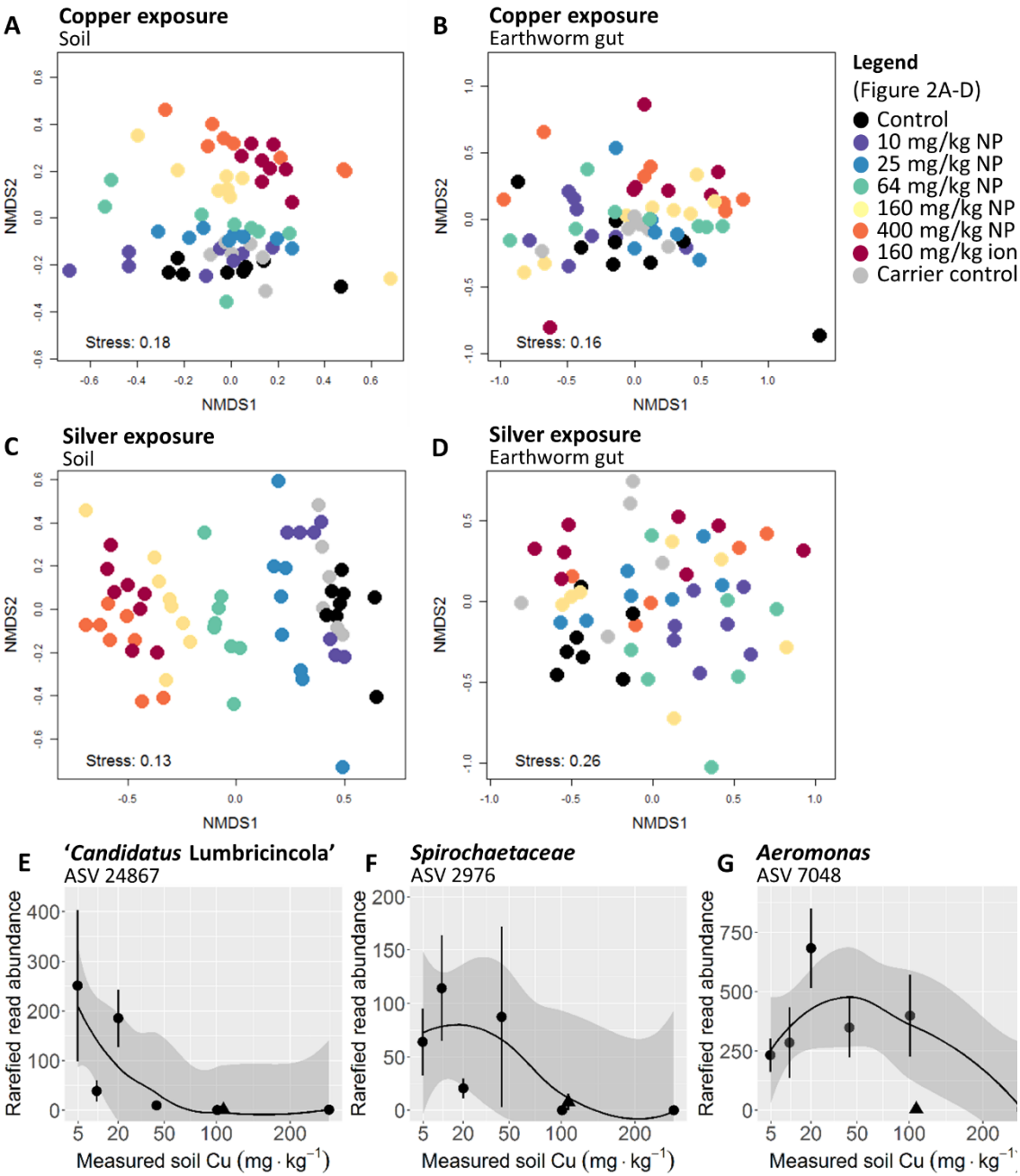


Figure 4.5: Impact of copper and silver forms on the bacterial community structure and abundance of *E. fetida* symbionts. **(A-D)** Non-metric dimensional scaling plots of soil and *E. fetida* gut bacterial communities in controls and replicates exposed to copper and silver forms. Different colours indicate different treatments. **(E-G)** Mean (±SD) rarefied read abundance in the *E. fetida* guts of three copper sensitive bacteria per measured copper soil concentration. Circles indicate NP samples; triangles indicate ionic copper samples; solid line indicates loess curve plotted through the NP samples.

Table 4.5: The total number of differentially abundant ASVs per treatment and the percentage of the total number of ASVs in bacterial community that is differentially abundant.

Treatment	Nominal conc. (mg/kg)	Copper exposures				Silver exposures			
		Soil		Gut		Soil		Gut	
		No.	%	No.	%	No.	%	No.	%
Carrier ctrl.	*NA*	78	1.30	11	1.07	59	1.57	2	0.13
NP	**10**	60	1.32	2	0.19	80	2.36	16	2.58
NP	**26**	83	1.65	22	1.86	160	3.95	4	0.47
NP	**64**	105	2.12	26	1.97	290	7.86	16	2.66
NP	**160**	149	2.95	19	1.57	374	10.39	6	0.80
NP	**400**	238	4.94	29	2.07	414	11.80	6	0.83
Ionic ctrl.	**160**	230	4.73	18	1.34	423	11.66	16	1.94

4.3.5 Effects of copper treatments on the E. fetida resident midgut microbiome

At the phylum level, control animals were dominated by *Proteobacteria* (66.5±20.6%), followed by *Tenericutes* (10.4±16.4%), *Bacteriodetes* (9.5%±8.9%), *Actinobacteria* (3.7±3.5%), *Firmicutes* (3.0±2.9%), *Spirochaetes* (2.9±3.8%) and *Verrucomicrobia* (2.0±2.7%) (**Figure 4.4C**). At the ASV level, the gut bacterial community from control animals was dominated by 11 ASV comprising 63% of the total community. Most of these bacterial taxa belonged to the genera and species *Aeromonas* (ASV 7048, 7034 and 7052), *Verminephrobacter* (ASV 16376), and *Pseudomonas alcaligenes* (ASV 14072), *Flavobacterium* (ASV 16495) and '*Candidatus* Lumbricincola' or the families *Microbacteriaceae* (ASV 5796), *Spirochaetaceae* (ASV 2976) and *Microscillaceae* (ASV 20298 and 20299) (**Figure S4.2**). Most of these gut associated taxa were either absent from the respective soil microbial communities or around the limit of detection (**Appendix 2B**).

Quantitative PCR analysis showed that the bacterial load of the highest NP treatments and the ionic treatment were not significantly different from the control samples ($X^2(4)=2.45$, $p=0.635$) (**Figure 4.7**). Shannon index and species richness were also not affected by any copper exposure treatments or the carrier control, with an average Shannon index and species richness across all treatments of 2.8 (±1.0) and 135 (±92), respectively (**Table 4.4**). NMDS, however, indicated that the microbiomes in the highest soil NP concentrations and single ionic treatment were distinct from the negative control and the lower soil NP concentration treatments (**Figure 4.5B**). Multivariate analysis showed that at the ASV level, soil copper concentration had a significant effect on *E. fetida* gut microbiome structure (Db-RDA: $F(1,56)=1.828$, $p=0.001$).

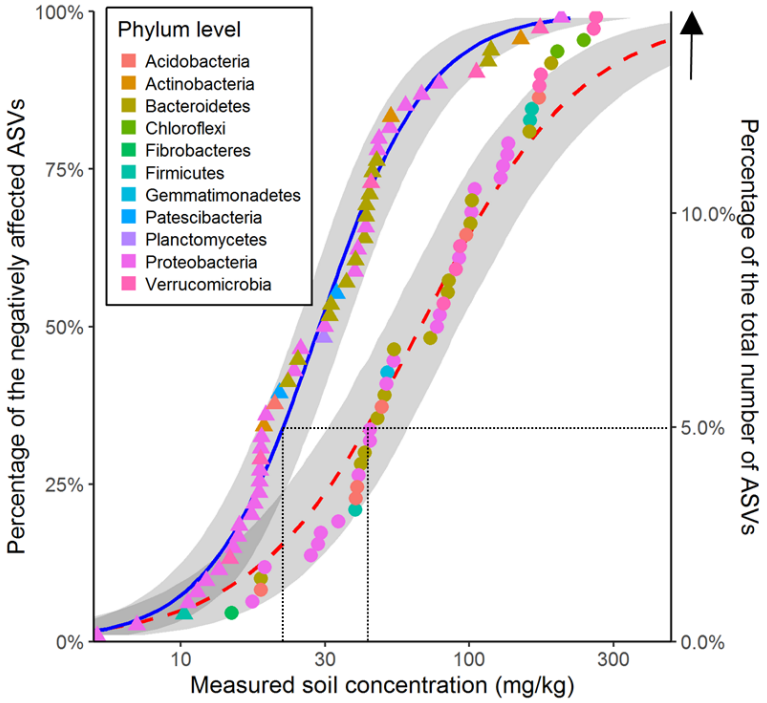


Figure 4.6: Species sensitivity distributions of soil bacterial communities exposed to CuO-NPs and Ag-NPs. Dots indicate the EC_{50} values of ASVs that are significantly negatively affected by CuO-NPs. Triangles indicate the EC_{50} values of ASVs that are significantly negatively affected by Ag-NPs. The first y-axis indicates the percentage of the negatively affected ASVs. The second y-axis indicates the percentage of the total bacterial community that is affected. The black arrow indicates that the second axis extends further than shown in the graph. The colour of the dots indicate the phylum to which these ASVs belong. The dashed red and solid blue line indicate the SSD model fit for the CuO-NPs and Ag-NPs EC_{50} values, respectively. Grey ribbons indicate the model fit ± the upper and lower confidence limit. Dotted lines indicate the HC_5 values based on the total bacterial community. HC_5 value for CuO-NPs and Ag-NPs amount to 44.0 (±SE 7.5) and 22.6 (±SE 2.4) mg metal/kg. This graphs only shows the EC_{50} values of ASVs that were negatively affected by the NP exposures. However, the HC_5 values were calculated based on the total number of ASVs for which a log-logistic model could be fitted, irrespectively of the significance and the direction of the log-logistic model.

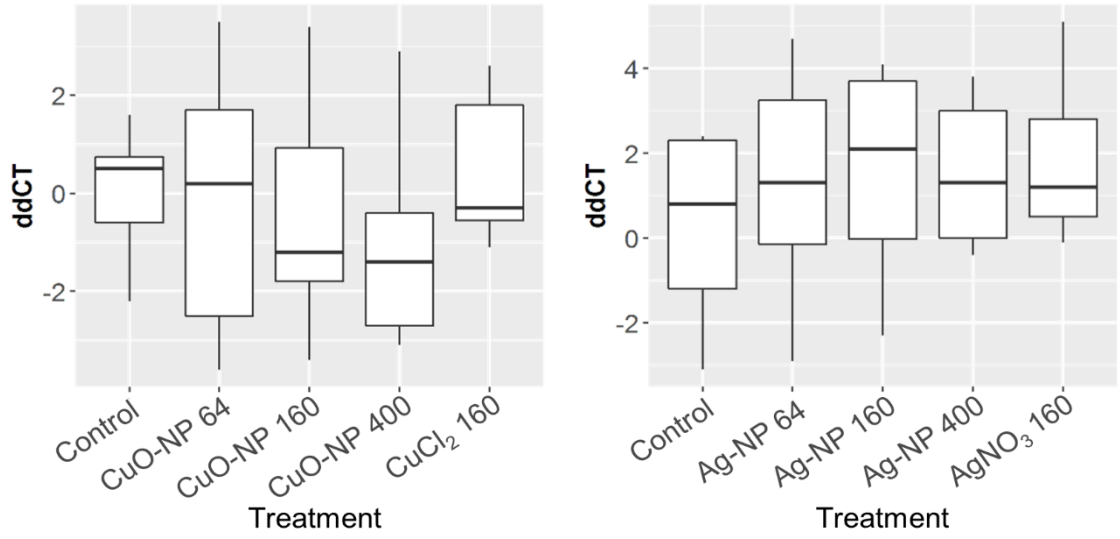


Figure 4.7: Boxplots of ddCT values based measured abundance of the 18S-rRNA gene and the 16S-rRNA gene in DNA extracted from gut tissue from *E. fetida* exposed to copper treatments (left) and silver treatments (right).

Linear modelling further indicated that of the 23 dominant ASVs (ASVs with a relative abundance of >2% in any of the treatments), the read abundance of ten were significantly positively related to NP concentration (**Appendix 2C**). These taxa belonged to the genera *Flavobacterium* (ASV 22134, 22131, 22141, 23563 and 23538), *Pseudomonas* (ASV 14103 and 14063), *Algoriphagus* (ASV 20480), *Luteolibacter* (ASV 24805) and *Aeromonas* (ASV 7034). For only one ASV (*Aeromonas*, ASV 7048) a significant negative linear relation between the read abundance and NP concentration was found (**Figure 4.5G**). Although no significant linear relationship was found for '*Candidatus* Lumbricincola' (ASV 24867) and a member of the *Spirochaetaceae* family (ASV 2976), both of these taxa were abundant in the control *E. fetida* (10.3±17.6%, 2.6±3.6%, respectively) and close to or below the limit of detection at the highest NP concentration and ionic control (**Figure 4.5E-F**).

At the highest NP test concentration, 2.1% of the ASVs were differentially abundant compared to the controls but no concentration related response in ASV number was observed (**Table 4.5**) (Pearson's r=-0.097, p=0.837). No significant negative log-logistic models could be fitted on any of the ASVs and no SSD and HC_5 value could therefore be calculated.

4.3.6 Effects of silver treatments on the soil bacterial community

The control soils in the silver experiment were dominated by the same phyla as the control replicates in the copper experiment (**Figure 4.4**). The most abundant phyla in control soils were *Proteobacteria* (40.1±SD 2.6%) and *Bacteroidetes* (23.2±4.7%), followed by *Firmicutes* (13.6±2.6%), *Actinobacteria* (7.2±1.6%), *Verrucomicrobia* (6.5±1.9%), *Patescibacteria* (2.6±1.5%) and *Acidobacteria* (2.3±0.6%). The relative abundance of *Firmicutes* in control soils in the silver experiment was, however, significantly higher than in the copper experiment control replicates (Mann-Witney test: p<0.01; **Table S4.2**). In addition, the relative abundance of *Acidobacteria* was significantly lower in the silver experiment controls than in the copper experiment controls (Mann-Witney test: p<0.01; **Table S4.2**). The controls from both experiment thus provide a different base line for the microbiome analysis. Differences may have been driven by a small difference in soil pH between the controls of the experiments.

Ag-NP exposure concentration had a significant negative effect on the relative abundance of *Firmicutes* (F(1,45)=14.6, p<0.001) and *Patescibacteria* (F(1,45)=32.7, p<0.001) with relative abundance of these taxa reduced to 9.2% (±SD 2.0) and 0.02% (±SD 0.02) at the highest NP treatment, respectively. Ag-NP exposure concentration had a significant positive effect on the relative abundance of *Bacteroidetes* (F(1,45)=36.2, p<0.001) (**Table S4.3**) with the relative abundance of this taxa increased to 35.9% (±SD 3.1) at the highest NP treatment.

Shannon index and species richness of the control soils were 6.1 (±0.2) and 840 (±275) (**Table 4.6**). Both diversity indices were negatively related to NP concentration (Shannon: $F(1,45)=37.8$, $p<0.001$; richness: $F(1,45)=10.370$, $p<0.01$) with Shannon index and species richness being 0.67 and 283 lower at the highest NP treatment than at the negative controls. Soil silver concentration had a significant effect on the soil bacterial community at the ASV level (Db-RDA: $F(1,59)=7.744$, $p<0.01$). Soil pH was also significantly related to soil bacterial community composition (Db-RDA: $F(1,59)=7.770$, $p<0.01$). NMDS indicated that the highest NP concentrations and ionic treatment clustered away from the controls, the two lowest NP concentrations and the carrier control (**Figure 4.5C**).

Table 4.6: Mean Shannon index and ASV richness (± SD) per treatment group in the soil and gut microbiomes at 28 day of the silver experiment.

Treatment	Nominal Ag conc. (mg/kg	Soil		Gut	
		Shannon	Richness	Shannon	Richness
Negative ctrl.	**0**	6.1 (± 0.2)	840 (± 275)	2.2 (± 1.0)	108 (± 37)
NP	**10**	6.0 (± 0.3)	721 (± 199)	1.4 (± 0.6)	39 (± 14)
NP	**26**	6.1 (± 0.3)	896 (± 306)	2.6 (± 1.1)	79 (± 30)
NP	**64**	5.8 (± 0.1)	714 (± 95)	1.6 (± 0.7)	41 (± 14)
NP	**160**	5.6 (± 0.3) *	603 (± 125)	2.1 (± 1.0)	72 (± 43)
NP	**400**	5.4 (± 0.2) *	557 (± 117)	1.6 (± 0.8)	54 (± 16)
Ionic ctrl.	**160**	5.5 (± 0.2) *	612 (± 92)	2.1 (± 1.1)	72 (±40)
Carrier ctrl.	**NA**	6.0 (± 0.3)	699 (± 129)	3.1 (± 0.9)	111 (± 77)

* Asterisk indicates statistical significance with negative control based on Tukey's HSD test at $\alpha = 0.05$.

The number of differentially abundant ASVs was correlated with NP concentration (Pearson's $r=0.903$, $p<0.01$) with 11.8% of ASVs being affected by treatment at the highest NP concentration (**Table 4.5**). A majority of the differentially abundant ASVs in the ionic control were also differentially abundant in the two highest NP treatments (**Figure S4.1**). Log-logistic models could be fitted for 389 ASV. Only 57 of these models showed a significant negative concentration response relation and allowed calculation of an ASV specific EC_{50}. The calculated HC_5 (the hazardous concentration for 5% of the taxa) amounted to 22.6 (±SE 2.4) mg Cu/kg (**Figure 4.6**). The most Ag-NP sensitive taxa are listed in **Appendix 2D**.

4.3.7 Effects of silver treatments on the E. fetida resident midgut microbiome

At the phylum level, the gut community composition of the controls in the silver experiment was dominated by *Proteobacteria* (69.8%±SD 26.6), followed by *Tenericutes* (10.2%±14.9), *Bacteriodetes* (7.6%±16.1), *Firmicutes* (5.7%±4.2), *Spirochaetes* (2.9%±1.7) *Actinobacteria* (2.1%±1.8) and *Verrucomicrobia* (1.5%±2.5) (**Figure 4.4D**). There were no significant differences in the relative abundance of these phyla between the controls from the copper experiment and the

silver experiment (**Table S4.2**). At the ASV level, the gut bacterial community from control animals was dominated by nine ASV comprising 74% of the total community. These bacterial taxa belonged to the genera and species *Aeromonas* (ASV 7048, 7034 and 7052), *Verminephrobacter* (ASV 16376), *Cellvibrio* (ASV 16868) and '*Candidatus* Lumbricincola' (ASV 24867), and the *Spirochaetaceae* family (ASV 2976) (**Figure S4.3**). As in the copper controls, most of these gut-associated taxa were either absent from the respective soil microbial communities or around the limit of detection (**Appendix 2E**).

No statistically significant differences in the bacterial load between treatments were observed ($X^2(4)=1.51$, $p=0.825$) (**Figure 4.7**). Diversity indices were not affected by any of the treatments (Shannon: $F(1,51)=1.279$, $p=0.26$; richness: $F(1,51)=0.148$, $p=0.702$) with an average Shannon index and species richness of 2.1 (±1.0) and 72 (±41) across all treatments (**Table 4.6**). Also at the ASV level, silver concentration had no statistically significant impact on the *E. fetida* gut microbiome composition (Db-RDA: $F(1,46)=1.689$, $p=0.081$) (**Figure 4.5D**). Linear modelling indicated that none of the most dominant ASVs (taxa with relative abundance of >2% in any of the treatments) were significantly affected by NP treatment (**Appendix 2F**).

No concentration dependent response in the number of differentially abundant ASVs was recorded (Pearson's $r=0.628$, $p=0.131$) (**Table 4.5**). In addition, a significant negative log-logistic model could not be fitted for any of ASVs and therefore no HC_5 value for the *E. fetida* microbiome could be derived.

4.3.8 Effects of TiO_2-NP on soil and E. fetida microbial communities

The TiO_2-NP exposure negatively affected the relative abundance of *Acidobacteria* (from on average 2.3% to 1.6%) ($F(1,12)=6.16$, $p<0.05$) and *Verrucomicrobia* (from 6.5% to 4.8%) ($F(1,12)=5.00$, $p<0.05$) compared to the negative control (from the silver experiment) (**Figure S4.4**). However, no significant effect of TiO_2-NP addition on the microbial diversity indices was found (Shannon: $F(1,12)=3.721$, $p=0.078$; richness: $F(1,12)=1.613$, $p=0.22$) and the NMDS showed that TiO_2-NP exposed soils clustered with control soils (**Figure 4.8)**. However, a significant difference with the control soils was found at the ASV level (Db-RDA: $F(1,12)=1.555$, $p<0.05$).

TiO_2-NP exposure did not significantly affect the Shannon index ($F(1,11)=3.688$, $p=0.11$) or the relative abundance of the dominant bacterial phyla in the *E. fetida* gut (1-way ANOVA: $p>0.05$, for all dominant phyla) (**Figure S4.5**). Bacterial richness in the gut of *E. fetida* exposed to TiO_2 was significantly higher (i.e. 231±35) than in the control animals (i.e. 113±97) ($F(1,12)=1.555$, $p<0.05$). Db-RDA analysis indicated a significant difference in the gut bacterial community composition at the ASV level between TiO_2-NP exposed and control animals ($F(1,11)=1.724$, $p<0.05$) (**Figure 4.8**).

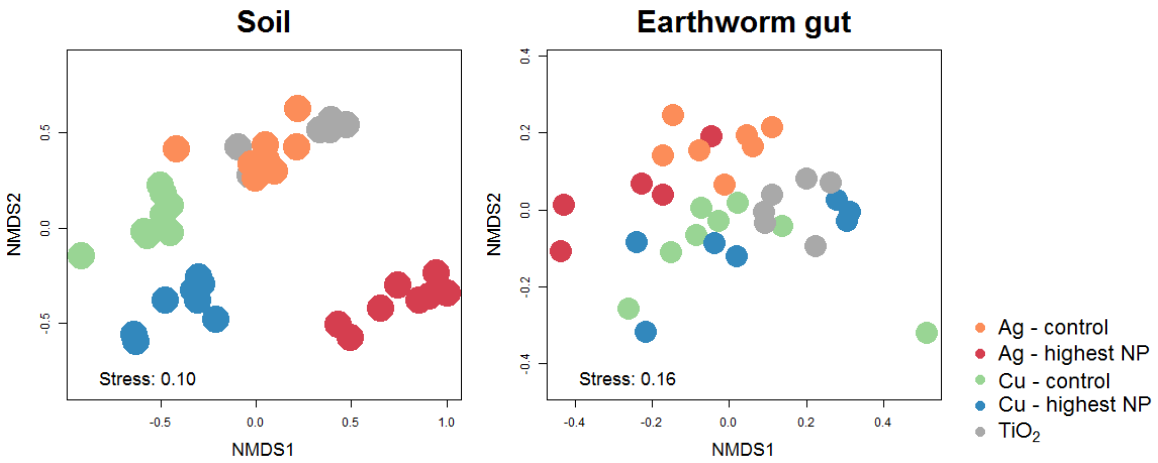


Figure 4.8: NMDS plots showing the ordination of soil (left) and *E. fetida* gut (right) samples derived from control replicates, the highest Ag-NP treatment replicates, highest CuO-NP treatment replicates and TiO2 NP treated replicates.

4.4 Discussion

Microbiomes are essential for the normal functioning of animals. Studying the impact of pollutants on animal microbiomes is therefore crucial to understand the full impact of pollutants on animals (see (Motta et al., 2018). Microbiomes are often characterized by high temporal, spatial and individual variability (Falony et al., 2016; Thompson et al., 2017; Wong et al., 2013) therefore sufficient replication across a concentration range is crucial to elucidate patterns of change (Harris et al., 2014; Prosser, 2010; van Gestel & Selonen, 2018). In this paper, we studied the effects of two biocidal NPs and ionic, carrier and particle controls on the soil bacterial community and the resident gut microbiome (the bacterial community that remains in the gut after all gut contents have been cleared through depuration and rinsing) of the earthworm *E. fetida* across concentrations and with high replication.

4.4.1 Ag-NPs are more toxic to the soil bacterial community than CuO-NPs

Concentration dependent effects of both the CuO-NPs and Ag-NPs on the soil bacterial community were found. Multivariate analysis, differential abundance analysis and SSD analysis indicated that Ag-NPs had a greater effect on the soil bacterial community than CuO-NPs, in line with previous studies (Bondarenko et al., 2013; Garner et al., 2015). Moreover, ionic silver and ionic copper were more toxic to the soil bacterial community than their NP counterparts, as previously reported (Rousk et al., 2012; Zhai et al., 2016). In both experiments, multivariate analysis indicated that the metal salt controls clustered closely with the two highest NP treatments, indicating that the effects observed in these treatments are most likely related to the cations and not the anions of these metal salts.

In the silver experiment, soil pH and soil Ag concentration were correlated and both variables were significantly related to community structure. Both Ag and pH are known drivers of the soil bacterial communities (Colman et al., 2013; Doolette et al., 2016; Griffiths et al., 2011; Rousk et al., 2010). Due to the correlation between these two variables, it is however difficult to determine which variable is responsible for the changes in bacterial community structure observed in this study. In most natural soils, pH starts affecting bacterial community composition most drastically below a pH 5 (Griffiths et al., 2011; Rousk et al., 2010). The pH of soils in the silver experiment range between 5.6 and 6.3. In addition, the pH in the carrier control (i.e. 5.9) was also higher than in the negative control (i.e. 5.6). However, this increase of pH was not reflected in changes in the bacterial community composition between these two treatments (**Figure 4.5**). Based on these observations, we hypothesise that most of the changes in the soil bacterial community structure are driven by Ag. However, an effect of pH cannot be excluded.

Ag-NP concentration had a positive impact on the relative abundance of *Bacteroidetes* and a negative impact on the relative abundance of *Firmicutes* and *Paterscibateria*. A positive relation between Ag-NPs and *Bacteroidetes* has also previously been reported by others (McGee et al., 2017, 2018; J. Wang et al., 2017). However, insights into which phyla are sensitive to Ag-NPs are divergent, with some studies indicating these to be *Firmicutes* (Asadishad et al., 2018; Xiao et al., 2017) and others *Acidobacteria*, *Verrucomicrobia* and *Plantomycetes* (G. Liu et al., 2017; McGee et al., 2017, 2018; J. Wang et al., 2017). CuO-NP exposure had a positive effect on the relative abundance of *Proteobacteria* and *Actinobacteria* and a negative effect on *Verrucomicrobia*, *Firmicutes, Acidobacteria* and *Patescibacteria*. Experimental studies investigating effects of copper forms on soil microbial communities have mostly focussed on enzymatic activities (Simonin et al., 2018), although some studies have looked at structural endpoints (Azarbad et al., 2015; Berg et al., 2012; J. Li et al., 2015; Rocca et al., 2019). These studies indicated the copper sensitivity of *Acidobacteria* (Frenk et al., 2013; J. Li et al., 2015; Sun et al., 2016) and *Verrucomocrobia* (Azarbad et al., 2015; Berg et al., 2012) and the copper tolerance of *Actinobacteria* (Frenk et al., 2013; J. Li et al., 2015; Rocca et al., 2019), which is consistent with our observations. The lack of further identifiable sensitive phyla may be linked to the large functional diversity in copper homeostasis within bacterial clades (Hernández-Montes et al., 2012). Further, we did not measure the total bacterial abundance in the soils. Therefore, the changes in the relative abundance of some bacterial phyla may in fact be driven by an increase or decrease in the total bacterial abundance of another bacterial group.

Addition of TiO_2-NP to soils had a significant negative impact on the relative abundance of *Verrucomicrobia* and *Acidobacteria* in the soil community. Previous studies have shown that TiO_2-NP amendments to soils can have a negative impact on the abundance of nitrifying bacteria and soil nitrification (Simonin et al., 2016; Zheng et al., 2011), as well the total bacterial abundance (Ge et al., 2011) and community structure (Ge et al., 2011; Moll et al., 2017; Simonin et al., 2016), even at low, environmentally realistic doses (Simonin et al., 2016). The reduction of the relative abundance of *Acidobacteria* as reported here, has also been demonstrated in other studies (Simonin et al., 2016), however other reports also indicate the sensitivity of *Actinobacteria* and *Chloroflexi* to TiO_2-NPs (Moll et al., 2017). Even in the absence of light, TiO_2-NPs can still induce cellular oxidative stress, possibly due to the disruption of membrane integrity by the particles, which may explain the effects on the relative abundance of some bacterial phyla (Adams et al., 2006; Gurr et al., 2005). Others have suggested that physical interactions between TiO_2-NPs and soil particles can change soil structure and thereby alter the microbial community (Moll et al., 2017). The NMDS analysis indicated that the effects of CuO-NPs and Ag-NPs on the soil bacterial community are greater than the effects of the TiO_2-NPs (**Figure 4.8**). Exposure to the TiO_2-NPs at a particle concentration equivalent to the highest CuO-NP treatment, cannot therefore explain the effects recorded in the CuO-NP and Ag-NP treatments. This may indicate that the effects of the CuO-NP and Ag-NP on the bacterial communities are not related to physical interactions between the NPs and the bacteria. However, the shape and size of the TiO_2-NPs were different to those of the CuO-NP and Ag-NP. Previous studies indicate that different shapes of NP can have different effects on bacteria (Zhai et al., 2016). Therefore, it cannot be ruled out that some of the effects in CuO-NP and Ag-NP on the bacterial communities are linked to the physical interaction with the NPs. In both experiments, multivariate analysis as well as differential abundance analysis, indicated that the ionic control treatments had similar effects on the bacterial community as the two highest NP treatments. Therefore, it is likely that in this case the effects on the bacterial community are largely driven by ionic toxicity.

We used SSD models to calculate HC_5-values for soil bacterial communities. SSD models are commonly used in environmental risk assessment (Posthuma et al., 2001) and conventionally use EC_x or no observed effect concentrations (NOEC) derived from single species toxicity assays. A significant limitation of SSD modelling based on endpoints derived from single-species toxicity assays is that it treats species as separate independent entities. In field communities, however, each species is part of a network of species that interact with each other and so SSD modelling based on such single-species data does not take into account the complexity of ecological communities (Forbes & Calow, 2002; van den Brink et al., 2001). SSD models are however also

applied using endpoint-values derived from (semi-) field experiments. Such field-SSD modelling approaches may provide a more realistic insight into the effects of chemicals on field populations. Recently this approach has been extended to microbiome metabarcode data (Doolette et al., 2016; Yang et al., 2017, 2018). A major challenge for such microbiome data is that these datasets are compositional (Gloor et al., 2017). This means that a change in the actual total abundance of one ASV can influence the relative abundance of other ASVs. The impact of the compositionality on microbiome data analysis is largely dependent on the number of species and the evenness of the community and can be expressed by the Inverse Simpson index (Weiss et al., 2016). The Inverse Simpson index for the soil bacterial communities after rarefication were 248 and 164 in the copper and silver experiments, respectively, which indicates that the effects of compositionality are likely to be relatively small (H. Li, 2015). SSD modelling using microbiome data has the potential to improve the environmental risk assessment of chemicals for the soil microbial communities. However, we recognize that this approach needs further development and validation and therefore the results derived from microbiome SSD modelling should be interpreted with care.

Yang et al.(2018) studied effects of copper on sediment microbiomes and found an HC_5 value (i.e 76 mg/kg) derived from SSD modelling, close to the value reported here (i.e. 44.0 mg/kg). An SSD model derived HC_5 value for Ag-NPs in soils found by Doolette et al.(2016) is, however, two magnitudes lower than the reported value here (i.e. 22.6 mg/kg). Doolette and colleagues, however, excluded all taxa that did not show a negative concentration response from HC_x calculations resulting in a negative bias for the HC_5 value that explains the difference with the value reported in here. Current predicted modal environmental concentrations for Ag-NPs in soils are 7-5000 ng/kg depending on the exposure and fate scenarios (Giese et al., 2018). The HC_5 value for Ag-NPs reported here is, therefore, three orders of magnitude higher than the highest current predicted environmental concentration (PEC), suggesting a negligible risk at current release rates at large-scale. However, most predictive models do not take local points sources into account and so concentrations in some environments can be higher than these predicted values and may reach effect thresholds. Modelled environmental concentrations for CuO-NPs have been predicted to be in the ppb range (Garner et al., 2017). This assessment was based on sewage sludge addition as the main route to soil. Direct application as pesticides may be an important future release mode (Keller et al., 2017) and it is known that the use of conventional copper as a fungicide has resulted in significant soil pollution (e.g. in vineyards). Total copper concentrations in soil in the European Union is on average 16.68 (±SD 21.9) mg/kg, but can reach 91.29 mg/kg in some agricultural regions where Cu-based fungicides may be applied (Ballabio et al., 2018). Environmental concentrations of

copper in many cases thus exceed the HC_5 for CuO-NPs reported here, which implies that soil bacterial communities may be at risk due to copper exposure.

4.4.2 E. fetida microbiome buffered from effects of metal exposure

The earthworm gut bacterial community is composed of both transients (Zeibich et al., 2019b) (associated with ingested soil and food) as well as bacteria closely associated to the gut wall (Singleton et al., 2003; Thakuria et al., 2010). In this study, the midgut of *E. fetida* was cleared of the gut content to remove transient bacteria. Results here suggest that the resident gut bacteria are much less sensitive to soil copper and silver concentrations than soil bacteria.

Previous research on the effects of biocidal NPs on host microbiomes give contrasting results, as reviewed elsewhere (Bouwmeester et al., 2018; Jin et al., 2017; Rosenfeld, 2017), with some studies indicating a clear negative effect of biocidal NPs on host microbiomes (Chen et al., 2017; Williams et al., 2015) and other studies indicating towards an absence of effects (Javurek et al., 2017; Wilding et al., 2016). Cases of higher stability of host associated microbiomes compared to surrounding free living microbes has also previously been reported in corals (Glasl et al., 2019) The lack of clear biocidal effects on the *E. fetida* gut microbiome is still somewhat surprising given the high biocidal potential of the tested NPs as shown by the impacts on the soil community

The lack of toxicity of biocidal NPs towards gut microbes may be linked to the specific environmental conditions in the earthworm gut. The relatively neutral pH in the earthworm intestinal tract, for example, may slow down NP dissolution (Sekine et al., 2017) and reduce exposure of gut microbes to toxic ions. The earthworm gut is also a nearly anoxic environment (Horn et al., 2003). In such anoxic conditions, silver and copper, in both nano-particulate and ionic form, may rapidly undergo chemical transformations like sulfidation (Gogos et al., 2017; Hashimoto et al., 2017), leading to the production of less toxic metal species (Levard et al., 2013). The reduced impact of silver or copper forms could further be driven by the interaction of these metals with drilodefensins. Earthworms produce and release these sulfonated metabolites in high quantities into the gizzard (drilodefensins can make up ~1.3% of earthworm dry weight biomass) which protects the earthworm against humic acids by acting as a surfactant (Liebeke et al., 2015). The interaction of these sulfonated surfactants with silver and copper metals and the resulting impact on toxicity warrants further investigation. Finally, the intestine of earthworms is covered by numerous folds. The embedment of bacterial cells into these intestinal folds and their physically attached to host tissue may further reduce exposure of these embedded bacterial cells to chemical stressors.

4.4.3 Soil copper concentration negatively affects some resident bacterial taxa

Although generally, effects of CuO-NPs and Ag-NPs on the *E. fetida* gut microbiome are smaller than on the soil community, copper exposure did negatively affect the relative abundance of some gut bacterial taxa. These changes occur at lower concentrations than effects on *E. fetida* reproduction becomes apparent, meaning that the responses of microbiomes can be used as a sensitive indicator for effects of chemicals on soil invertebrates, as previously reported by others (L Ma et al., 2017; Zhu et al., 2018a). We found no significant effect of treatment on the total bacterial load in the *E. fetida* gut (**Figure 4.7**). Therefore, the changes in the relative abundance of some ASVs are likely the results of changes in the actual overall abundance of these ASVs. The pattern of change may be the result of the higher sensitivity of some taxa towards copper, but could also be driven by changes in competition between microbes. The exact mechanisms that drive the changes in the *E. fetida* gut microbiome in the copper treatments thus remain unclear and require further investigation. Full genome analysis of the affected taxa may help to explain patterns of differential sensitivity towards copper.

ASVs that responded negatively to copper treatment were '*Candidatus* Lumbricincola' (*Mollicutes*), a member of the *Spirochaetaceae* family (*Spirochaetes*) and an ASV belonging to the genus *Aeromonas*. '*Candidatus* Lumbricincola' is closely related to the woodlouse symbiont '*Candidatus* Hepatoplasmata' (Nechitaylo et al., 2009). In woodlice this symbiont likely plays a role in the degradation of cellulose (Fraune & Zimmer, 2008; Y. Wang et al., 2004). Recent studies show that the relative abundance of 16S-rRNA gene of the earthworm symbiont '*Candidatus* Lumbricincola' in bio-reactors increases upon supplementation with chitin and xylan suggesting a possible role of this symbiont in the metabolism of these polysaccharides (Zeibich et al., 2019a). The metal sensitivity of earthworm gut associated *Mollicutes* has also been demonstrated by others (Šrut et al., 2019) indicating a general sensitivity of these earthworm symbionts to metal pollutants. *Spirochaetaceae* are well known earthworm associated microbes. Although mostly described as a vertically transmitted nephridial symbiont (Davidson et al., 2006) some studies indicate presence in the gut (Pass et al., 2015; Procházková et al., 2018; Swart et al., 2020). *Spirochaetaceae* are mostly described as pathogens, however, they can also act as beneficial symbionts as well (Haselkorn et al., 2013). For example, bacteria belonging to the *Spirochaetaceae* genus *Treponema* present in the hindgut of termites and are part of a lignocellulose metabolic network (Brune, 2014; Köhler et al., 2012; Rosenthal et al., 2011). The role of *Spirochaetaceae* microbes in the earthworm is, however, unknown. *Aeromonas* (or *Aeromonadaceae*) have frequently been reported to be associated to the earthworm gut (Byzov et al., 2009; Rieder et al., 2013; Zeibich et al., 2019a). Roles of these symbionts are unknown, although recent studies indicate that earthworm gut *Aeromonadaceae* are

involved in anaerobic digestion of polysaccharides (Zeibich et al., 2019a). The TiO_2-NP treatment did not lead to the loss of the abovementioned taxa. In contrast, exposure to ionic copper did negatively affect these taxa. Therefore, it seems likely that the effects of the CuO-NP are due to the toxicity of the released ions and not due to the physical interaction of the NPs with the resident gut microbiome. However, as the shape and size of the CuO-NPs were different to the TiO_2-NP control, we cannot fully rule out that some of the effects of the CuO-NPs are due to the physical interactions with resident gut bacteria. The loss of symbionts occurs at total copper concentrations frequently reported in agricultural fields (Ballabio et al., 2018), indicating that specific *E. fetida* symbionts may be at risk due to copper exposure.

4.5 Conclusions

Agricultural practices such as biocide application may pose a threat to free-living microbes as well as host-associated microbes, which can provide crucial ecosystem services. This study demonstrates that soil bacterial communities can show a dose dependent response to exposure to biocidal NPs. In the case of CuO-NPs, many soil bacteria are negatively affected at total copper concentrations frequently observed in the environment. Given the widespread application of biocides in agricultural landscapes, these results indicate the need for further system wide investigations into the effects of biocides on soil microbial communities. We show that the *E. fetida* resident gut microbiome is comparatively resilient to exposure to both CuO-NPs and Ag-NPs. The effects of pollutants on bacterial communities are, thus, system specific. Despite the overall resilience of the resident *E. fetida* gut microbiome, some core symbionts are sensitive to CuO-NP and are negatively affected at concentrations lower than those at which adverse effects on the host become apparent, indicating that microbiomes can be used as sensitive endpoints for the effects of pollutants on invertebrates. In this paper we have characterized the effects of biocides on the composition and the structure of the *E. fetida* gut microbiome and surrounding soil bacterial communities. Future studies should focus on enhancing our understanding of the functionality of the earthworm gut microbiome and the impact of pollutants on microbiome provided functions. Defining the effects of pollutants on microbiome structure, as reported here, can form the basis for such work.

5

The effects of *in vivo* exposure to copper oxide nanoparticles on the gut microbiome, host immunity and susceptibility to a bacterial infection in earthworms

5.1 Introduction

Nanomaterials (NMs) are increasingly used in various applications including surface coatings, biocide pesticides and electronics (Keller et al., 2013; Sun et al., 2016). The potential risks of NMs to human health and the environment have long been identified (Colvin, 2003; Handy et al., 2008; Moore, 2006). Over the last decade, research has provided vast amounts of toxicity data that have reduced many of the initial uncertainties around NM risk. There are, however, still some aspects that need further investigation. One of these remaining issues relates to the immuno-safety of NMs (Boraschi et al., 2011, 2020; Dobrovolskaia & McNeil, 2007). Owing to their particulate nature, NMs have an increased potential to interact with the innate immune system of organisms (Alsaleh & Brown, 2018; Boraschi et al., 2017; Fadeel, 2019; Pallardy et al., 2017) and to induce both pro- and anti-inflammatory responses (Bhattacharya et al., 2017). Most of the current research to investigate such effects has used *in vitro* models to characterize NM-immune interactions. Although these studies have provided crucial information on how immune systems may interact with NMs, it remains unclear how responses *in vitro* will translate to *in vivo* effects. Further, immuno-modulation by NMs does not necessarily indicate that the immune system is being compromised. In fact, immune reactions are part of a healthy response by the host towards foreign objects. In order to assess whether NMs actually compromise host immunity, co-exposure with infectious pathogens are necessary (Boraschi et al., 2011, 2020).

A major application of NMs is as antimicrobial agents in pesticides and coatings. Effects of biocidal NMs on soil microbial communities have been relatively well studied (Courtois et al., 2019; McKee & Filser, 2016; Simonin & Richaume, 2015). There are, however, uncertainties concerning the impact of NMs on microbes that are associated with plants and animals (commonly referred to as 'microbiome') (Judy et al., 2015). Common roles of the microbiome in host health include the provision of essential nutrients and aiding in digestion, whilst the role of the microbiome in host immunity is now increasingly recognized (Brestoff & Artis, 2013; Buffie & Pamer, 2013; Nyholm & Graf, 2012). Microbes associated to mucosal surfaces can contribute to immunity by providing resistance against invading pathogens (Cirimotich et al., 2011; Dillon et al., 2005; Koch & Schmid-Hempel, 2011) and by stimulating the release of antimicrobial peptides by the host (Kwong et al., 2017). Disruption of the healthy microbiome by, for example, chemical exposure, can lead to reduced immune functioning and reduced survival of bacterial infections (J.K Kim et al., 2015; Motta et al., 2018; B.L. Weiss et al., 2012). Previous studies have shown that when NMs alter the microbiome of animals, the expression of host immune genes can also change (Auguste, Balbi, et al., 2019; Auguste, Lasa, et al., 2019; H. Chen et al., 2017; Williams et al., 2015). Host immunity and the microbiome are thus a complex and integrally linked system and it is therefore important to

include microbiome analysis in the immuno-safety assessment of NMs. Invertebrate animals such as earthworms, bivalves and sea urchins, provide a suitable models to study *in vivo* effects of NMs on immune functioning under more realistic environmental conditions (Alijagic & Pinsino, 2017; Auguste, Lasa, et al., 2019; Hayashi, Heckmann, et al., 2013).

Earthworms provide crucial ecosystem services in soils through mixing, organic matter degradation of plant material and nutrient cycling, and thereby contribute to enhanced crop production (Edwards & Bohlen, 1996). Living in soil, earthworms inhabit an environment with high microbial activity. To provide protection from pathogens, cellular immunity in earthworms is provided by immune cells called coelomocytes, which circulate the coelomic cavity. A crucial element of the innate immune system is the recognition of microorganism associated molecular patterns (MAMPs) by host pathogen recognition receptors (PRRs). A well described PRR in earthworms is coelomic cytolytic factor (CCF). This PRR, upon binding to specific MAMPs, induces the prophenoloxidase pathway, which ultimately leads to the production of antimicrobial factors (Beschin et al., 1998; Bilej et al., 2001; Šilerová et al., 2006). Earthworm pathogens are also controlled by various humoral factors. One of these is lysozyme, an enzyme that can hydrolyse components of the cell wall of Gram-positive bacteria (Josková et al., 2009). In the earthworms *Eisenia fetida* and *Eisenia andrei,* immunity is also supported by the humoral factors lysenin (Sekizawa et al., 1996) and fetidin (Cooper & Roch, 2003; Lassegues et al., 1997), the modes of antibacterial action of which are not fully understood (Bruhn et al., 2006; Lassegues et al., 1997). Recent work shows that changes in gene expression of these immune factors can be used as a marker of immune-modulation in earthworms (Dvořák et al., 2013, 2016; Josková et al., 2009). *In vitro* studies have shown that exposure to NMs can also alter the expression of earthworm immune genes (Hayashi et al., 2012, 2016). Effects of *in vivo* exposure to NMs earthworm immune system regulation have not been studied extensively (Hayashi, Heckmann, et al., 2013) and it remains uncertain whether NM exposure can compromise earthworm immunity by affecting the host susceptibility to infections.

The earthworm gut microbiome has been relatively well described. Gut communities have been identified as being composed of both transient bacteria associated with ingested soil and food (Zeibich et al., 2019a) and resident bacteria more closely associated to intestinal surfaces (Singleton et al., 2003; Thakuria et al., 2010). Loss of some core earthworm symbionts can lead to reduced host fitness and juvenile development (Lund, Holmstrup et al., 2010; Viana et al., 2018). Environmental pollutants can alter the microbiome of earthworms (L. Ma et al., 2017; Šrut et al., 2019; Yausheva et al., 2016) and lead to the loss of core symbionts important to host health (Pass et al., 2015). An increasing body of literature now indicates that also exposure to NMs can disrupt the microbiome of soil invertebrates (J. Ma et al., 2019, 2020; B.L. Zhu et al., 2018). In the

earthworm *Enchytraeus crypticus*, for example, exposure to CuO-NPs can significantly reduce the abundance of core intestinal Plantomycetes bacteria (J. Ma et al., 2019). The interplay between microbiome, NMs and host immunity in earthworms has however not been studied. Disruption of host-microbiome interactions can be expected for chemicals that are designed to target microbes (biocides). In agriculture, copper based NM formulations are being developed for biocidal applications (Keller et al., 2017). In widespread application there is therefore the potential for such NMs to negatively affect earthworms through alterations to their microbiome structure.

This paper aims to study whether a pre-exposure to copper oxide nanoparticles (NPs) affects the susceptibility of the earthworm *E. fetida* to a bacterial infection. *E. fetida* were exposed in soil to concentrations of copper forms known to alter the *E. fetida* gut microbiome for a duration of 28 days. The *E. fetida* were subsequently removed from soils and challenged with the bacterium *Bacillus subtilis* for a further four days. Effects of the bacterial challenge were assessed by looking at survival, tissue damage and by measuring mRNA levels of known immune markers. An analysis of the gut microbiome was concurrently conducted through a metabarcoding approach to link effects on microbiomes to immune responses. Effects of NPs were compared to those of metal salts, to test whether any effects were attributed to particles or ions. It is hypothesized that (i) *E. fetida* that have their microbiome changed through exposure to CuO-NPs and copper salts are more susceptible to a bacterial infection. And that (ii) exposure to CuO-NPs, copper salts and the proceeding bacterial challenge will have an effect on the gene expression of tested immune markers, in line with previous studies (Dvořák et al., 2016; Mincarelli et al., 2019).

5.2 Materials and Methods

5.2.1 Test organism, test chemicals and soil spiking

Eisenia fetida were reared at 20°C in a medium consisting of loamy top soil, composted bark and garden compost in 1:1:1 ratio by volume basis. *E. fetida* were fed with field collected horse manure from horses grazing on unpolluted pastures and free from recent medical treatment. All *E. fetida* used in the experiment had a stripe patterned outer body characteristic of *E. fetida* with fully developed clitella and were within a weight range between 300 and 600 mg.

Molecular grade $CuCl_2 \cdot 2H_2O$ was supplied by Sigma-Aldrich (Poole, UK). CuO-NPs were manufactured by Promethean Particle Ltd (Nottingham, UK) and were dispersed in water. Nanoparticles were cuboid in shape, with a stated mean dimension of 20 by 50 nm. Size distributions of NPs were determined with nanoparticle tracking analysis using a Nanosight (Malvern Instruments, Salisbury, UK). Derived mean and modal dimensions were 183 nm (±SE 5.2).

Zeta potential of CuO-NPs (33mV ±SD 0.3mV) were determined using phase analysis light scattering using a Malvern Zetasize Nano ZS.

All exposures in soil were conducted in LUFA 2.2 natural soil (LUFA-Spreyer, Germany), a sandy loam soil widely used in ecotoxicological testing. Test soils were spiked with a nominal concentration of CuO-NPs (160 mg kg^{-1} d.w. soil) or $CuCl_2$ (160 mg kg^{-1} d.w. soil) or a negative control (0 mg kg^{-1} d.w. soil). Test concentrations were based on a previous study that showed changes in the microbiome structure and loss of core symbionts at these copper concentrations (**Chapter 4**). CuO-NPs treated soils were spiked one day before the initiation of the exposure. $CuCl_2$ treated soils were spiked five days before the start of the exposure to allow the metal speciation in the soil to reach a quasi-equilibrium (Smit & Van Gestel, 1998). A control consisting of the liquid carrier of the CuO-NP dispersion was not included as previous work has established that the liquid carrier of this NP dispersion is not toxic to *E. fetida* and does not alter the *E. fetida* microbiome structure (**Chapter 4**).

Mixing of the chemicals with the soil was done for each treatment following Waalewijn-Kool et al. (2012). Briefly, the total amounts of CuO-NPs and $CuCl_2$ required for all replicates were dissolved in 60 ml of de-ionised water. These stock solutions were then each mixed with 250 g of d.w. LUFA 2.2 soil using a spatula. The mixture was then thoroughly mixed with the remaining soil and subsequently wetted with de-ionised water to reach 55% of the water holding capacity (WHC) before final mixing. The soil mixtures were divided into replicates each consisting out of a 60 g w.w. aliquot in a 100 ml clear plastic round tub.

5.2.2 Overview of the experimental design

E. fetida were initially exposed to a pre-treatment of either CuO-NPs, $CuCl_2$ or a negative control for 28 days after which time they were removed from soil and challenged with either *Bacillus subtilis* or a negative control for 4 days (**Figure 5.1**). Up to date, no true earthworm bacterial pathogen has been described in literature. Previous studies have shown that exposure to a high dose of various soil bacteria can induce immune responses in earthworms and lead to an infection which can result in the death of infected earthworms (Dvořák et al., 2013; 2016). To identify the best bacterium to use for the bacterial challenge, a pilot experiment was conducted in which the growth curves of three different bacteria common in soils (i.e. *B. subtilis*, *B. thuringiensis* and *Citrobacter rodentium*) were measured by culturing these bacteria in a medium containing coelomic fluid of *E. fetida*. The results showed that the coelomic fluid had inhibiting effects on the growth of *B. subtilis*, possibly indicating an effective immune response by cellular or humoral components (**Figure 5.2**). This pilot experiment was conducted by Dr. Petra Procházková at the Institute of Microbiology (Prague, Czech

Republic). After the bacterial challenge, *E. fetida* were returned to their original soil for recovery for another 28 days during which time they were periodically sampled for analysis. In total six replicates were prepared for each treatment per time point, except at 'bacterial challenge day 4' for which only a single replicate per treatment was prepared. Details of each of the three experimental steps are described below.

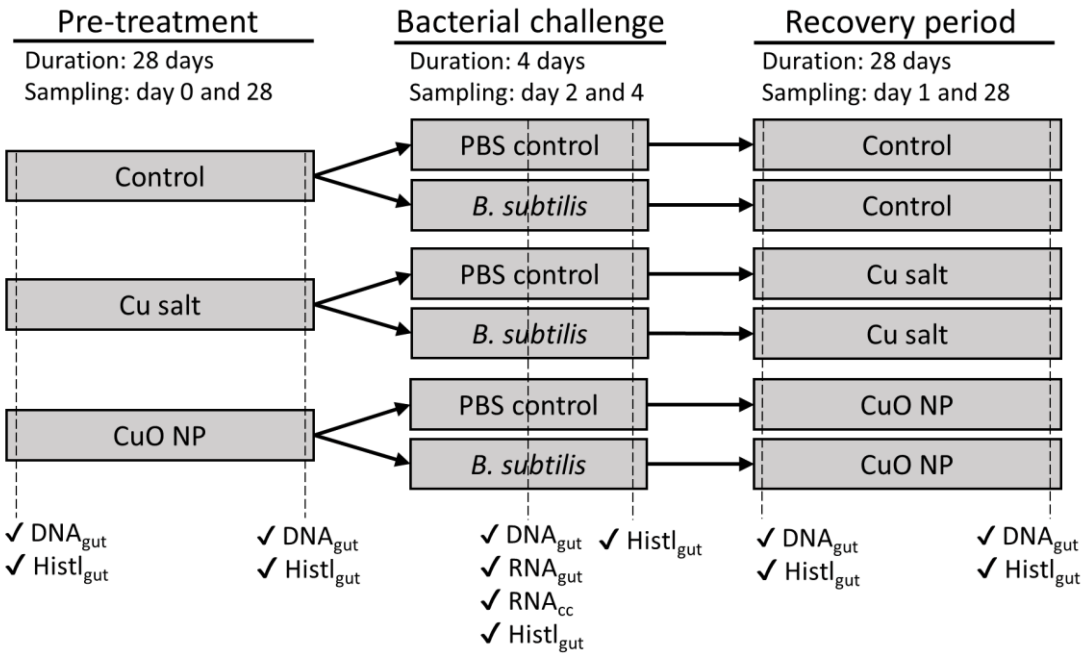


Figure 5.1: Schematic overview of the study design and collected samples. Dashed line indicates sampling point. Tick marks indicate the samples collected at the respective sampling point. 'DNA_{gut}' indicates sampling of DNA from gut tissue for microbiome analysis, 'RNA_{gut}' and 'RNA_{cc}' indicate sampling of RNA for gene expression analysis from gut tissue and coelomic fluid respectively, '$Histl_{gut}$' indicates sampling of gut tissue for histological analysis. NP, nanoparticle, PBS, phosphate buffered saline.

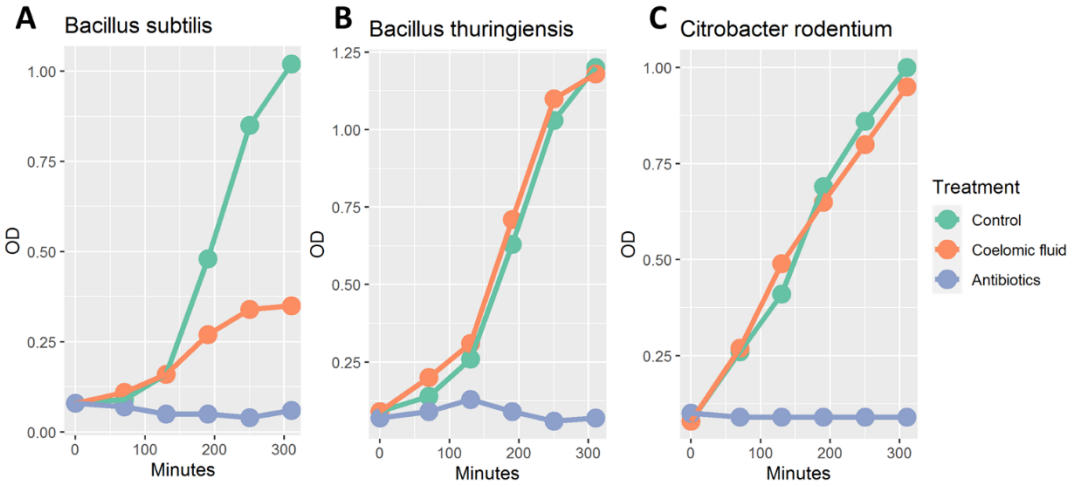


Figure 5.2: Growth over time of (**A**) *Bacillus subtilis*, (**B**) *Bacillus thuringiensis* and (**C**) *Citrobacter rodentium* in LB medium (i.e. ' Control'), filter sterilized culture medium consiting out of 800 µl LB medium and 200 µl 10x diluted coelomic fluid ('Coelomic fluid') or culture medium containing both diluted coelomic fluid and antibiotics ('Antibiotics'). In the antibiotics treatments, Kanamycin (1µM) was used for *B. thuringiensis* and *B. subtilis* and Streptamycin (1 µM) was used for *C. rodentium*.

5.2.3 Pre-treatment exposure

Prior to the start of the pre-treatment exposure, *E. fetida* were acclimatized to LUFA 2.2 soil for two weeks under the same conditions as the main exposure assay. Before exposure initiation, *E. fetida* were rinsed and weighed. To start the test, one adult *E. fetida* was added to each test replicate. The pre-treatment exposure was conducted at 20°C for 28 days. Once a week, each replicate received 0.5 g of spiked horse manure on a d.w. basis as food. At the end of the exposure, *E. fetida* from all replicates were removed from soil, rinsed and weighed. Collected *E. fetida* were subsequently depurated on wetted filter paper to allow egestion of their gut content for two days before bacterial challenge.

5.2.4 Bacterial challenge

Following depuration, each *E. fetida* earthworm was challenged in a petri dish with either *Bacillus subtilis* (*B. subtilis* subsp. *subtilis*, CCM2217; Czech Collection of Microorganisms, Brno, Czech Republic) or a negative control in a medium consisting of re-wetted paper pellets. The pellets were subsequently re-wetted using either 10 ml of phosphate buffered saline (PBS) containing 5×10^8 *B. subtilis* cells per ml or 10 ml PBS only. *B subtilis* culture used for the challenge were in exponential growth phase at the time of the start of the challenge. Cell concentration was determined by measuring OD_{600}. The bacterial challenge was initiated by placing a depurated *E. fetida* into the prepared Petri dish. The bacterial challenge was conducted at 20°C in dark conditions for four days. Survival was monitored at day one, two and four.

5.2.5 Recovery period

After the bacterial challenge, *E. fetida* were rinsed, weighed and subsequently returned to the original soil exposure replicates to assess response to exposure after challenge. The recovery exposure was conducted at 20 °C and lasted 28 days. *E. fetida* were fed with 0.5 g of d.w. spiked horse manure every week.

5.2.6 Sample points

Six sampling points were used: 'pre-treatment day 0', 'pre-treatment day 28', 'bacterial challenge day 2', 'bacterial challenge day 4', 'recovery period day 1' and 'recovery period day 28' (**Figure 5.1**). At every sampling point, except 'bacterial challenge day 4', gut tissue and coelomic fluid from five animals were collected (see below). At every sampling point, one additional animal was collected for histological analysis (see below). All *E. fetida* collected from soil exposure replicates (i.e. 'pre-treatment day 0', 'pre-treatment day 28', 'recovery period day 1' and 'recovery period day 28') were depurated for two days prior to sampling. *E. fetida* collected at day two of the bacterial

challenge were rinsed in de-ionised water but not depurated and immediately dissected. At the end of the pre-treatment exposure, 10 g w.w. soil was collected for metal analysis.

5.2.7 Sampling of gut tissue and coelomic fluid

Each depurated *E. fetida* was placed in a petri dish containing 500 µl PBS and coelomic fluid was extruded by electrification for 5 seconds using a 4.5 V battery. The mixture of coelomic fluid and PBS was collected and mixed with 500 µl of 2× RNA/DNA Shield (Zymo Research, Irvine, CA, USA) and placed in a lysis tube. The extruded *E. fetida* was subsequently euthanized in pure ethanol and a midgut sample (spanning 20 segments posterior to the clitellum) was collected according to **2.1.1**.

5.2.8 Histological analysis

E. fetida collected for histological analysis were fixed and processed according to Dvorak *et al.* 2016. Briefly, a whole body sample spanning a 10 segment region posterior to the clitellum was fixed in 4% paraformaldehyde overnight, dehydrated and embedded in paraffin. For each sample, three 2 µm sections were cut using a microtome, dried overnight, deparaffinised using xylene, rehydrated and stained using hematoxylin/eosin following Kiernan (Kiernan, 2008). Sections were visually inspected for tissue integrity using a light microscope. Damage to the gut epithelium and chloragogen tissue was scored using an ordinal scoring method with four categories (1. no effects, 2. mild effects, 3. moderate effects and 4. severe effects) following Gibson-Corley et al. (Gibson-Corley et al., 2013). This histological analysis was conducted by Dr. Jiří Dvořák at the Institute of Microbiology (Prague, Czech Republic).

5.2.9 Soil metal measurements

Soil copper concentrations were measured in 130 mg of d.w. soil which was mixed with a 4:1 mixture of nitric acid and hydrochloric acid on a volume basis and digested for seven hours at 150 °C. Copper concentration was determined using atomic absorbance spectrometry at the Vrije Universiteit Amsterdam (the Netherlands). Copper recovery from exposure soils were on average 60.4% and 71.3% for CuO-NP and $CuCl_2$ spiked soils, respectively. Lack of full recovery may be linked to loss of copper during preparation of stocks solutions and due to heterogeneity in the distribution of copper forms in soils.

5.2.10 DNA and RNA extraction and cDNA synthesis procedure

DNA was extracted from gut tissue and soil according to the procedure described in **2.2.1**. RNA was extracted from gut tissue and coelomic fluid using Quick-RNA™ Miniprep Kit (Zymo Research) following the protocol supplied by the manufacturer and included a DNA removal step using DNase. Visual inspection through agarose gel electrophoresis under denaturing conditions verified that the

RNA in all samples was not degraded. Extracted RNA was subjected to a further clean-up using a Clean and Concentrator™-5 kit (Zymo Research). RNA quantity of the cleaned samples was determined using Qubit™ RNA HS Assay Kit (ThermoFisher Scientific, Waltham, MA, USA). Per sample 250 ng of RNA was reverse transcribed to cDNA using Reverse Transcription System A3500 (Promega, Madison, WI, USA) following the standard protocol supplied by the manufacturer.

5.2.11 E. fetida genotyping

Gene expression analysis relies on accurate binding of primers to target genomic regions. Genetic variation in binding sites between different individuals is likely to reduce the efficacy to elucidate patterns of gene expression. Within species genetic diversity in earthworms is high (Anderson et al., 2017; King et al., 2008; Novo et al., 2010). On the basis of cytochrome oxidase I (COI) sequence similarities, two distinct genetic *E. fetida* clades have so far been recognised (Pérez-Losada et al., 2005). To screen whether *E. fetida* used in this experiment were part of single genetic clade, *E. fetida* sampled at day 2 of the bacterial challenge were genotyped by amplification and sequencing of the mitochondrial COI DNA. PCR reactions were set up using forward primer COI_1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and reverse primer HCO_2189 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al., 1994) using One*Taq*® Hot Start polymerase and reaction buffer (New England Biolabs) using the following programme: initial denaturation at 94 °C for 2 minutes followed by 35 cycles of 1. denaturing at 94 °C for 30 seconds, 2. annealing at 47 °C for 30 seconds and 3. extension at 68 °C for 1 minute, followed by a final extension step at 68° for 10 minutes. Amplification of a single fragment was verified through gel electrophoresis. PCR products were cleaned using a QIAquick PCR purification kit (QIAGEN) and DNA quantity was assessed using a Qubit dsDNA HS Assay Kit (ThemoFisher Scientific). 7.5 ng of PCR product was sequenced using Sanger sequencing using 3.2 pg of the forward primer at the University of Birmingham (UK). Sanger sequences were submitted to NCBI BLASTn for taxonomical assignment. Pairwise alignment of sequences was performed using MUSCLE alignment in Geneious 9.1.8. Ambiguous bases and erroneous inserts were manually resolved and low quality ends of sequences were trimmed. The remaining 632 bp alignment was used as input for genetic analysis in MEGA software v7. Gamma-distributed Hasegawa, Kishino and Yano model was calculated to best fit the data and used to calculate a maximum-likelihood phylogenetic tree using 500 bootstraps. Pairwise between groups genetic distance was calculated in MEGA.

Phylogenetic analysis on the COI gene revealed the existence of three separate genetic clusters. From each cluster five samples were selected and subjected to further genetic analysis through random amplification of polymorphic DNA (RAPD). The RAPD reactions were conducted using the

primer 5'-CAGGCCCTTC-3' (Sharma et al., 2010) and One*Taq*® polymerase and reaction buffer (New England Biolabs) following the thermal cycling programme: initial denaturation at 94 °C for 2 minutes followed by 35 cycles of 1. denaturing at 94 °C for 1 minute, 2. annealing at 37 °C for 1 minute and 3. extension at 68 °C for 2 minutes, followed by a final extension step at 68° for 10 minutes. Genomic DNA extracted from the earthworm *Lumbricus rubellus* was used as outgroup. PCR product was run on a 1.5% agarose gel for three hours at 120 V using 1kb HyperLadder (Bioline, London, UK) as reference. Band patterns were manually scored in a blind manner. Rooted Neighbourhood-Joining tree were calculated in R using the package "ape" (Paradis & Schliep, 2019).

5.2.12 Gut and soil 16S-rRNA gene sequencing metagenomics bioinformatics

The prokaryotic community in genomic DNA extracted from soil and gut tissue was determined by PCR amplification and sequencing following the method outlined by Kozich et al. (2013) and described in detail in **2.2.1-3**. Briefly, a ~555 bp fragment spanning the V3-V4 region of the 16S-rRNA gene was amplification using the forward primer 5'-CCTACGGGAGGCAGCAG-3' and reverse primer 5'-GGACTACHVGGGTWTCTAAT-3'. PCR product was normalized, cleaned and subsequently sequenced on a MiSeq using MiSeq Reagent Kit v3 - 600 cycles (Illumina, Inc., San Diego, CA, USA). The Illumina demultiplexed sequences were processes using the DADA2 bioinformatics pipeline (Callahan et al., 2016) to generate an ASV tables. ASVs assigned to mitochondria, chloroplasts, Archaea, Eukaryotes and ASVs with unknown kingdom or phylum were removed from the dataset. Nucleotide sequence data have been submitted to NCBI and are available under submission number SUB7500125 as part of BioProject number PRJNA610159.

5.2.13 Quantitative PCR

Quantitative PCR was used to determine differential levels of mRNA of several *E. fetida* immune genes in gut tissue and coelomic fluid (**Table 5.1**). Both tissues were screened for coelomic cytolytic factor (CCF), lysozyme and lysenin/fetidin. Primer pairs were mapped against reference transcriptomes of both *E. fetida* and *E. andrei* to estimate the binding potential to all known allelic variants. Primer pairs targeting CCF were designed to both *E. fetida* and the *E. andrei* versions of the CCF gene using Primer 3. Amplification efficiency of primer pairs was verified through serial dilution and was between 90 and 110 % for all pairs. Amplification of the target fragment was verified by Sanger sequencing of the PCR products. qPCR reactions were conducted using GoTaq® qPCR Master Mix (Promega) in a 20 µl reaction volume using 6.25 ng of cDNA as input. qPCR was performed using a Roche LightCycler® 480II with PCR conditions: initial denaturation at 95°C for 3 minutes, followed by 40 cycles of 1. denaturation at 95°C for 10 seconds and 2. annealing and extension at 60 °C for 30 seconds. Melt curve analysis was conducted to verify single PCR product.

Changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). EF1α was used as a reference gene for the normalization of the target immune genes. Log2 fold change was expressed in relation to the negative control (*E. fetida* exposed to control soils in the pre-exposure and PBS in the bacterial challenge).

Table 5.1: Details of primers targeting housekeeping gene (EF1α) and target immune system genes

Primer name	Target gene(s)	F/R	Sequence (5' → 3')	Amplicon length (bp)
EF1α_F	Elongation factor 1 alpha	F	ATCGGTCATGTCGATTCCGG	213
EF1α_R	Elongation factor 1 alpha	R	GGCAGTCTCGAACTTCCACA	
CCF_721F	Coelomic cytolytic factor	F	ACGACAACCGATACTGGCTG	193
CCF_914R	Coelomic cytolytic factor	R	CTCCCAGAAATCCACCCACC	
Lysfet_F	Lysenin/fetidin	F	TGGCCAGCTGCAACTCTT [a]	177
Lysfet_R	Lysenin/fetidin	R	CCAGCGCTGTTTCGGATTAT [a]	
Lysozyme_F	Lysozyme	F	GCCATTCCAAATCAAGGAAC [a]	129
Lysozyme_R	Lysozyme	R	TAGGTACCGTAGCGCTTCAT [a]	

[a] From Dvorak et al. (2013)

5.2.14 Statistical analysis

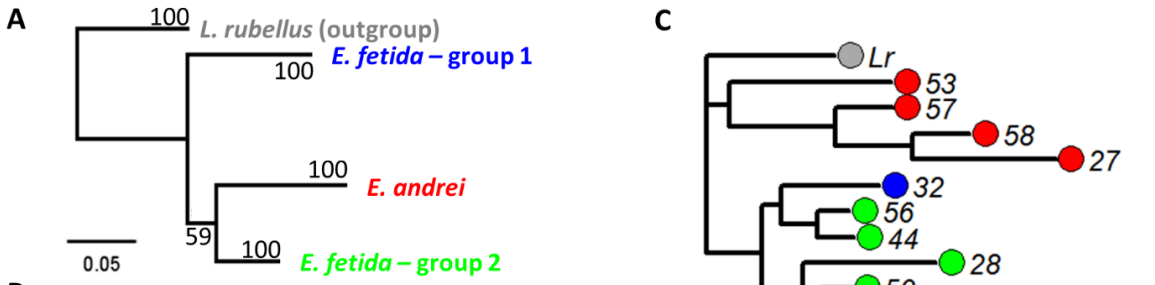
All data analysis was done in R. Non-metric dimensional scaling (NMDS), distance-based redundancy analysis (using Bray-Curtis distance matrix) (Db-RDA), permutational analysis of variance (Permanova) and calculation of diversity indices were conducted using the R package 'vegan' (Oksanen et al., 2018) by using datasets rarefied to 4959 reads per sample with removal of samples below this threshold. Differences between treatments in diversity indices and gene expression values were tested using two-way analysis of variance (2w-ANOVA) and Tukey's post hoc test. Differential abundance analysis of bacterial taxa was done using Kruskal-Wallis Rank Sum Test and Mann-Whitney' test using datasets rarefied to 2448 reads. For the differential abundance analysis, rarefication to this lower read number was done to prevent losing replicates and therefore statistical power.

5.3 Results

5.3.1 E. fetida population genotypes

Sequencing of the COI gene from *E. fetida* sampled during the bacterial challenge indicated three separate genetic clusters (**Figure 5.3A**). Local alignment using NCBI BLASTn, indicated that one of those clusters was most similar to *E. andrei* COI whilst the COI of the two other clusters aligned best with *E. fetida* COI. The two *E. fetida* COI sub-clusters did not group together in the phylogenetic tree. However, the overall genetic distance between the two *E. fetida* COI clusters was smaller (0.160) than that between the *E. andrei* COI cluster and *E. fetida* COI cluster 1 (0.186) (**Figure 5.3B**). RAPD analysis based on 26 polymorphic markers indicated the existence of two genetic clusters (**Figure 5.3C**). One of these clusters consisted of individuals carrying an *E. andrei* COI gene copy. The

other cluster comprised individuals carrying an *E. fetida* COI copy and one COI assigned *E. andrei* individual.



B

	Ea	Ef1	Ef2	Lr
***E. andrei* (Ea)**		.186	.236	.354
***E. fetida* (group 1) (Ef1)**	.186		.160	.310
***E. fetida* (group 2) (Ef2)**	.236	.160		.314
***L. rubellus* (Lr)**	.354	.310	.314	

Figure 5.3. Phylogenetic trees showing relationship between cytochrome oxidase I (COI) cluster and genetic distances between COI clusters. (**A**) Maximum-likelihood phylogenetic tree of showing phylogenetic relation between the three COI clusters. Samples are collapsed to COI cluster level, see **Figure S5.1** for full tree. Bootstrap values are derived using 500 bootstraps. (**B**) Table with genetic distances between the three COI clusters. (**C**) Rooted Neighbourhood-Joining tree based on RAPD profiles with *L. rubellus* (*Lr*) as outgroup. Different number indicate sample number. Colours indicate COI grouping.

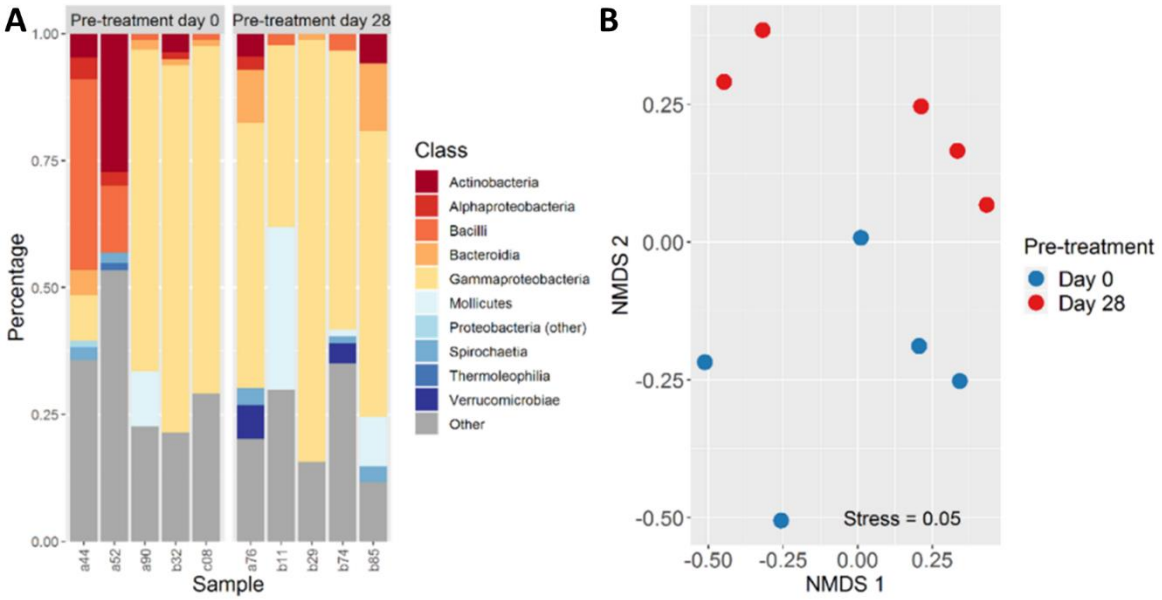


Figure 5.4: Bacterial community composition and structure of *E. fetida* gut samples from control replicates at start and end of pre-treatment exposure (**A**) Relative abundance of dominant ASV at Class level per sample. All ASV with a relative abundance <1% of the total community are grouped under "Other". (**B**) Plot of NMDS at ASV level.

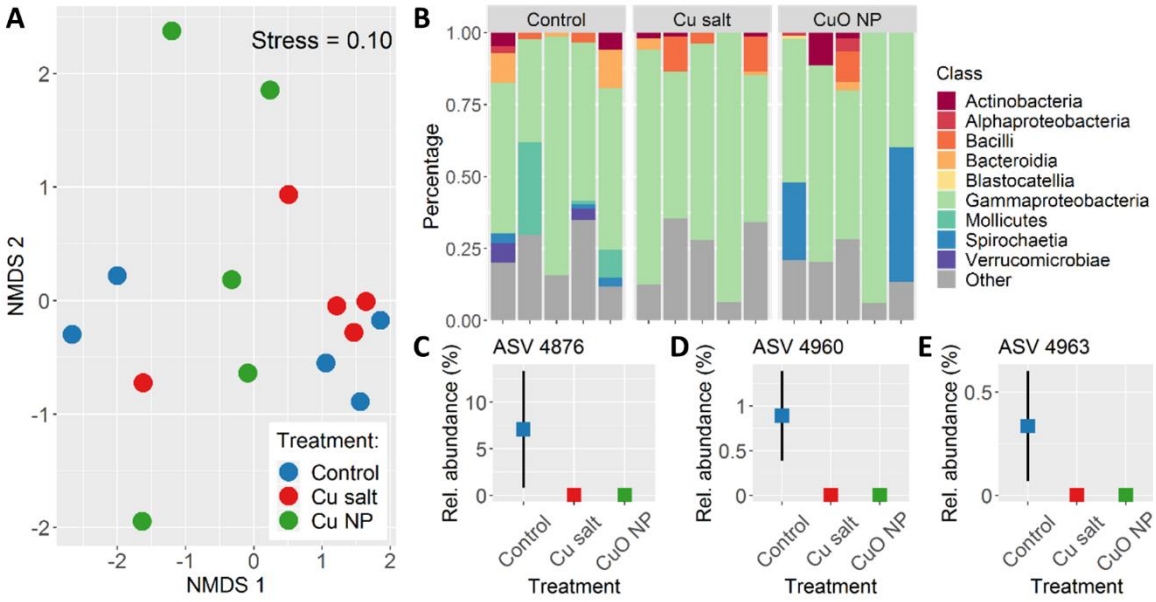


Figure 5.5: Bacterial community composition and structure of *E. fetida* gut samples at the end of the pre-treatment exposure (i.e. 'Pre-treatment day 28'). (**A**) Plot of NMDS showing ordination of samples at ASV level. (**B**) Relative abundance of dominant ASV at Class level per sample. All ASV with a relative abundance <1% of the total community are grouped under "Other". Mean relative abundance (±se) per treatment as percentage of total community of (**C**) '*Candidatus* Lumbricincola' (ASV 4876), (**D**) *Luteolibacter pohnpeiensis* (ASV 4960) and (**E**) *Luetolibacter* (ASV 4963).

5.3.2 The effects of pre-treatment exposure and bacterial challenge on the gut microbiome

The *E. fetida* gut community at the start of the pre-treatment composed of a consortium of bacteria comparable to that found in previous studies (Procházková et al., 2018). The gut community was dominated by *Verminephrobacter* (*Proteobacteria*), '*Candidatus* Lumbricincola' (*Mollicutes*), a member of the *Spirochaetaceae* family (*Spirochaetes*) and multiple ASV belonging to the genus *Aeromonas* (*Proteobacteria*) (**Figure 5.4A**). Transfer of earthworms from culture soil ('pre-treatment day 0') to LUFA control soils ('pre-treatment day 28') did not significantly alter the total community structure in the gut (Permanova: $F(1,8)=1.059$, $p=0.356$) (**Figure 5.4B**) nor Shannon diversity ($F(1,8)= 0.882$, $p=0.375$) or species richness ($F(1,8)= 0.074$, $p=0.375$) (**Table 5.2**). Average Shannon diversity and richness across all replicates were 3.0 (±SD 1.1) 239 (±SD 146), respectively.

At the end of the pre-treatment exposure, (i.e. 'pre-treatment day 28'), there were no significant differences in overall community structure between the treatment groups (Permanova: $F(2,12)=1.252$, $p=0.256$) (**Figure 5.5A**). Bacterial diversity was also not affected by pre-treatment exposure (Shannon: ($F(2,12)=0.176$, $p=0.84$; richness: $F(2,12)=1.695$, $p=0.225$) and was on average 2.5 (±0.9) (Shannon) and 189 (±114) (richness) (**Table 5.2**). Among the most abundant gut bacteria (ASV with a relative abundance >1% in any of the samples), three ASV were significantly negatively affected by exposure to both copper forms (Kruskal-Wallis: $p<0.05$). These ASV included

'Candidatus Lumbricinola' (ASV 4876) and two ASV belonging to *Luteolibacter* (ASV 4960 and 4963) (**Figure 5.5C-E**). These taxa together comprised an average of 8% of the total community in controls, but were at or below the limit of detection in the copper treated *E. fetida* .

In *E. fetida* that were sampled at 'bacterial challenge day 2', treatment (i.e. pre-treatment exposure followed and bacterial challenge combined) had a nearly statistical significant effect on the bacterial community composition in *E. fetida*, with treatment explaining 24% of the total variance (Permanova: $F(5,23)=1.423$, $p=0.075$) (**Figure 5.6B**). Pre-treatment exposure and bacterial challenge treatment alone explained 10% (Permanova: $F(2,26)=1.453$, $p=0.108$) and 4% of the total variance (Permanova: $F(2,26)=1.222$, $p=0.265$), respectively (**Table 5.3**). No significant effect of treatment on diversity indices which averaged (1.95 (±0.6) (Shannon) and 57 (±22) (richness) across all samples, was found (**Table 5.2**). Among the most dominant gut bacteria (ASVs with relative abundance >1%), '*Candidatus* Lumbricincola' (ASV 4876) relative abundance was significantly negatively affected copper treatment (**Figure 5.6-D**) (Kruskal-Wallis: $X^2(5)=12.1$, $p<0.05$) whilst *Aeromonas* (ASV 10149) was positively affected (**Figure 5.6D**) (Kruskal-Wallis: $X^2(5)=14.0$, $p<0.05$).

Table 5.2: Mean Shannon index and ASV richness (±SD) per sampling point and treatment group.

Diversity index		Bacterial treatment:					
		Control			*B. subtilis*		
		Pre-treatment:			Pre-treatment:		
	Sampling point	Ctrl	NP	Ion	Ctrl	NP	Ion
Shannon diversity	Pre-treatment (day 0)	3.3±1.2					
	Pre-treatment (day 28)	2.6±0.9	2.3±1.1	2.6±1.2			
	Bacterial challenge (day 2)	1.8±0.8	2.3±0.6	1.5±0.6	1.9±0.6	2.0±0.4	2.3±0.3
	Recovery period (day 1)	2.6±0.3	3.3±1.1	2.2±0.7	2.2±0.8	2.6±1.1	2.7±0.6
	Recovery period (day 28)	2.6±1.2	2.1±1.1	2.5±1.1	2.5±1.3	1.6±0.7	2.6±0.5
ASV richness	Pre-treatment (day 0)	225±167					
	Pre-treatment (day 28)	226±226	115±44	225±118			
	Bacterial challenge (day 2)	62±16	68±41	49±16	51±10	53±12	65±31
	Recovery period (day 1)	118±22	280±192	134±89	186±137	184±104	143±96
	Recovery period (day 28)	210±130	140±105	154±80	215±231	78±57	146±19

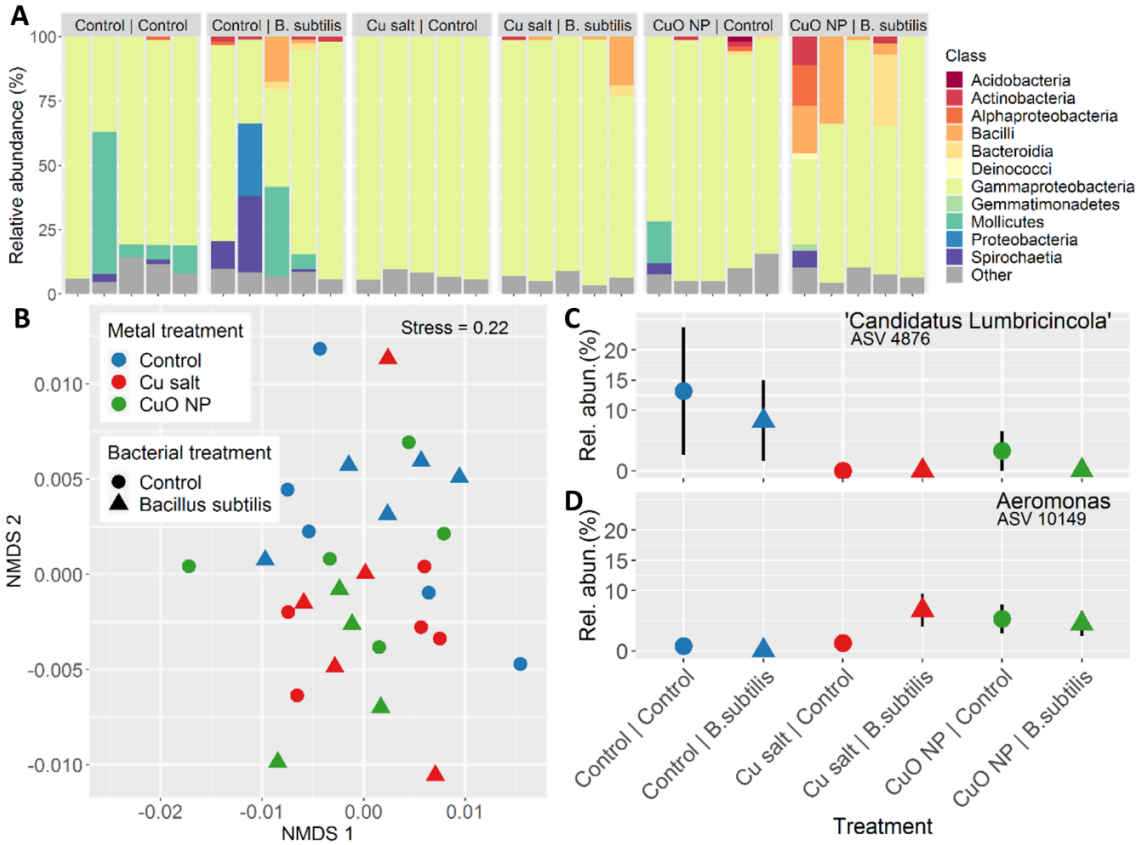


Figure 5.6: Bacterial community composition and structure of *E. fetida* gut samples during bacterial challenge (i.e. 'Bacterial challenge day 2'). (**A**) Relative abundance of dominant ASV at Class level per sample. All ASV with a relative abundance <1% of the total community are grouped under "Other". (**B**) Plot of NMDS showing ordination of samples at ASV level. (**C-D**) Mean relative abundance (±se) per treatment as percentage of total community of '*Candidatus* Lumbricincola' (ASV 4876) and *Aeromonas* (ASV 10149).

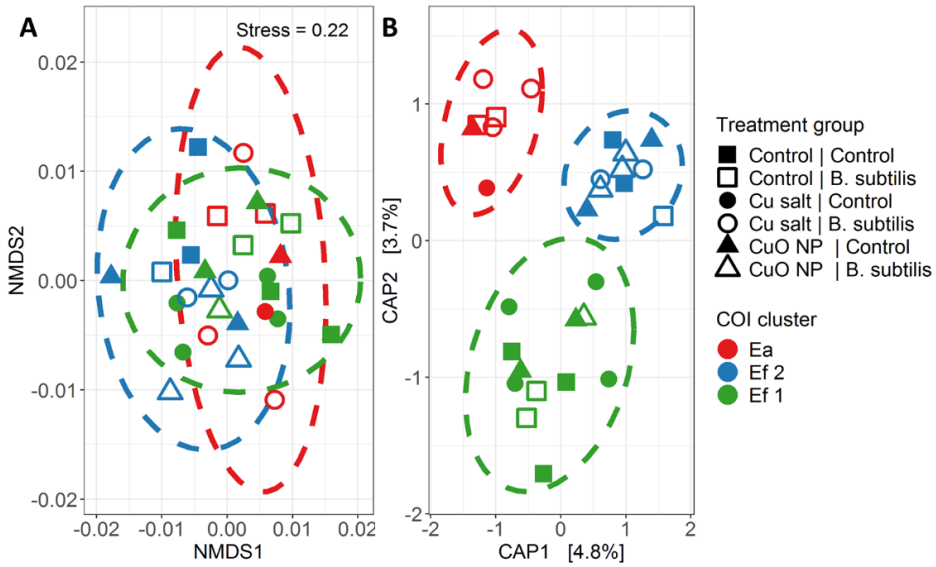


Figure 5.7: (**A**) NMDS plot showing ordination of 'Bacterial challenge day 2' samples. (**B**) First two-axis of distance based redundancy analysis (Db-RDA) including 'pre-treatment', 'bacterial challenge treatment' and 'genotype' as explanatory variables. Percentage following axis labels in **B** indicate percentage of total inertia explained by respective axis. In both figures, different colours indicate different COI genotypes, different shapes and filling, indicate different treatments. Ellipses indicate 90% CI of respective COI cluster. In legend, text before vertical bar indicates 'pre-treatment' and text following vertical bar indicates 'bacterial challenge treatment'. 'Ea' (*E. andrei*), 'Ef1' (*E. fetida* 1) and 'Ef2' (*E. fetida* 2) indicate COI genotype.

5.3.3 Relation between E. fetida genotype and bacterial community structure

Permanova and Db-RDA indicated that genotype was a better predictor for the bacterial community composition than either pre-treatment exposure or bacterial challenge treatment (**Table 5.3**) with samples clustering primarily by COI genotype (**Figure 5.7**). After removal of the variation associated to COI genotype using partial Db-RDA models, the effect of pre-treatment and bacterial challenge treatment on community composition was still not significant (**Table 5.3**).

5.3.4 Impact of treatments on Bacillus subtilis abundance in gut tissue and E. fetida survival, tissue integrity and immune responses

All control animals exposed to the PBS for four days survived. The survival of *E. fetida* exposed to *B. subtilis* was on average 79% at day four of the bacterial challenge, with no statistically significant effects of pre-treatment on the survival rate observed ($X^2(2)=0.875$, $p=0.646$) (**Figure 5.8A**). Abundance of *Bacillus* in gut tissue from *E. fetida* challenged with *B. subtilis* was significantly higher than in control animals indicating successful inoculation (**Figure 5.8B**). No differences in the abundance of *Bacillus* in gut tissue was observed during the recovery period. Histological analysis indicated a possible effect of pre-treatment exposure with copper (in both forms) on integrity of the gut epithelium and longitudinal muscle tissue. Average integrity scores in copper treatment between 0.8 and 1.3 points higher than controls (**Table 5.4**). Effects of copper treatment were manifested as the thinning of the gut epithelium tissue lining as well as the thinning of muscle fibres (**Figure S5.2**). Gene expression levels were assessed through qPCR analysis targeting several know earthworm immune genes using EF1α as reference gene (**Figure S5.3**). No significant effect of treatments was found on immune gene expression in both tissue types (2w-ANOVA: $p>0.05$) (**Figures 5.8C-H**).

5.3.5 Relation between E. fetida genotype and gene expression

No significant relation between gene expression and COI genotype was found with the exception of lysozyme expression in coelomic fluid samples. For this gene, expression in the *E. andrei* COI genotype was marginally but significantly higher than the *E. fetida* group 2 (2w-ANOVA: $F(2,26)=4.646$, $p<0.05$; Tukey's post hoc test: $p<0.05$) with the difference in mean fold change of 1.4 indicating a small magnitude effect (data not shown).

Table 5.3: Outcomes of models testing the relationship between bacterial community composition in the gut of *E. fetida* sampled at 'Bacterial challenge day 2' and different combinations of explanatory variables. Db-RDA: distance based redundancy analysis using Bray-Curtis distance matrix and applying square root transformation and Wisconsin double standardization. Permanova: Permutational multivariate analysis of variance using Bray-Curtis distance matrix and 999 permutations. 'Expl.' and 'Un-expl.' represent in Db-RDA models the proportion of inertia either 'explained' or 'unexplained' by explanatory variables. In brackets (in the 'Explained' column) the fraction of the inertia that is conditioned (i.e. removed). In Permanova models, 'Explained' and 'Unexplained' refer to the model R^2 and residual R^2 values.

Model type	Model	Model outcomes				
		Explanatory variables	Expl.	Un-expl.	*F*	*p*
Db-RDA	Community ~ Pre-treatment + Bacterial treatment + Genotype	All	0.202	0.798	1.167	0.008 **
	Community ~ Pre-treatment + Bacterial treatment	(change this)	0.118	0.882	1.114	0.084
	Community ~ Pre-treatment + Bacterial treatment + conditioned(Genotype)	Pre-treatment + Bacterial treatment	0.085 (0.117)	0.798	1.130	0.079
	Community ~ Pre-treatment	Pre-treatment	0.041	0.959	1.159	0.103
	Community ~ Bacterial treatment	Bacterial treatment	0.077	0.924	1.077	0.189
	Community ~ Genotype	Genotype	0.085	0.915	1.205	0.021*
Permanova	Community ~ Pre-treatment + Bacterial treatment + Genotype	Pre-treatment	0.101	0.899	1.614	0.074
		Bacterial treatment	0.044	0.956	1.428	0.173
		Genotype	0.139	0.861	2.229	0.019 *
	Community ~ Pre-treatment + Bacterial treatment	Pre-treatment	0.101	0.899	1.470	0.128
		Bacterial treatment	0.044	0.956	1.300	0.220
	Community ~ Pre-treatment	Pre-treatment	0.101	0.899	1.453	0.108
	Community ~ Bacterial treatment	Bacterial treatment	0.043	0.957	1.221	0.265
	Community ~ Genotype	Genotype	0.167	0.833	2.598	0.004 **

* indicates p<005, ** indicates p<0.01

Table 5.4: Integrity scores of gut epithelium and chloragogen tissue per sample point and treatment. Values are means of the scores of three sections from a single replicate.

	Bacterial challenge treatment:					
	Control			*B. subtilis*		
	Pre-treatment:			**Pre-treatment:**		
Sampling point	*Control*	*Cu salt*	*CuO NP*	*Control*	*Cu salt*	*CuO NP*
Pre-treatment (day 0)	1					
Pre-treatment (day 28)	1.3	1.3	1.0			
Bacterial challenge (day 2)	1.0	4.0	2.3	1.0	1.0	2.3
Bacterial challenge (day 4)	1.0	1.0	3.0	1.0	1.3	2.3
Recovery period (day 1)	2.0	2.0	1.7	2.0	3.3	3.3
Recovery period (day 28)	1.3	4.0	2.0	2.0	3.0	1.0
Average (across all sampling points)	1.3	2.5	2.0	1.5	2.2	2.2

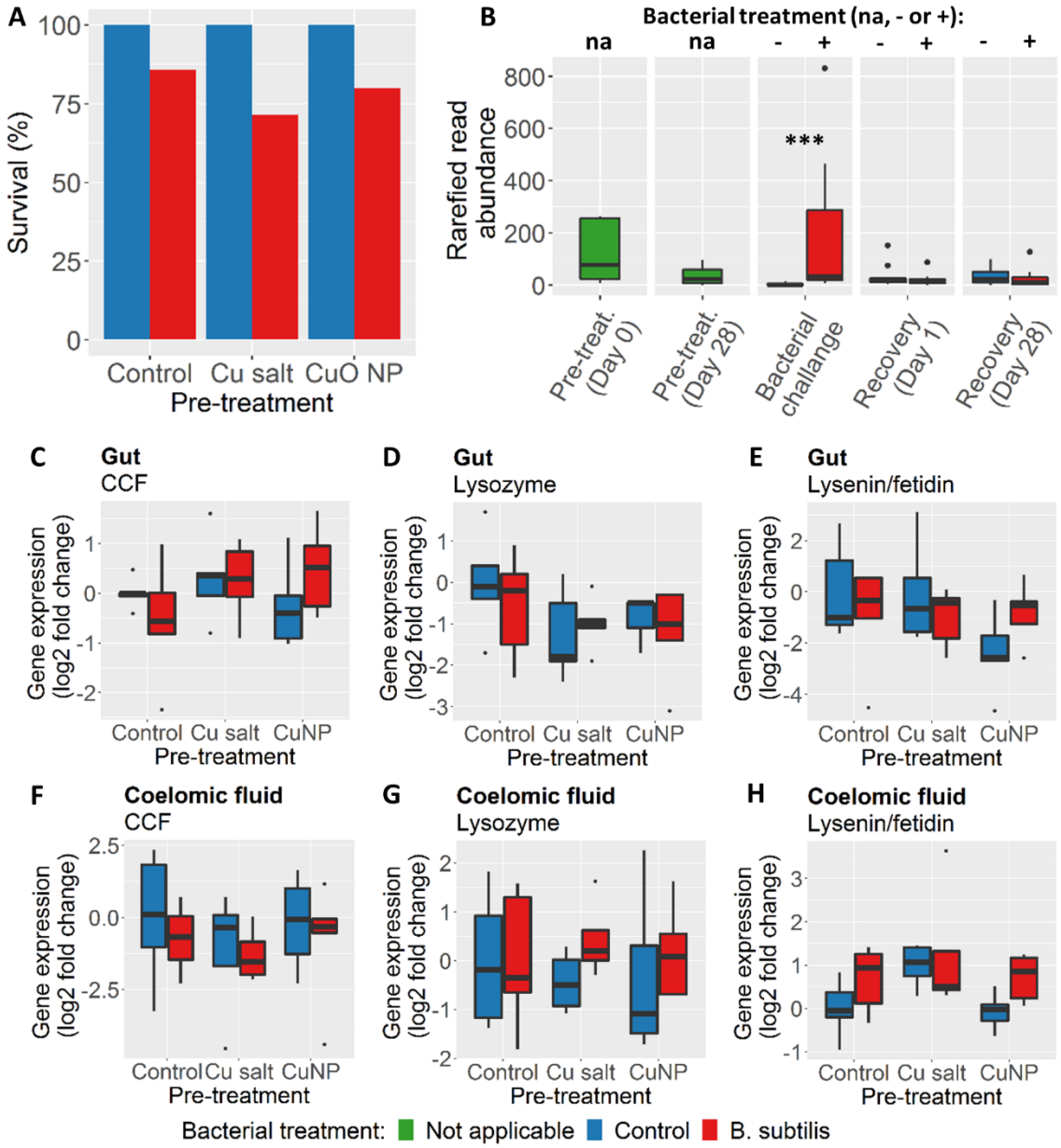


Figure 5.8: (A) Survival at day four of the bacterial challenge. (**B**) Boxplots of rarefied read abundance of *Bacillus* per sample point. Annotation at top of graph indicates bacterial treatment: na (not applicable), - (PBS control) and + (*B. subtilis* treatment). Triple asterisks indicate statistical significant between bacterial challenge control and bacterial challenge treatment with $p<0.001$. Relative high abundance of *Bacillus* at 'Pre-treatment day 0' is driven by the two samples in that sample group (**Figure 5.4**). Boxplots of fold change in gene expression of *E. fetida* immune responses in (**C-E**) gut tissue and (**F-H**) coelomic fluid at 'Bacterial challenge day 2'. All gene expression values are log2 fold change derived through $2^{-\Delta\Delta Ct}$ method. Expression values represent fold changes of treatments compared to control animals (control pre-treatment + control bacterial challenge) and are normalized to the expression of the housekeeping gene EF1α. There were no significant differences in gene expression between groups for all tested genes ($\alpha=0.05$). Different colours in all panels indicate different bacterial challenge treatments.

5.4 Discussion

Innate immunity provides a first line of defence against invading pathogens. NMs are known to interact with the immune system of organisms and can induce both pro- and anti-inflammatory responses (Bhattacharya et al., 2017; Boraschi et al., 2020). NMs are developed and applied as antimicrobial agents in personal care products and in an agricultural setting. In many animals, microbial symbionts play an important role in host defence. Therefore, when animals are exposed to biocidal NMs, disruption on their microbiome can be expected which, accordingly, may lead to effects on host immunity. It remains, however, unclear whether NM exposure can compromise host immunity through effects on the microbiome when hosts are infected by pathogens. This paper aimed to study the impact of biocidal CuO-NPs and its ionic counterpart on the gut microbial community, host immune responses and infection susceptibility in an *E. fetida*.

Previous *in vitro* studies have shown that NPs can be taken up by earthworm immune cells (coelomocytes) (Bigorgne et al., 2012; Hayashi et al., 2012; Hayashi, Miclaus, et al., 2013), and can alter the expression of earthworm immune markers (Hayashi et al., 2012, 2016) leading to cellular toxicity (Garcia-Velasco et al., 2019; Patricia et al., 2017). In this study, *in vivo* exposure to metal biocides (in both metal salt and NP form) caused changes to microbiome structure with several bacterial symbionts being negatively affected by exposure to copper. Following these exposures and microbiome changes, survival rates were unaffected when *E. fetida* were challenged with a high dose of the soil bacterium *B. subtilis*. Histological analysis indicated possible tissue damage due to copper exposure, although this analysis is based on observations for a limited number of samples and so further assessment of this response is needed. Overall, we found no evidence for altered infection susceptibility or altered immune gene regulation at biocide concentrations where the gut microbiome is already affected.

The lack of an immune response after NP exposure contrasts the results of previous studies (Hayashi et al., 2012, 2016). Hayashi and colleagues, for example, found that *in vitro* exposure to Ag-NPs by earthworm immune cells alter the temporal expression of immune genes (Hayashi et al., 2012). Also in other invertebrates, such as mussels and sea urchins, both *in vivo* and *in vitro* exposure to NMs can modulate the immune system of these animals (Alijagic et al., 2020; Auguste, Balbi, et al., 2019; Auguste, Lasa, et al., 2019). NMs that are released into the environment are likely to undergo transformations (Levard et al., 2012; C. Meier et al., 2016; Sekine et al., 2017). Uptake by organisms may further modify shape, size and form of NMs (Baccaro et al., 2018; Peng et al., 2015). The NMs that earthworm immune systems are exposed to *in vivo* are therefore different to the pristine forms that have often been used in *in vitro* studies. The discrepancy between the impact

of NMs on earthworm immune reactivity *in vivo* and those *in vitro* may therefore be linked to the transformations of NM in soil media and resulting change in the immuno-reactivity of NMs.

Previous research has shown that the microbiome of animals can be altered by NM exposure. In rodents for example, exposure to Ag-NPs can negatively affect the abundance of *Firmicutes* and *Lactobacillus* and induce histological damage to intestinal tissue (D. Chen et al., 2017; Williams et al., 2015). Also in soil invertebrates, NP exposure can alter intestinal microbiomes (J. Ma et al., 2019, 2020; B.K. Zhu et al., 2018). Exposure to Ag-NPs in the springtail *Folsomia candida*, for example, negatively affects the abundance of *Firmicutes* and *Actinobacteria* in the gut of these soil invertebrates (Zhu et al., 2018). Although implications on host functioning were not further studied, other research shows that microbiome dysbiosis induced by environmental pollution can be associated to changes in the isotopic composition of springtails, suggesting that in these animals microbiome dysbiosis is linked to altered nutrient turnover (Xiang et al., 2019). In this study, we found that the earthworm symbiont '*Candidatus* Lumbricincola' is negatively affected by an exposure to copper forms. '*Candidatus* Lumbricincola' is a bacterium exclusively associated to earthworms and has a possible role in the degradation of polysaccharides (Nechitaylo et al., 2009; Zeibich et al., 2019a). The implications of the near loss of this symbiont for the health and functioning of earthworms requires further investigation.

Contrary to our hypothesis, a two day exposure to a high bacterial level did not change the expression of known earthworm immune markers. Successful inoculation with the bacterium was confirmed by the mortality data and the abundance of *Bacillus* in gut tissue. The lack of an immune response at concentrations of bacteria at which 21% of the exposed individuals die, is therefore unexpected. Earthworms can show large variation in the expression of immune genes over time (Hayashi et al., 2012; Tak et al., 2015). Timing of expression of components of the immune system can be gene specific (Dvořák et al., 2016) but can also depend on the specific pathogen to which the earthworm is exposed (Tak et al., 2015). For example, in *E. andrei*, lysozyme is expressed within several hours of exposure to *Escherichia coli*, but only after 16 hours following exposure to *B. subtilis* (Josková et al., 2009). Similarly, in *E. andrei* lysenin/fetidin has been reported to be upregulated after six hours in response to a *Staphylococcus aureus* exposure but down regulated when exposed to *E. coli* (Opper et al., 2013). The methodology adopted in this study was based on studies by Dvorak and colleagues who showed that exposure to high levels of *B. subtilis* can induce changes in immune regulation of earthworms (Dvořák et al., 2013, 2016). Discrepancy between measured immune responses in *E. fetida* as reported in this study and those measured in previous studies in a related species under similar condition shows that earthworm immune responses are

also species dependent. Investigations into the molecular structure of the earthworm immune gene CCF in eight different species has shown that some earthworm species have a wider recognition capacity than others (Šilerová et al., 2006). Even within closely related *Eisenia* spp., there are differences in the reactivity of immune genes and immunity related enzymatic activity (Dvořák et al., 2013). These differences in immune reactivity between related earthworms may reflect differences in microbial environments which may require niche-specific immune responses and lead to differing basal immune reactivities. Time as a factor in earthworm immune responses is, thus, not fully understood (Hayashi, Heckmann, et al., 2013) and requires further species-specific investigation. Sufficient sampling over a time-course is needed to fully elucidate patterns of immune expression in earthworms under various environmental stressors and in particular to identify the specific points of highest upregulation for key genes.

Earthworm coelomocytes are composed of three subpopulations, each with a unique function (Adamowicz, 2005; Engelmann et al., 2011) and molecular immune-expression profile (Bodó et al., 2018; Opper et al., 2013). This cellular complexity means that it is possible for different cell subpopulations to have different sensitivities to pollutant exposure (Irizar et al., 2015; Patricia et al., 2017). Exposure to metals and xenobiotics, but also immunostimulants like LPS (Homa et al., 2013) have been demonstrated to change the ratio between the different coelomocyte cell subpopulations (Irizar et al., 2015; Olchawa et al., 2006; Patricia et al., 2017) and to alter the expression of earthworm immune markers (Mincarelli et al., 2019). In this study, coelomic fluid was extruded and sampled without separation of different cell subpopulations. Accordingly, the measured immune responses to *B. subtilis* exposure, are an average of the expression levels of these genes across these different subpopulations. This, in combination with high variation between individuals in expression of some of the tested genes as previously reported (Procházková et al., 2006), may limit the ability to elucidate differences in the patterns of gene expression within any individual cell subpopulations (Patricia et al., 2017).

In this study we found that seven out of the 30 genotyped earthworms carried an *E. andrei* COI copy. Moreover, for the remaining *E. fetida* individuals, two COI clades were recorded. COI genotype did, however, not affect measured immune responses. The finding of clade structure for earthworms from the *E. fetida*/*E. andrei* complex is in agreement with previous studies (Martinsson & Erséus, 2018; Pérez-Losada et al., 2005; Plytycz, Bigaj, Osikowski, et al., 2018; Römbke et al., 2016). *E. fetida* are phenotypically characterized by their stripped pigmentation pattern, whereas *E. andrei* are classically more uniformly red coloured. These two species were formally described as two different subspecies (e.g. *E. fetida fetida* and *E. fetida andrei*) (Bouché, 1972), but were on the

basis of crossbreeding experiments and differences in biochemical markers classified as separate species (Domínguez et al., 2005). More recent research has shown that in laboratory conditions, *E. fetida* and *E. andrei* can hybridize and produce fertile hybrid offspring (Plytycz, Bigaj, Osikowski, et al., 2018; Plytycz, Bigaj, Panz, et al., 2018). Field studies also confirm that gene flow between these two species does occur (Martinsson & Erséus, 2018). *E. fetida* in this study were characterized by typical *E. fetida* pigmentation. Previous studies, however, report that pigmentation is not always a good predictor for COI genotype (Plytycz, Bigaj, Osikowski, et al., 2018; Römbke et al., 2016). Here, RAPD profiling suggests that COI genotype does not always predict genomic variability, as indicated by the presence of an individual carrying an *E. andrei* COI copy within a clade consisting out of *E. fetida* COI carrying individuals. The COI genotype was shown to be a better predictor for the bacterial community composition than any treatment. Host genetics is one of the components shaping the human gut microbiome (Goodrich et al., 2016; Kolde et al., 2018) but similar relationships have also been observed in other animals such as mice (Buhnik-Rosenblau et al., 2011) and invertebrates. In the water flea *Daphnia manga*, for example, host genotype not only shapes the structure but also the functionality of the gut microbiome in particular its ability to respond to toxic cyanobacteria (Macke et al., 2017). The gut microbiome of *Eisenia* spp. are dominated by a consortium of bacterium that are vertically transmitted from parental animal to offspring (Davidson et al., 2006; Procházková et al., 2018). The relation between COI genotype and gut microbiome structure may thus be linked to the concurrent maternal transmission of both mitochondria and bacterial symbionts.

5.6 Conclusions

This research shows that the microbiome of *E. fetida* can change when exposed to a copper (in both NP and salt form). However, these biocide mediated changes of the microbiome do not lead to altered susceptibility to a bacterial infection. Even though *B. subtilis* has so far not been described as a natural earthworm pathogen, exposure to a high dose of this bacterium is known to cause mortality and induce immune responses in exposed individuals. In this study, despite the mortality when challenged with *B. subtilis*, no effects of treatment on the measured *E. fetida* immune markers were observed. The absence of any effect on immune function needs to be further validated by studies of gene expression using a greater time resolution of immune responses in *E. fetida* and further identification of markers of immunity through, for example, full transcriptomic analysis. The methodological approach applied in this paper may guide future studies to improve assessment of immuno-safety of NMs.

6

Long-term effects of metal and polymeric nanoparticles on the microbiome of earthworms in soil mesocosms

6.1 Introduction

Nanomaterials (NMs) are increasingly produced and used in various applications including coatings, pesticides and electronics. NMs that are released into the environment may ultimately end up in soils through the application of NM containing sewage sludge onto agricultural land (Keller et al., 2013; Sun et al., 2016) or through the application of NM-based pesticides and fertilizers (Kah et al., 2018; Keller et al., 2017). In soils, NMs may exert toxicity in exposed soil invertebrates which can lead to reduced reproduction and growth in exposed animals (Gomes et al., 2015; Heckmann et al., 2011; Shoults-Wilson et al., 2011). Many animals rely on the microbial communities associated to dermal or intestinal surfaces (commonly referred to as the microbiome) for functions such as the provision of essential nutrients and the protection against pathogens (Akman Gündüz & Douglas, 2012; B.L. Weiss et al., 2012). Chemicals that adversely affect microbial symbionts may, thereby, indirectly affect the health of animals through the disruption of the functions provided by these microbes (Motta et al., 2018). The impacts of chemicals on symbiotic microbial communities should therefore be included in the hazard assessments of chemicals.

Effects of NMs on microbiomes of soil invertebrates are now increasingly studied. Recent work has shown that short-term exposures to metal NMs can alter microbiomes of soil organisms (Ma, Chen, et al., 2020; Sánchez-López et al., 2019). In the earthworm *Enchytraeus crypticus* exposure to CuO-nanoparticles (NPs) led to a significant reduction of *Plantomycetes* bacteria in the gut microbiome after 21 days (Ma, Chen, et al., 2020). Similarly, Ag-NPs perturbed the gut microbiome of springtails causing significant reductions in the relative abundance of bacteria belonging to the phyla *Firmicutes* and *Actinobacteria* (Zhu, Zheng, et al., 2018). In the Lumbricidae earthworm *Eisenia fetida*, exposure to CuO-NPs led to the near loss of the earthworm symbionts '*Candidatus* Lumbricincola' and an unknown *Spirochaetacea* (**Chapter 4**). Increasingly soil invertebrates are also exposed to polymeric NPs. Similar to metal NPs, these polymeric particles may be released into soil system through the application of sludges and fertilizers (Corradini et al., 2019; Weithmann et al., 2018; L. Zhang et al., 2020; Zubris & Richards, 2005) but may also be formed in soils through fragmentation of plastic debris and plastic mulch (Astner et al., 2019). Some studies indicate that at concentrations lower than at which effects on life-history traits become apparent, micro-sized polymeric particles ('microplastics') can disrupt the gut epithelium, induce inflammation responses (Jiang et al., 2020; Rodriguez-Seijo et al., 2017). and alter the gut microbiome of soil organisms (Ju et al., 2019; Xiang et al., 2019; Zhu, Chen, et al., 2018). Effects of polymeric NPs on soil microbiomes are however much less studied. A recent report indicates that a short-term exposure to polymeric NPs can alter the structure of the gut microbiome of *E. crypticus* by altering the ratios between the relative abundances of some core bacterial families (Ma, Sheng, et al., 2020)). With likely future

increase of the release of NPs into the soil system, there is a need for further research on the potential of NPs to cause harm to soil invertebrates through the disruption of host microbiomes.

Up to date, most studies have assessed the effects of metal and polymeric NPs on animal microbiomes using as manufactured "pristine" NPs. Although some NPs are likely to enter the soil system in pristine forms (e.g. copper-based nano-pesticides), other NPs are likely to be chemically or functionally transformed "aged" in the environment before they enter the soil. NPs that are introduced into the soil system through the application of sewage sludge (such as Ag-NP and polymeric NPs entering waste-streams from different consumer applications), for example, have gone through wastewater treatment plants (WWTP). During passage through WWTP, NPs can undergo transformations such as heteroagglomeration, sulfidation and surface functionalisation by organic matter interactions (Kaegi et al., 2011; Lombi et al., 2013; Lowry et al., 2012; C. Meier et al., 2016). These transformations processes can reduce the biological availability and toxicity of the NPs (Doolette et al., 2016; Levard et al., 2013; Reinsch et al., 2012). In addition to a focus on only pristine NPs, most research so far has focussed on short-term effects of NPs on microbiomes (see McKee at al 2016). The short-term effects are however not always a good predictor of the effects of extended exposure (Diez-Ortiz et al., 2015a). Long-term studies are, therefore, needed to improve the hazard assessment of metal and polymeric NP effects in terrestrial environments.

To address the aim of establishing the effects of chronic exposure, here we study the long-term of exposure to three different NPs (i.e. uncoated CuO-NPs, PVP-coated Ag-NPs and uncoated polystyrene (PS) NPs) in outdoor mesocosms over a six month period as single exposures and in combinations on the gut microbiome of two earthworms (*Lumbricus terrestris* and *Aporrectodea calignosa*). The two species were selected as representatives of two earthworm ecotypes that may be exposed to NPs in agricultural soil. *L. terrestris* are anecic earthworms forming deep vertical burrows, whilst *A. calignosa* are endogeic earthworm feeding on mineral topsoils. Owing to their importance in the functioning of soils, both ecotypes are considered important for soil functioning. As such, they are of significant interest for ecotoxicological research (Bart et al., 2018). The three test NPs were specifically chosen to reflect a range of biocidal potentials (high: CuO-NPs and Ag-NPs; low: PS-NPs) and different routes of entry to the soil system (sludge application: Ag-NPs and PS-NP; direct application: CuO-NPs). In total five different NP treatments were tested. These included three singular exposure of the three different NPs (i.e. CuO-NPs, Ag-NPs and PS-NPs) along with a single combined exposure of two NPs (i.e. Ag-NPs + PS-NPs, herein, 'AgPS') and a single combined exposure of all three tested NPs (i.e. CuO-NPs + Ag-NPs + PS-NPs, herein 'All'). To reflect environmentally realistic entry routes, prior to spiking of the soils, Ag-NPs and PS-NPs were aged in sewage sludge in reaction containers mimicking the environmental conditions in WWTP. CuO-NPs

were spiked in pristine form NPs to reflect its major release mode as a pesticide. It was hypothesized that i) chronic exposure to NPs cause effects on the earthworm gut microbiome; ii) the higher the exposure complexity the greater the effects on the microbiome (i.e. effects on singular exposures < double exposure < triple exposure); and iii) the earthworm symbionts '*Candidatus* Lumbricincola' and *Spirochaetaceae* are negatively affected by exposure to CuO-NP, in line with previous studies (**Chapter 4** and **5**; Srut et al 2018).

6.2 Methods

6.2.1 Invertebrate animals and plants

Aporrectodea calignosa (Lumbricidae) were collected from a site under pasture land-use in Wales (UK) (UK Ordnance Survey Grid reference ST 14021 72214) and were maintained on soil collected from the same site. *Lumbricus terrestris* (Lumbricidae) were supplied by Worms Direct (Ulting, UK) and maintained in a culture soil combining equal volumes of a loam soil, composted bark and garden compost for 60 days before to the start of the test. Earthworms were reared indoors at 6°C until the initiation of the exposure. Winter wheat (*Triticum aestivum*) seeds were supplied by the University of Copenhagen, Department of Plant and Environmental Science, Denmark. Woodlice (*Porcellio scaber*) were collected from a woodland site in Wales (UK) (UK Ordnance Survey Grid reference ST 14628 71694) and kept in plastic containers and fed with a mixture of dried leaves until the start of the experiment. *Enchytraeus crypticus* (Enchytraeidae) and *Folsomia candida* (Collembola) were supplied by the Vrije Universiteit Amsterdam (the Netherlands). *E. crypticus* were maintained in agar and feed with oats until the start of the experiment. Springtails were cultured in plastic contained and fed with baker's yeast until initiation of the experiment.

6.2.2 NP characterizations

Uncoated 50×30 nm CuO nano-rods (CuO-NPs) and 50 nm PVP-coated Ag nano-spheres (Ag-NP) were manufactured and supplied by Promethean Particles Ltd. (Nottingham, UK) and uncoated 100 nm polystyrene NPs (PS-NPs) from Sigma-Aldrich (Poole, UK). All NPs were supplied dispersed in water. Primary size distributions of pristine NPs (Ag: 54±5.6 nm; PS: 87±25 nm) were determined using transmission electron microscopy (TEM) at Oxford University Department of Materials (UK). For this purpose, a drop of the NP dispersions were placed on a TEM grid and dried for 1 hour until estimation of size distributions using a JEOL 2010 analytical TEM equipped with Oxford Instruments LZ5 windowless energy dispersive X-ray spectrometer (EDS). A small volume of sludge from the 'All' treatment was sampled at the end of the sludge ageing procedure (as described below) in order to estimate the surface sulphidation level of the aged Ag-NP at that start of the NP exposure experiment. Sulphidation levels were estimated by measuring the Ag and S content of the aged Ag-

NP using EDS at Oxford University Department of Materials (UK). Analysis indicated that nearly all Ag at the surface of the aged Ag-NPs was present as Ag_2S as indicated by a mean Ag to S spectral ratio of 2.2 (±SD 0.2). TEM characterizations were conducted by Dr. Kerstin Jurkschat at the Department of Materials, University of Oxford, UK.

6.2.3 Sludge-soil and NP mixtures

Sewage sludge (3.5% w/w solids) was collected from an anaerobic digester at the Finham sewage treatment plant, Coventry, UK. For each treatment (i.e. five NP treatments and a negative control) 3 L of sewage sludge was added to a 5 L jar. Ag-NP and PS-NP were then added as required per treatment and the jars sealed (**Figure 6.1A**). The sludges were then incubated in water baths at 35°C for 12 days under constant stirring. In the UK, prior to application to agricultural land, sewage sludge is often treated with lime in order to inactivate pathogens (Defra, 2018). Following UK guidelines, at the end of the incubation period, lime was added to raise the sludge pH to above 12. After the lime addition, un-aged CuO-NPs were mixed to the aged sludges of the 'CuO' and 'All' treatment jars. The mesocosm exposure medium was prepared by mixing the spiked sludge with clean soil. The clean topsoil was a sandy loam soil (Clay: 12%, Silt: 28%, Sand: 60%, pH: 7.6, TOM: 5.0% w/w) supplied by Bailey's of Norfolk (Norfolk, UK). For each treatment, five exposure replicates (i.e. individual mesocosm cylinders, see **Figure 6.1C**) were prepared. Mixing of the soil and sludges was done per replicate. For each replicate, 600 ml of the prepared sludge was thoroughly mixed with the w.w. equivalent of 7 kg d.w. the sandy loam top soil (**Figure 6.1A**). Final concentration of sludge solids in the mixtures was 0.3% (w/w). This concentration was equal to the maximum amount of sludge that can be added to agricultural land per year by area according to United Kingdom regulations (Defra, 2009). Final nominal concentrations of Cu, Ag and PS in the soil/sludge mixtures were 40, 20 and 100 mg/kg, respectively. The amount of Cu added as CuO-NP was based on the maximum recommended yearly application rate of the copper fungicide KIOCIDE® 2000 (DuPont™) for potato farming and equivalent of eight years application at maximum rate. For Ag the concentration was selected based on a survey conducted by United States Environmental Protection Agency, which found Ag concentrations in sewage sludges ranging between 2 to 195 mg Ag/kg, with the 95th percentile being 57 mg Ag/kg (U.S. EPA, 2009). The amount of Ag-NP added to the sludge was based on this latter concentration and equivalent to eight years of the maximum allowable sludge application to agricultural soils in the UK at maximum rates (Defra, 2009). The current emission rates for microplastics to soils are estimated to range between 0.6 and 60 g/ha/year (Kawecki & Nowack, 2020). Owing to the lack of data on polymeric NP concentrations in sewage sludges and unknown current release rates to soils, the amount of PS-NP added was not

based on any measured or predicted values but represented a hypothetical future high concentration scenario.

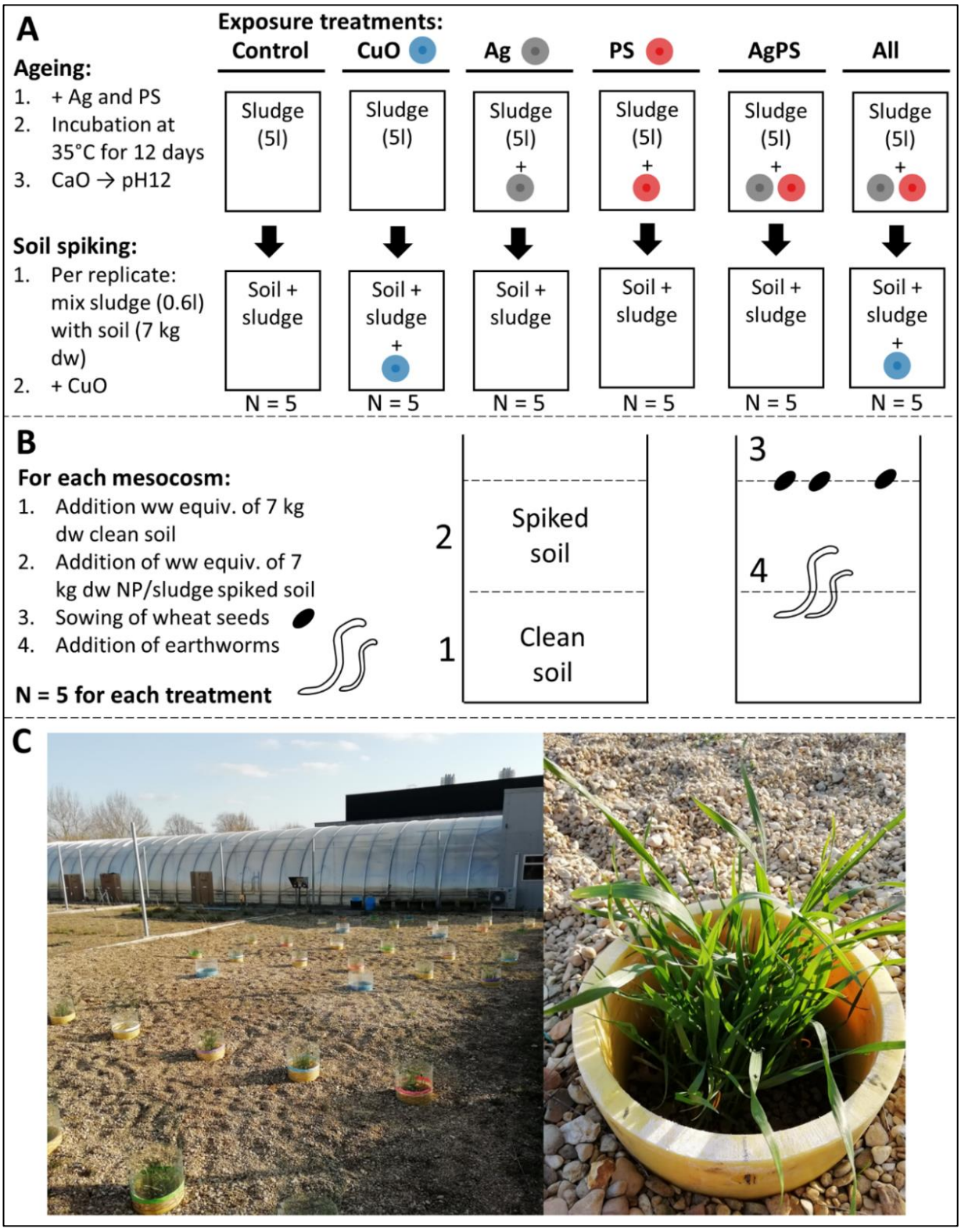


Figure 6.1: Schematic overview of the ageing procedure of NPs in sludge and the soil spiking (**A**), set-up of the exposure replicates (**B**) and photographs of mesocosms in outdoor gravel beds (**C**). PS, polystyrene. Ageing and soil spiking was done by mixing of PS-NPs and/or Ag-NPs with sludges, followed by a incubation at 35 °C for 12 days and lastly the addition of CaO to bring the pH to 12. Sludges were then mixed with soils and CuO-NPs were subsequently added to the relevant exposure replicates and further mixed.

6.2.4 Set up of mesocosms

The mesocosms consisted of 20.5 by 38 cm cylinders and were placed in an outdoor gravel bed in a randomised layout (**Figure 6.1B**). Each mesocosm was filled with two distinct layers of soils. First, each mesocosm received the w.w. equivalent of 7 kg d.w. the unpolluted sandy loam topsoil. This layer represented the agricultural soil that is not affected by ploughing and was thus not spiked with sludge or NPs (herein, 'bottom soil'). The mesocosms were then topped with the w.w. equivalent of 7 kg of the respective soil/sludge mixture (described above). This layer represented the layer of an agricultural soil which is mixed through ploughing and which may receive sludges as well as pesticide applications (herein, 'top-soil'). The mesocosms were then left to settle for two weeks to allow soils to stabilize. After this time, five g of top-soil was collected from each mesocosm and subsequently dried and stored for metal concentrations measurement. Each mesocosm then received ten *Lumbricus terrestris* (Lumbricidae) (eight animals without clitellum and two animals with fully developed or developing clitella) and fifteen fully clitellated *Aporrectodea caliginosa* (Lumbricidae) earthworms. In addition, each mesocosm received 20 *E. crypticus*, 18 woodlice (*P. scaber*) and 50 springtails (*F. candida*). Ten wheat seeds (*Triticum aestivum*) were sowed in the top layer of the sludge. Every two weeks a handful of dried mixed leaves were added to the mesocosms as food for earthworms. The total duration of the exposure was six months. Total metal concentrations of soils were determined using inductively coupled plasma mass spectrometry at the UK Centre for Ecology and Hydrology, Lancaster (UK), according to Lahive et al. (2017). Briefly, for each sample, 0.75 g of air-dried soil or soil/sludge mixture was refluxed with a 3:1 mixture of hydrochloric and nitric acids. After digestion, the solutions allowed to cool and subsequently filtered through an 8-µm paper filter. Digests were then diluted to a 50 ml volume using 0.5% v/v nitric acid and measured using a Perkin Elmer Nexion 300D. The impact of the various treatments on woodlice, springtails and Enchytraids were assessed but are not further discussed in this document.

6.2.5 Earthworm sampling

At the end of the exposure, mesocosms were removed from the gravel beds and earthworms were subsequently collected by destructive sampling. For each mesocosm, up to three *L. terrestris* earthworms were anesthetized by submerging the animals in carbonated water for 2.5 minutes. Earthworms were first dissected by making a dorsal incision spanning a 40-segment region between segment 40 and 80 using sterile equipment. In this study, two types of earthworm microbiome samples were collected: i) the midgut tissue that was thoroughly cleaned to remove soil particles (for both *A. calignosa* and *L. terrestris* earthworms) (herein, 'resident gut' sample/microbiome) and ii) the contents of the midgut (but only for *L. terrestris*) (herein, 'faecal' sample/microbiome). Guts

contents of the dissected region were removed using a sterile spatula. Using a sterile pipet tip, ~50 mg of the gut contents were sampled and placed in a bead beating tube containing 500 µl RNA/DNA shield™ (ZymoReseach, Irvine, CA, USA) ('faecal' sample). The midgut between segment 40 and 60 was dissected and placed in a microcentrifuge tube containing 1 ml of phosphate buffered saline (PBS). The gut tissue was then cleaned by vortexing the tube for 30 seconds repeated twice. Previous studies have shown that rinsing of the midgut is an efficient method to remove residual soil particles and can help to identify the resident gut microbiome independent from soil (**Chapter 3**). The cleaned midgut ('resident gut' sample) was placed in a bead beating tube containing 500 µl ml of RNA/DNA shield. Upon collection from the soil, per replicate, up to three *A. calignosa* were each placed on wetted filter paper in a Petri dish to depurate their gut contents for two days. After two days, *A. calignosa* were euthanized in 100% ethanol and the midgut spanning a 20-segment region posterior to the clitellum was dissected using sterile equipment following **2.1.1.** The midgut was centrifuged in 1 ml of PBS two times for 30 seconds as described above. The cleaned midgut was placed in a bead beating tube 500 µl of RNA/DNA Shield™ ('resident gut' sample). Immediately after collection, all resident gut samples and faecal samples were homogenized two times for one minute using a FastPrep-24™ (MP Biomedicals, Santa Ana, CA, USA), incubated at 4°C overnight and stored at -20°C until DNA extraction.

6.2.6 Sampling of soils for microbiome analysis

During the take-down of the mesocosms, soil samples were collected for microbiome analysis. For this purpose, a 2 ml cryo-vial was filled with soil collected from the top five cm of the soil ('top-soil' sample). From the layer of soils at the bottom half of the mesocosm that did not receive any sludges, an additional soil sample was collected in a 2 ml cryo-vial ('bottom-soil' sample). The vials were stored at -80 °C until DNA extraction.

6.2.7 DNA extractions, amplicon sequencing and bioinformatics

Genomic DNA from midgut tissue and faecal samples were extracted following the procedure in **2.2.1**. Soil genomic DNA was extracted using a DNeasy PowerSoil HTP 96 Kit using 250 mg of w.w. soil as input material and by following the manufacturer's protocol. Integrity of extracted genomic DNA was verified via agarose gel electrophoresis and purity and concentration were determined through spectrophotometry. The prokaryotic community in the genomic DNA extracted from midgut tissues, faecal samples and soil samples was determined by PCR amplification and sequencing of a ~550 bp fragment spanning the V3-V4 region of the 16S-rRNA gene, following the method outlined by Kozich et al. (2013) and described in detail in **2.2.2**. The earthworm sequencing library and soil sequencing library were sequenced on separate Illumina MiSeq flow cells. The

Illumina demultiplexed sequences were processes using the DADA2 bioinformatics pipeline (Callahan et al., 2016) as described in **2.2.3**. DNA extractions and PCR amplification and sequencing of the 16S-rRNA gene of soil samples was conducted by Dr. Melanie Gibbs and Tim Goodall at the UK Centre for Ecology and Hydrology.

6.2.8 Data analysis of earthworm microbiomes

All data analysis was conducted in R. Microbiome analysis of the bacterial community associated to *A. calignosa* midgut samples (herein, 'total resident gut microbiome of *A. calignosa*') indicated that some of the *A. calignosa* samples were likely contaminated with residual soil as indicated by the high abundance of soil specific taxa in these samples (see **6.3.2**). The soil contamination in these samples was likely a major driver for the community structure, which compromised the ability to test for effects of treatments. To remove the effects of soil contaminants and to allow testing of the research hypotheses, a list of core ASVs that likely represented part of the true resident gut microbiome of *A. calignosa* was identified. Selection of these core ASV was done by first selecting 20 samples with likely lower levels of soil contamination (any sample with a value <0 on the NMDS1 axis in **Figure 6.2**). Using this subset of samples, core ASV were identified using the R package 'microbiome' (Lahti et al., 2017) and defined as any ASV with a prevalence over 1/5 in this subset of samples. All ASVs that were not part of this set of core ASVs were then removed from the original dataset. The resulting subset (herein, 'core gut microbiome of *A. calignosa*') was then subjected to analysis for treatment effects, as described below.

All statistical microbiome analysis was done using rarefied datasets. Rarefication was performed using the R package 'phyloseq' (McMurdie & Holmes, 2013) with removal of samples below the rarefaction threshold (**Table 6.1**). Calculation of diversity indices (total number of observed ASVs and Shannon diversity index) and non-metric dimensional scaling (NMDS) was conducted using the R package 'vegan' (Oksanen et al., 2018). In this study, up to three individuals (per earthworm species) were sampled from every mesocosm. The earthworms sampled from the same mesocosm are, thus, not independent but are nested within mesocosm. Mesocosms represent the true biological replicate. In all univariate and bivariate data analysis applied in this study, the nested design was taken into account by using (generalized) linear mixed-effects models. These mixed-effects models were run using treatment, total Cu concentration or total Ag concentration as fixed effects and mesocosm as random effect. In multivariate analysis of variance, nested permutational analysis of variance (Permanova) were used to take into account the nested design.

Differences in bacterial diversity were calculated using linear mixed-effects (lme) models using the R package 'lme4' (Bates et al., 2015). Multivariate homogeneity of groups dispersions were calculated and tested for significance using the 'vegan' functions 'betadisper' and 'permutest' applying 9999 permutations. Nested Permanova models, applying 9999 permutations, were used to test the significance of the relation between the bacterial community structure and the explanatory variables (i.e. treatment as factor, total Cu concentration at the start of the experiment (herein, 'T0') and total Ag concentration at T0)

Table 6.1: Number of mesocosms (N) and samples (Obs.) included in each dataset before and after rarefication and the rarefication threshold applied per data set.

	Before rarefaction		Rarefaction	After rarefaction	
Microbiome	**N**	**Obs.**	**threshold**	**N**	**Obs.**
A. calignosa - total	29	52	4361	27	46
A. calignosa - core	29	52	4217	25	40
L. terrestris - resident	30	82	8086	30	80
L. terrestris - faecal	30	82	10544	30	81

For datasets with a significant effect of treatment on the community structure, analysis of similarity percentages (SIMPER) was performed using the 'vegan' function 'simper'. SIMPER analysis identifies the contribution (in %) of each ASV to the Bray-Curtis dissimilarity metric between two groups and was used to identify the ASV that explain most of the variance between groups. SIMPER analysis was conducted using a size-reduced dataset for computational reasons. Size reduction was performed by removing all ASV with a prevalence equal to or under 1/10. After removal of these taxa, 829 taxa remained which were subjected to SIMPER analysis applying 100 permutations. Taxa that explained more than 1% of the between-group variance were selected (herein, 'greatest drivers'). The relation between the read abundance of these 'greatest drivers' and the explanatory variables (i.e. treatment as factor, total Cu concentration at T0 and total Ag concentration at T0) was tested using generalized linear mixed-effects models (glme) using the R package ('lme4'). Read abundances of the 'greatest drivers' were Poisson distributed but were strongly over-dispersed (data not shown). Accordingly, negative binomial (NB) models were used instead of Poisson based models to test relationships between read abundances and the various fixed effects, following literature (Lindén & Mäntyniemi, 2011; Ver Hoef & Boveng, 2007).

6.2.9 Testing the relation between earthworm microbiomes and bacterial communities of the soil

To test whether differences between treatment and samples in the earthworm microbiome composition were related to differences in the composition of the soil bacterial communities, matrix correlation analysis was conducted using Mantel tests. Each earthworm microbiome was

compared to both the soil bacterial community of the top-soil of the mesocosms and soil bacterial community of soil sampled at the bottom half of the mesocosms. For this purpose, pseudo-replicates were merged into single values using the 'phyloseq' function 'merge_samples'. Earthworm and soil bacterial community datasets were then rarefied to a single rarefaction threshold to remove any library size biases (**Table 6.2)**, with the exception for the comparison between the core *A. calignosa* and the soil bacterial communities. In this case, after selecting the core *A. calignosa* ASVs, for many of the soil samples, the total number of reads was so low that rarefication was not a useful method to remove library size biases. However, the distance matrixes that were used for the Mantel tests were calculated using Bray-Curtis as distance metric. This non-parametric distance metric is less sensitive to library sizes differences and is likely to mitigate some of the effects of unequal sample sums. Mantel correlation analysis was conducted using the package 'vegan' and statistical significance was tested using 9999 permutations.

6.3 Results:

6.3.1 Metal recovery and sulphidation levels of Ag-NPs in aged sludge

The mean (±SD) Cu and Ag recovery of the expected exposure concentration in the top-soils after correction for metal concentrations in the controls was on 91.7 % (±SD 5.1) and 75.11% (±7.2) for Cu and Ag, respectively. The Ag and Cu concentrations of the single NP exposures were equal to the Ag and Cu concentrations in the combined exposures (t-test: $p>0.05$).

Table 6.2: Pearson correlation coefficients and statistical significance of Mantel tests testing the correlation between different combinations of community distance matrices. Bold *p*-values indicate statistical significance

Pairwise comparison			Pearson's *r*	*p*-value
Distance matrix	**Distance matrix**	**Rarefaction threshold**		
A. calignosa – total resident	Top soil	6691	-0.013	0.540
	Bottom soil	6691	0.068	0.226
A. calignosa – core resident	Top soil	na[a]	-0.099	0.900
	Bottom soil	na[a]	0.068	0.226
L. terrestris - resident	Top soil	13240	-0.112	0.875
	Bottom soil	9926	-0.108	0.819
L. terrestris - faecal	Top soil	10544	0.022	0.411
	Bottom soil	9926	-0.028	0.556
L. terrestris - resident	*L. terrestris* - faecal	8086	0.241	**0.001*** **

*** indicates p<0.001, [a] For the comparison between the core *A. calignosa* and soil bacterial communities, datasets were not rarefied, as discussed above in **6.2.10**.

6.3.2 The relation between earthworm microbiomes and the bacterial communities of soils

Matrix correlation analysis was conducted using Mantel tests to test whether differences between treatments and samples in the composition of the earthworm microbiomes were related to the

differences in the bacterial communities of the surrounding soils. None of the earthworm microbiome communities displayed a significant correlation with the bacterial communities of the soils (**Table 6.2**). The faecal bacterial community did correlate significantly to the resident microbiome of *L. terrestris*.

6.3.3 The total resident gut microbiome of A. calignosa

NMDS analysis indicated the existence of two clusters of samples separated along NMDS axis one (**Figure 6.2A**). Differential clustering of these samples was suspected to be due to soil contamination. To test this, we defined two sample clusters: one consisting out of all samples with NMDS axis 1 value lower than zero ('Cluster A') and the other consisting of the samples densely clustered around the high end of the NMDS axis 1 (>0.038 of NMDS axis 1) ('Cluster B'). The observed number of taxa in 'Cluster B' was significantly higher than in 'Cluster A' (lme: $X^2(1)=76.6$, $p<0.001$) (**Figure 6.2C**). In addition, the read abundance of the bacterial phyla *Acidobacteria* and *Gemmatimonadetes* in 'Cluster B' were higher than in 'Cluster A' (glme: $z=3.07$, $p<0.01$ and glme: $z=6.96$, $p<0.001$, respectively) (**Figure 6.2B** and **6.2D**). Both phyla are typical for soil bacterial communities and are not common in earthworm resident microbiomes (**Chapter 3**; Prochazkova et al, 2018). The higher abundance of soil specific bacteria and the higher observed number of taxa in 'Cluster B' compared to 'Cluster A' both indicated that samples in 'Cluster B' were likely contaminated with soil. This was further supported by observations made during the dissection of the *A. calignosa* tissues. After the 48h depuration period, the gut of four animals still contained visible amounts of soil. Most of the visible soil in these samples was however removed during the cleaning procedure. However, in the NMDS plot, three of these samples were clustered in 'Cluster B' whilst the ordination of one sample was just outside of the 'Cluster B' perimeter indicating that even despite cleaning some residual soil was likely to have remained.

6.3.4 The core resident gut microbiome of A. calignosa

To remove the effects of soil contamination as the major influence on microbiome NMDS ordination, a list of core resident ASVs was identified (see **6.2.8**). The mean alpha-diversity (±SD) of this core microbiome across all samples for observed no. of taxa was 32 (±7) and Shannon diversity index 1.9 (± 0.7) (**Figure 6.3A**). There was no evident clustering of samples in the core gut microbiome by the sample clusters identified in the total gut microbiome (as described above) (**Figure 6.3B**). The core gut microbiome of *A. calignosa* was dominated by *Proteobacteria* (mean ±SD 66.2% ± 31.2%), *Actinobacteria* (14.5 ± 17.6%), *Firmicutes* (9.5 ± 12.5%), *Tenericutes* (5.9 ± 17.5%) and *Chloroflexi* (3.5 ± 7.6%) (**Figure 6.3C**). There were no *Acidobacteria* or

Gemmatimanodetes present in the *A. caligniosa* core gut microbiome, suggesting that by selecting only ASVs with a prevalence >1/5 soil contamination was efficiently removed

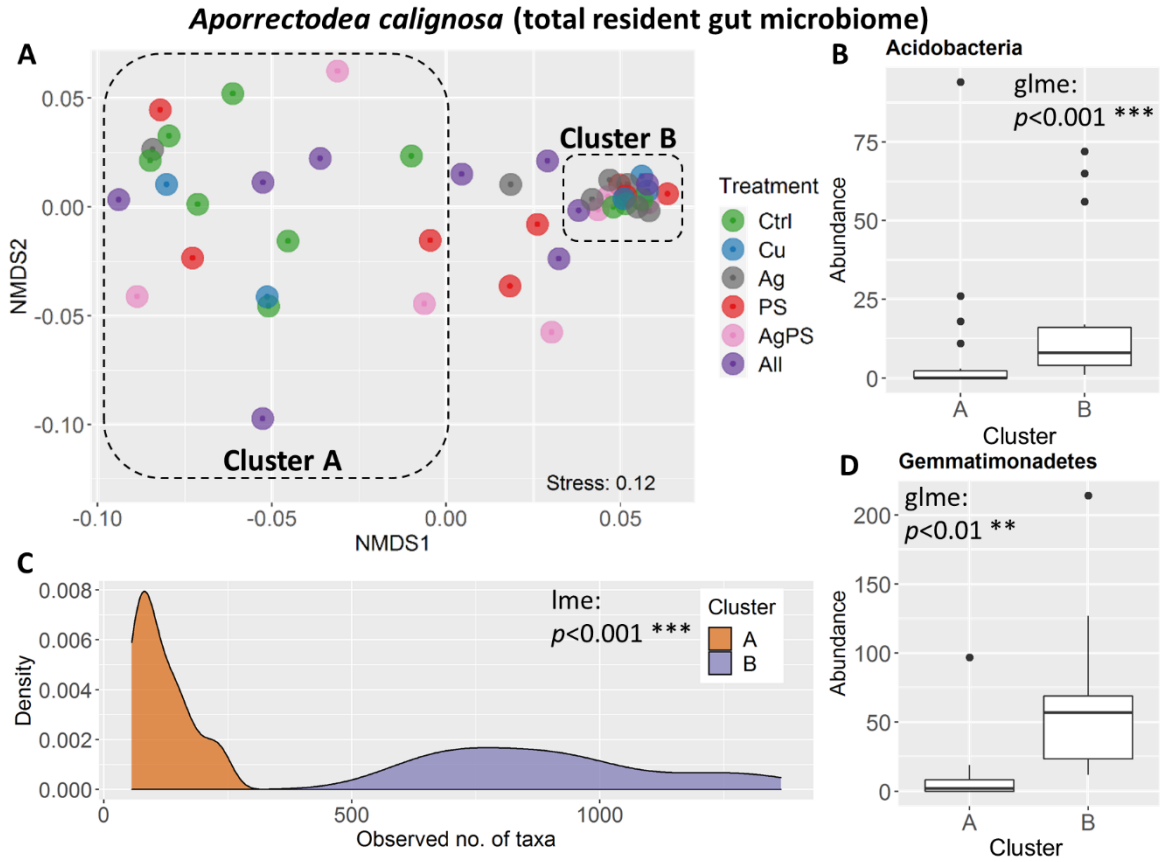


Figure 6.2: Effects of six-month NP exposures on the total microbiome of the earthworm *A. caligniosa*. (**A**) NMDS plot showing the ordination of samples and the two identified clusters. Different colours indicate different treatments. (**B**) Total read abundance of *Acidobacteria* per identified sample Ccuster (**C**) Smoothed kernel density estimation of observed number of taxa per identified cluster. (**D**) Total read abundance of *Gemmatimonadetes* per identified cluster. *P*-values in **B, C** and **D** are derived using glme- or lme-models using 'Cluster' as fixed effect and mesocosm as random effect, ** indicates $p<0.01$, *** indicates $p<0.001$. Points indicate outliers (CI>95%).

Table S6.1 lists the top 30 most abundant taxa in the core resident gut microbiome of *A. caligniosa*. The relative abundances of most of these taxa were at least one order of magnitude higher in the gut microbiome than in the top-soil (19 out of 30) or bottom soil (21 out of 30). The core microbiome was dominated by a relatively small number of ASVs with six ASVs together comprising >50% of all reads (**Table S6.1**). The most abundant ASV was an unknown *Enterobacteriaceae* (ASV 2) which comprised (± SD) 21% (± 29%) of the community, followed by the *Verminephrobacter* symbiont (ASV 10) (12.3 ± 23%), *Pseudarthrobacter* (ASV1) (8.8 ± 13%), '*Candidatus* Lumbricincola' (ASV 6) (6.0 ± 18%), *Aeromonas* (ASV 19) (4.2 ± 9.2%), and *Sphaerobacter thermophiles* (3.6 ± 7.5) (**Table S6.1**). Local alignment using NCBI BLASTn indicated that the 16S-rRNA gene sequence of *Enterobacteriaceae* (ASV 2) was most similar to that of an uncultured bacterium (GenBank: AB991318) isolated from the depurated gut of the ice glacier earthworm *Mesenchytraeus solifugus*

(Enchytraeidae) (Murakami et al., 2015). Sequence similarity between these two 16S-rRNA gene fragments was 97.9%. The ASV 2 sequence similarity also had an 89.7% similarity with the 16S-rRNA gene sequence of an *Enterobacteriaceae* isolated previously from the gut contents of *A. caliginosa* (Ihssen et al., 2003).

6.3.5 Impact of treatments on the core resident gut microbiome of A. caliginosa

Alpha-diversity of the core gut microbiome of *A. caliginosa* was not affected by treatment (Shannon: $X^2(5)=1.33$, $p=0.932$; observed no. of taxa: $X^2(5)=1.93$, $p=0.860$) (**Figure 6.3A**). Treatment also did not significantly affect the core bacterial community structure (nested Permanova: $F(5,21,19)=1.021$, $R^2=0.107$, $p=0.414$) (**Figure 6.3B**). The earthworm intestinal symbiont *'Candidatus* Lumbricincola' was affected by exposure to Ag-NPs and PS-NPs for which this symbiont was, in contrast to in other treatments, below detectable levels (glme: $z=-2.67$, $p<0.01$) (**Figure 6.3D**). Previous studies showed that the earthworm associated *Spirochaetaceae* were sensitive to copper exposure (**Chapter 4**). However, in the core microbiome of A. *caliginosa* no *Spirochaetaceae* were detected.

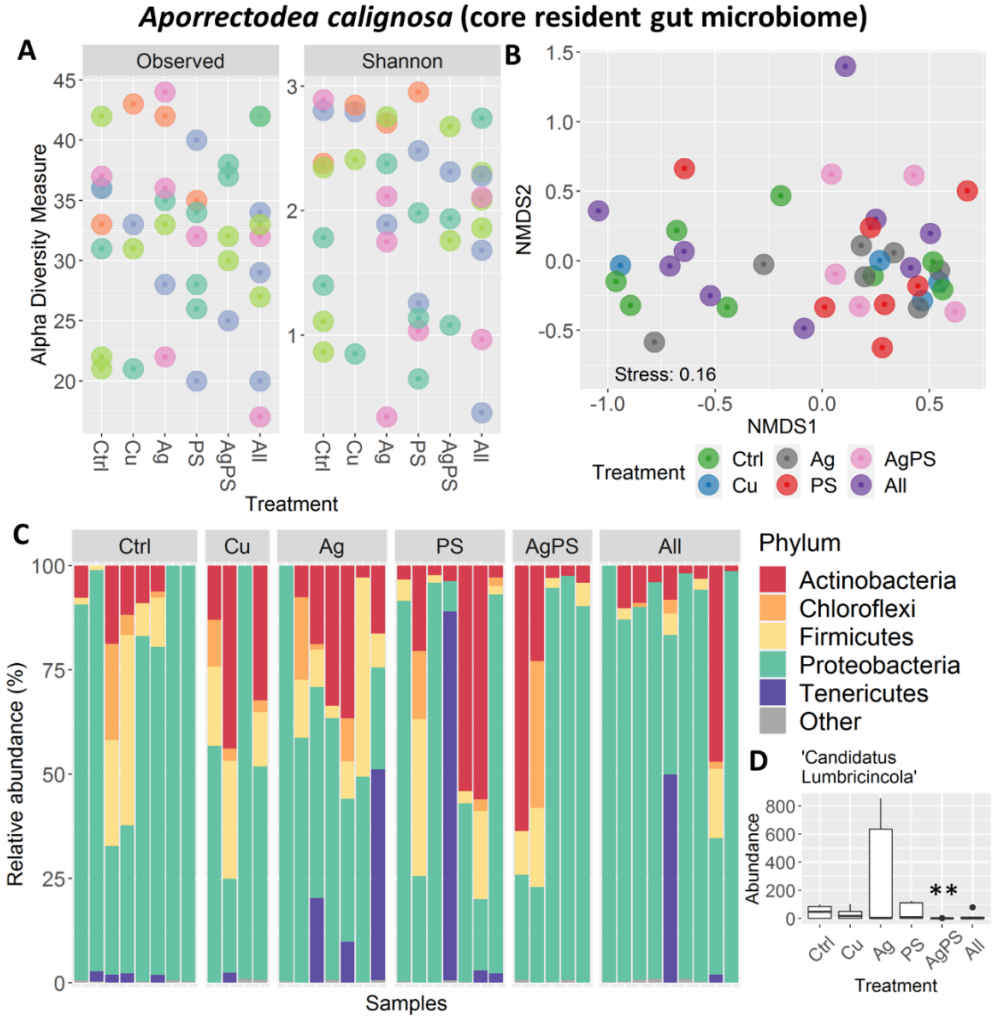


Figure 6.3: *description on next page*

Figure 6.3 (description): Effects of six-month NP exposures on the core microbiome of the earthworm *A. calignosa*. (**A**) Total number of observed taxa (left panel) and Shannon diversity index (right panel) per treatment. Different colours indicate different mesocosm replicates. (**B**) NMDS plot showing the ordination of samples. Different colours indicate different treatments. **(C**) Relative abundance in % of phyla per sample. All phyla with a relative abundance <2% are grouped under 'Other'. (**D**) Read abundance of '*Candidatus* Lumbricincola' per treatment; ** indicates a significant difference of the respective treatment compared to the control with $p<0.01$

6.3.6 The resident gut microbiome of L. terrestris

The mean alpha-diversity of the resident gut microbiome of *L. terrestris* across all samples was 684 (±SD 366) (observed no. of taxa) and 4.2 (±1.5) (Shannon index) (**Figure 6.4A**). This microbiome was dominated by *Proteobacteria* (36.5% ± SD 24.8%), *Actinobacteria* (21.6 ± 12.8%), *Firmicutes* (15.7 ± 9.0%), *Tenericutes* (13.8 ± 17.9%), *Bacteroidetes* (4.6 ± 5.8%) and *Chloroflexi* (2.4 ± 2.5%) (**Figure 6.4C**). The ASVs with the highest average abundances were an unknown *Proteobacteria* (ASV 4), the earthworm symbiont *Verminephrobacter* (ASV 8), two unknown *Mycoplasmataceaea* (ASV 7 and 9), *Pseudarthrobacter* (ASV 1) and *Deftia* (ASV 16) with these taxa contributing to nearly a third of the total community (**Table S6.2**). Most of the top ten most abundant resident gut taxa were below detectable levels in both the top soil and the bottom soil, the exception being *Pseudarthrobacter* (ASV 1) whose relative abundance in the gut tissue roughly matched those in the soil bacterial communities (**Table S6.2**). In addition to the *Verminephrobacter* symbiont ASV 8, eight other *Verminephrobacter* ASVs were present in the *L. terrestris* microbiome. Four of the nine *Verminephrobacter* ASVs, 8, 23, 28 and 81, together comprised of >99% of all reads from this genus.

Table 6.3: Outcomes of pairwise nested-Permanova tests testing the effects of each NP treatment on the faecal microbiome. Statistically significant *p*-values are in bold.

Pairwise comparison			F-value	R^2	Adjusted *p*-value[a]
Treatment	vs.	Treatment			
Control		CuO-NP	2.237	0.07	**0.016 ***
Control		Ag-NP	1.637	0.06	0.075
Control		PS-NP	1.424	0.05	0.347
Control		AgPS-NP	1.648	0.06	0.149
Control		All-NP	1.818	0.06	0.090

[a] *p*-value after Bonferonni correction; * p <0.05

6.3.7 Impact of treatments on the resident gut microbiome of L. terrestris

Alpha-diversity of the resident gut microbiome of *L. terrestris* was not affected by treatment for the observed no. of taxa ($X^2(5)=3.01$, $p=0.699$) and Shannon index ($X^2(5)=2.79$, $p=0.733$). Treatment also did not significantly affect the resident microbiome structure (nested Permanova: $F(5,24,50)=0.90$, $R^2=0.055$, $p=0.769$) (**Figure 6.4B**). In total 15 ASV taxonomically assigned to *'Candidatus* Lumbricincola' were found in the resident gut microbiome of *L. terrestris*. Three (ASV 6, 39 and 57) together comprise of >96% of all '*Candidatus* Lumbricincola' reads. There was no statistically

significant effect of any of the treatments on the read abundance of ASV6 and ASV 57, but not ASV 39 for which the Ag-NP treatment had a significant negative effect on read abundance (glme: z=-2.16, p<0.05) (**Figure 6.4D**). Measured soil Ag concentration was however not significantly related to the read abundance of ASV 39 (glme: z=3.14, p=0.536). Nine ASVs of the family *Spirochaetaceae* were found in the resident gut microbiome of *L. terrestris*. One (ASV 98) comprised of >97% of the all *Spirochaetacea* reads. The read abundance of this ASV was not statistically significantly affected by any treatments (glme: p>0.05).

Table 6.4: Parameter estimates of generalized linear mixed models testing the relationship between read abundance of selected ASVs (i.e. the greatest drivers the community structure) in the faecal microbiome and treatment as factor. Bold *p*-values indicate statistical significance.

Bacterial taxa	Fixed effects[a]	Beta (est.)	SE	z-value	*p*-value
Pseudarthrobacter (ASV 1)	Cu	0.58	-0.29	2.02	**0.042 ***
	Ag	0.50	-0.28	1.75	0.081
	PS	0.41	-0.28	1.45	0.148
	AgPS	0.55	-0.28	1.97	**0.049 ***
	All	0.62	-0.28	2.22	**0.027 ***
'Candidatus Lumbricincola' (ASV 6)	Cu	-1.49	-0.60	-2.42	**0.015 ***
	Ag	0.19	-0.61	0.31	0.759
	PS	-0.63	-0.59	-1.06	0.289
	AgPS	-0.30	-0.60	-0.50	0.617
	All	-0.72	-0.60	-1.21	0.228
Cellvibrio (ASV 24)	Cu	-0.63	-0.48	-1.41	0.160
	Ag	0.59	-0.45	1.30	0.194
	PS	-0.56	-0.45	-1.27	0.206
	AgPS	0.66	-0.46	1.43	0.154
	All	-0.37	-0.45	-0.83	0.408
Shewanella (ASV 42)	Cu	-1.21	-0.71	-1.69	0.091
	Ag	-0.87	-0.71	-1.23	0.220
	PS	-0.64	-0.71	-0.89	0.371
	AgPS	-1.18	-0.71	-1.65	0.099
	All	-0.80	-0.71	-1.13	0.260

[a] N observations: 81, N groups: 30; * p < 0.05.

6.3.8 The faecal microbiome of L. terrestris

The mean (± SD) alpha-diversity of the faecal microbiome of *L. terrestris* across all samples for observed no. of taxa was 1021 (±SD 274) and Shannon index 5.8 (±0.5) (**Figure 6.5A**). The faecal microbiome of *L. terrestris* was dominated by *Actinobacteria* (34.5 ± 8.0), *Firmicutes* (21.5±5.9), *Proteobacteria* (18.4 ± 6.1), *Bacteroidetes* (9.08 ± 5.2), *Chloroflexi* (5.0 ± 2.3), *Tenericutes* (4.7 ± 5.9) and *Verrucomicrobia* (3.0 ± 2.0) (**Figure 6.5C**). The six most abundant taxa in the faecal microbiome of *L. terrestris* were *Pseudarthrobacter* (ASV 1 and 13), '*Candidatus* Lumbricincola' (ASV 6), *Flavobacterium* (ASV 17) and *Cellvibrio* (ASV 24), these taxa comprising ~15% of the total community. **Table S6.3** lists the top 30 most abundant faecal microbiome taxa. For only seven of these taxa, the relative abundance in the faecal samples was at least an order of magnitude higher

than the relative abundance of these taxa in the top-soil bacterial community. Comparison of the faecal microbiome with the bottom-soil bacterial community shows a different trend. The relative abundance of most (i.e. 23) of these top 30 faecal taxa was at least one order of magnitude higher in the faecal microbiome compared to the bottom soil bacterial community (**Table S6.3**).

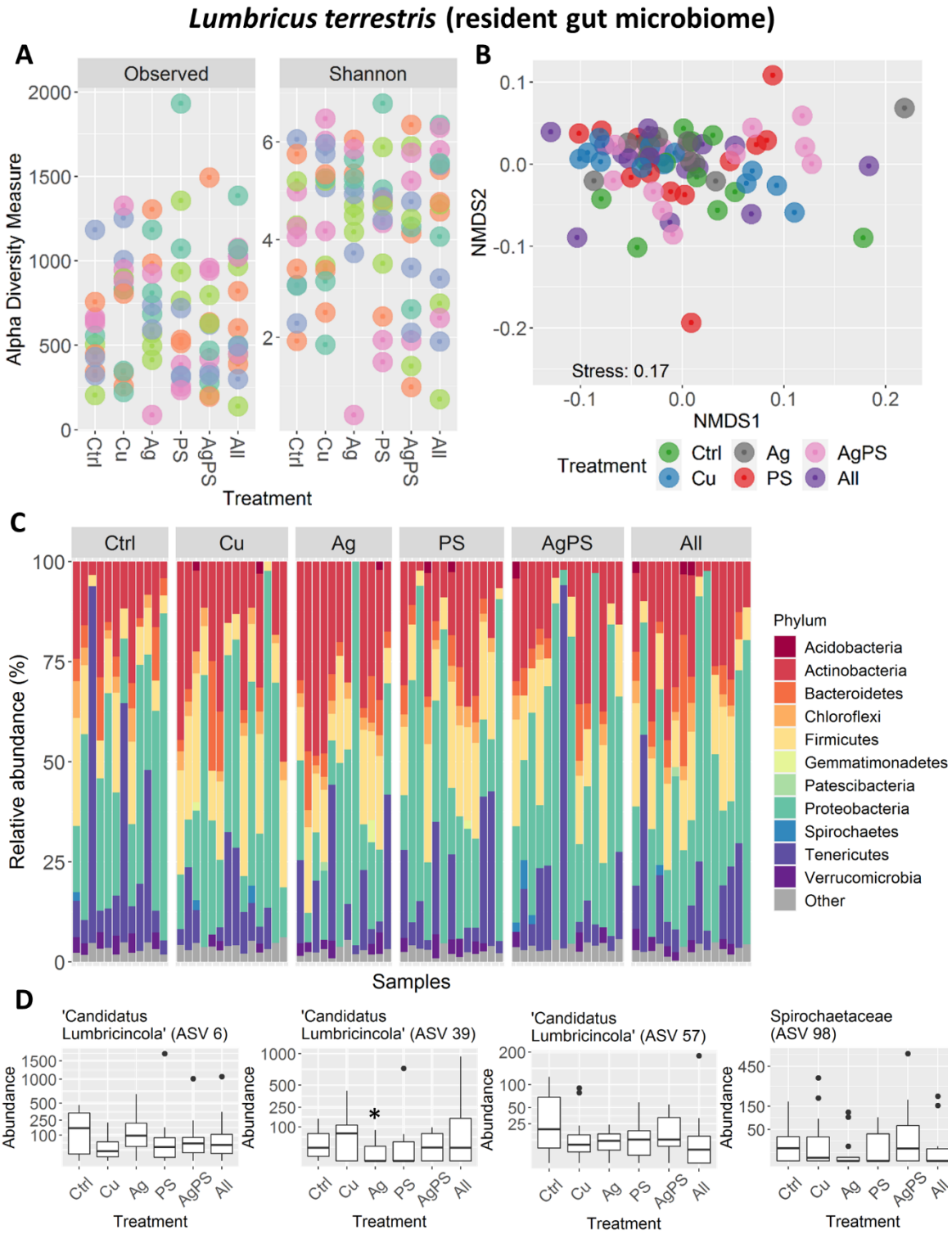


Figure 6.4: Effects of six-month NP exposures on the resident gut microbiome of the earthworm *L. terrestris*. (**A**) Total number of observed taxa (left panel) and Shannon diversity index (right panel) per treatment. Different colours indicate different mesocosm replicates. *Description continues on next page.*

Description Figure 6.4 continued: (**B**) NMDS plot showing the ordination of samples. Different colours indicate different treatments. (**C**) Relative abundance in percentage of phyla per sample. All phyla with a relative abundance <2% are grouped under 'Other'. (**D**) Read abundance of the most abundant '*Candidatus* Lumbricincola' and *Spirochaetaceae* per treatment; * indicates a significant difference of the respective treatment compared to the control with $p<0.05$. Points indicate outliers (CI>95%).

6.3.9 Impact of treatments on the faecal microbiome of L. terrestris

Alpha-diversity of the faecal microbiome of *L. terrestris* was not affected by treatment for observed no. of taxa ($X^2(5)=6.21$, $p=0.287$) and Shannon index ($X^2(5)=6.55$, $p=0.256$) (**Figure 6.5A**). Treatment did however have a significant effect on the faecal microbiome structure (nested Permanova: $F(5,24,51)=1.64$, $R^2=0.085$, $p<0.001$) (**Figure 6.5B**). Pairwise nested Permanova analysis with Bonferonni correction showed that only the CuO-NP treatment had a significant effect on the faecal microbiome structure compared to control (**Table 6.3**). There was no significant difference in the homogeneity of dispersion (i.e. variance) between the different treatments (permutation test: $F(5,75)=0.496$, $p=0.796$), indicating that treatments were likely driven by differences in means rather than group variances.

Analysis of similarity percentages (SIMPER) was used to assess which ASVs contribute most to the community structure. Only four ASVs contributed more than 1% of total Bray-Curtis dissimilarity metric, *Pseudarthrobacter* (ASV 1), '*Candidatus* Lumbricincola' (ASV 6), *Cellvibrio* (ASV 24) and *Shewanella* (ASV 42). Read abundance of *Pseudarthrobacter* (ASV 1) in the faecal microbiome was significantly higher in the 'CuO', 'AgPS' and 'All' treatments compared to controls (**Figure 6.5D**, **Table 6.4**). There was, however, no statistically significant relationship between the read abundance for this ASV and measured Cu and measured Ag concentration in the soil (**Table 6.5**). Candidatus Lumbricincola' (ASV 6) read abundance in the faecal microbiome was significantly lower than controls in the 'CuO' treatment (**Figure 6.5D, Table 6.4**). Also, this ASV was negatively related to measured total soil copper concentration (**Table 6.5**). The read abundance of *Cellvibrio* (ASV 24) and *Shewanella* (ASV 42) were not affected by any of the treatments (**Figure 6.5D, Table 6.4)**. However, the read abundance of *Cellvibro* was significantly positively related to measured Ag concentrations (**Table 6.5**).

In total 287 ASVs taxonomically assigned to '*Candidatus* Lumbricincola' were found in faecal microbiome of *L. terrestris*. Three of these (ASV 6, 39 and 57) together comprised of >94% of all '*Candidatus* Lumbricincola' reads but only one (ASV 6) was significantly affected by a NP treatment (see above). In total 18 *Spirochaetacea* ASV were recorded in the faecal microbiome, of which one ASV (i.e. ASV 98) comprised of >94% of all taxa reads. The read abundance of this ASV was not significantly affected by any of the treatments (glme: $p>0.05$, data not shown).

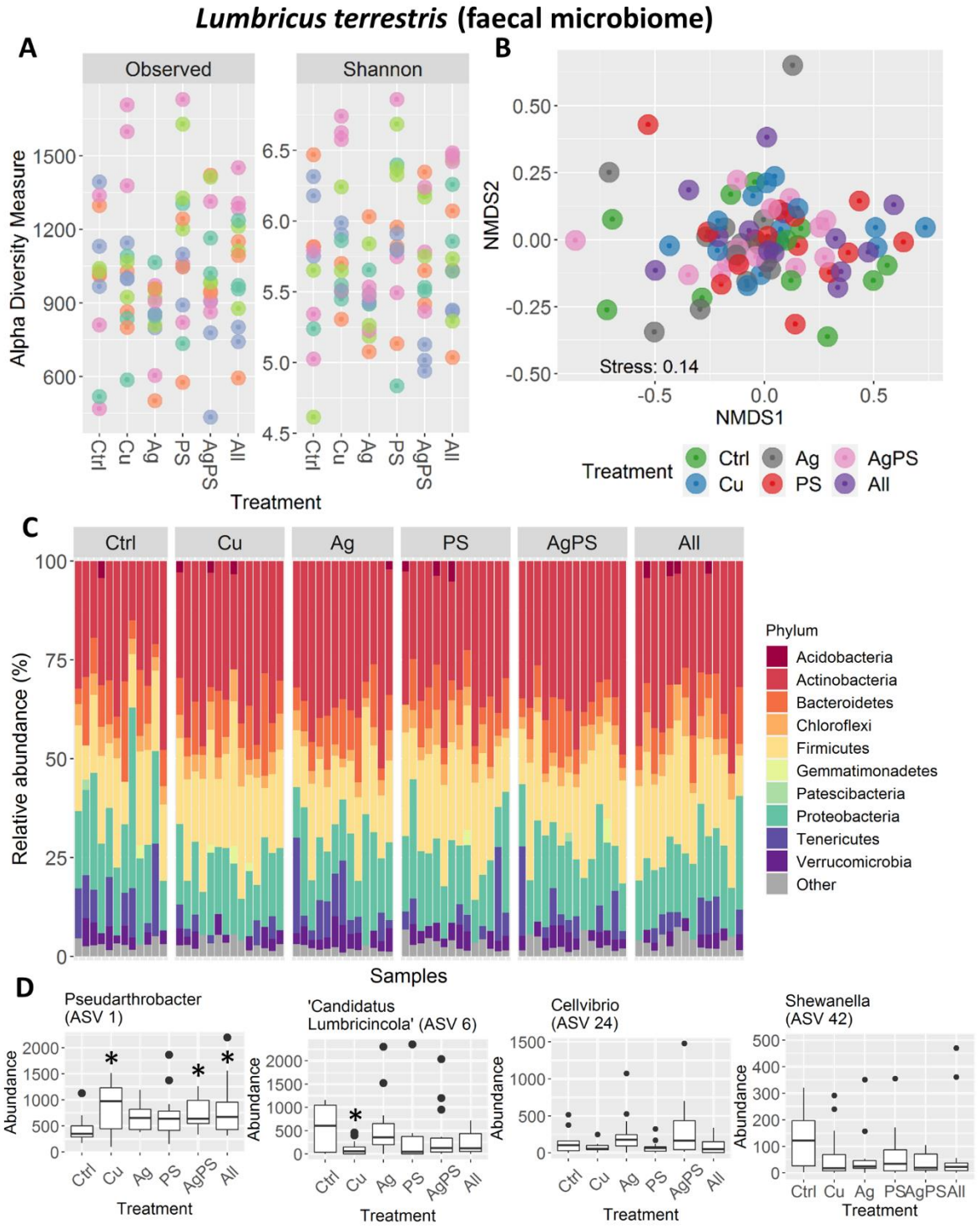


Figure 6.5: Effects of six-month NP exposures on the faecal microbiome of the earthworm *L. terrestris*. (**A**) Total number of observed taxa (left panel) and Shannon diversity index (right panel) per treatment. Different colours indicate different mesocosm replicates. (**B**) NMDS plot showing the ordination of samples. Different colours indicate different treatments. (**C**) Relative abundance in % of phyla per sample. All phyla with a relative abundance <2% are grouped under 'Other'. (**D**) Read abundance of the four 'greatest drivers' of the faecal microbiome structure; * indicates a significant difference of the respective treatment compared to the control with $p<0.05$. Points indicate outliers (CI>95%).

Table 6.5: Parameter estimates of generalized linear mixed models testing the relationship between read abundance of the selected ASVs (i.e. the greatest drivers the community structure) of the faecal microbiome and measured total silver and copper concentrations at start of the experiment. Bold p-values indicate statistical significance.

Bacterial taxa	Fixed effects[a]	Beta (est.)	SE	z-value	p-value
Pseudarthrobacter (ASV 1)	Total [Ag]	0.01	0.01	1.16	0.248
	Total [Cu]	0.01	0.00	1.35	0.178
'Candidatus Lumbricincola' (ASV 6)	Total [Ag]	0.03	0.02	1.09	0.276
	Total [Cu]	-0.02	0.01	-2.10	**0.036 ***
Cellvibrio (ASV 24)	Total [Ag]	0.04	0.02	1.99	**0.047 ***
	Total [Cu]	-0.02	0.01	-1.88	0.060
Shewanella (ASV 42)	Total [Ag]	-0.02	0.03	-0.81	0.417
	Total [Cu]	-0.01	0.01	-0.85	0.394

[a] N observations: 81, N groups: 30; * $p < 0.05$.

6.4 Discussion

Animals including invertebrates are colonized by many microbes. Over the last decade, the crucial role of these host-associated microbial symbionts in the health and functioning of animals has been increasingly recognized. The disruption of host and microbe interactions by, for example, an antimicrobial agent can enhance susceptibility to pathogens (J.K Kim et al., 2015; Motta et al., 2018), alter the nutrient turnover (Xiang et al., 2019) and in some cases can culminate to host death (Motta et al., 2018; B.L. Weiss et al., 2012). Owing to these critical interactions between host and microbe, it is important that environmental hazard assessments of chemicals consider microbiome impacts. Increasingly, the microbiome of animals has become a focus for ecotoxicological research. The large majority of animal microbiomes ecotoxicological studies have looked at effects over a relatively short period, often under artificial lab-conditions. Such short-term studies may not reflect the true impact of chronic long-term exposure. Currently, relatively little is known about the effects on microbiomes of long-term exposure to chemicals under more environmentally realistic conditions. In this study, we investigated the long-term effects under semi-field conditions in outdoor mesocosms of metal and polymeric NPs (as single exposures and in combinations) on the gut microbiomes of two different earthworm species.

6.4.1 The resident microbiome of A. calignosa

The resident microbiome *of A. calignosa* has not been studied extensively (Thakuria et al., 2010), with most of the research being done on cast and gut content microbial communities (Aira et al., 2010, 2019; Horn et al., 2006; Ihssen et al., 2003). Here we show that the identified core resident gut microbiome of *A. calignosa* is dominated by a relatively small set of bacterial strains. The most abundant ASV in the gut of *A. calginosa* (ASV 2) belongs to the bacterial family *Enterobacteriacaea*. This ASV was earthworm specific being below detectable levels in the soils from which the

earthworms originated. Previously, this ASV was also identified as part of the core resident gut microbiome of *Eisenia fetida* (**Chapter 3**). Local alignment using NCBI BLASTn showed that ASV 2 was most similar to a 16S-rRNA gene sequence that was collected from the gut of an Enchytraeidae earthworm inhabiting ice glaciers in North America (Murakami et al., 2015). Based on the sequence similarity, and following convention (Konstantinidis & Tiedje, 2005; Nguyen et al., 2016), these two taxa can be considered as part of the same operational taxonomical unit and are thus close relatives. The high abundance of ASV 2 in the gut of *A. calginosa* but apparent absence from soils (**Table S6.1**) suggests that this ASV may be part of an autochthonous microbiome of multiple earthworm species.

6.4.2 The resident gut microbiome and the faecal microbiome of L. terrestris

The gut microbiome of *L. terrestris* has been well studied (Jolly et al., 1993; A. B. Meier et al., 2018; Sampedro et al., 2006; Thakuria et al., 2010; Wüst et al., 2009, 2011; Zeibich et al., 2019a). Most of these studies assessed the bacterial communities of the cast or the gut contents. So far, few studies have investigated the resident gut bacterial communities that are separated from the gut content (Egert et al., 2004; Jolly et al., 1993; Thakuria et al., 2010) and to date none of these studies have used a next-generation sequencing approach. In this study, the community composition of the resident gut microbiome isolated from the gut content and assessed by Illumina sequencing was significantly correlated to the community composition of the faecal microbiome but not the soil bacterial communities. A significant component of the resident gut microbiome is, thus, a direct reflection of the faecal microbiome. However, there is also a component of the resident gut microbiome that is not detected in the faecal or soil microbiome. These ASVs are likely to be part of an autochthonous earthworm microbiome with the likelihood that these ASVs may be so closely associated with the gut epithelium that they do not release into the gut contents. The coelomic cavity of earthworms is not sterile and one possibility is that these ASVs could be associated to the outside surfaces of the intestine, which would explain their absence in the gut contents.

The faecal microbiome of *L. terrestris* was dominated at phylum level by *Actinobacteria*, *Firmicutes* and *Proteobacteria*, in line with previous studies of this community (A. B. Meier et al., 2018; Zeibich et al., 2018). Comparison between the relative abundances of the core faecal ASVs in the faecal and soil microbiomes, indicated that most core faecal ASVs likely originate from the top soil layer treated with sludge and representing the plough layer and not the bottom underlying soil. Although the top soil was a source of faecal bacteria, many of the most abundant top-soil bacteria, were either detected in the faecal microbiome but at much lower levels or not at all (**Figure S6.3**). This indicates that during gut passage, some soil bacterial are specifically filtered out, a finding is in line with previous research indicating bacteria selection or stimulation during gut passage (Fischer et

al., 1997; Khomyakov et al., 2007; Schönholzer et al., 2002). This selection process in the earthworm intestine is driven by a combination of the physical grinding and resulting lysis of bacteria in the earthworm crop and gizzard, as well as the specific environmental conditions in the intestine, which can stimulate and select against specific bacteria taxa (Horn et al., 2003; A. B. Meier et al., 2018).

6.4.3 The impact of NP treatments on the earthworm gut microbiomes

It was expected that with chronic NP exposures and with increasing exposure complexity that effects on the microbiomes would increase. This included a specific effect of the pristine CuO-NP on relative abundance of the earthworm symbiont *'Candidatus* Lumbricincola' and members of the bacterial family *Spirochaetaceae*, in line with previous studies (**Chapter 4**). Contrary to these expectations, NP treatments did not significantly alter the structure and the diversity of the resident gut microbiome of both *A. calginosa* and *L. terrestris*. Further, for all microbiomes, there was no indication for an increase in effects with increasing exposure complexity. *'Candidatus* Lumbricincola' in was not affected by CuO-NP exposure in both the *A. calginosa* and *L. terrestris* resident gut microbiome. Further, *Spirochaetaceae* were unaffected by copper treatment in the *L. terrestris* microbiomes (but not detected in the *A. calignosa* resident gut microbiome), indicating that under semi-field conditions at the concentrations tested in this experiment this group of bacteria are not at risk due to NP exposure. NP treatment did, however, have a small magnitude effect on the faecal microbiome of *L. terrestris*. Here the relative abundance of the earthworm symbiont *'Candidatus* Lumbricincola' was negatively related to soil copper concentrations and the relative abundance of *Cellvibrio* in the faecal microbiome was significantly positively affected by soil silver concentration.

A case of sensitivity of '*Candidatus* Lumbricincola' (class: *Mollicutes*) towards copper has previously been reported in short-term exposures under lab-conditions (**Chatper 4**). Earthworm gut *Mollicutes* were recently also found to be sensitive to cadmium pollution (Srut et al., 2019). However, exposure to mercury and the antibiotic tetracycline did not have a negatively impact on these earthworm symbionts (Chao et al., 2020; Rieder et al., 2013). The sensitivity by this earthworm symbiont to environmental pollutants thus appears to be chemical specific. The differential sensitivity to different pollutants may be a reflection of the specific resistance and metal homeostasis genes that this bacterium carries (Hernández-Montes et al., 2012) and so genome analysis of *'Candidatus* Lumbricincola' may help to explain patterns of sensitivity to pollutants. The tested concentrations of the CuO-NP treatments were based on expectations of build up over an 8-year application period for copper-based fungices. Further, in Europe, the total copper concentrations in some regions can reach several hundreds of mg/kg copper (Ballabio et al., 2018), possibly as the results of the application of copper-based plant protection products. The reduction of the relative abundance, of

the '*Candidatus* Lumbricincola' symbiont, as reported here, thus occurs at environmentally relevant copper concentrations.

The relative abundance of '*Candidatus* Lumbricincola' in the resident gut microbiomes of both *A. calginosa* and *L. terrestris* were unaffected by any of the treatments, which indicates that the symbiont populations more closely associated to the earthworm gut wall are less affected by the copper treatments than bacteria in the gut contents, perhaps reflecting a spatial separation from direct chemical exposure. Using transmission electron microscopy, Jolly and colleagues showed that the earthworm gut surface is most densely populated by bacteria in the midgut region (Jolly et al., 1993). In contrast to the foregut and the hindgut, which are smoother, the surface of the midgut of earthworms is characterized by numerous folds (Jolly et al., 1993). The physical attachment of symbiotic bacteria within these folds may protect these symbionts from adverse effects of environmental copper and may explain the different effects of copper on this symbiont in the different populations (i.e. resident gut microbiome and the faecal microbiome).

Recently, Liu and colleagues investigated the effects of heavy metal pollution on the earthworm (*Eisenia andrei*) faecal microbiome in earthworms from clean and polluted sites (P. Liu et al., 2020). The authors linked pollution to effects on the microbiome through correlation analysis and found that soil copper concentrations were negatively correlated to the relative abundance of the bacterial genus *Solibacter* and a bacterium from the phylum *Verrucomicrobia* (P. Liu et al., 2020). In another field study, an effect of metal pollution on the microbiome of *L. rubellus* was found for the earthworm symbiont *Verminephrobacter* which was among the most abundant earthworm microbiome taxa in references sites, but below detectable levels in metal-polluted sites (Pass et al 2015). In both of these field studies, the authors did, however, not report on the detection of *Mollicutes* in the microbiome of the collected earthworms. Previous studies, however, indicate that this earthworm symbiont is are part of the gut microbiomes of both *E. andrei* and *L. rubellus* earthworms reared in unpolluted soils (Pass, 2015; Procházková et al., 2018), and so it remains to be determined whether the adverse effects of copper pollution on this symbiont, as reported here, also occur in field populations.

Previous studies indicate that short-term exposures to pristine Ag-NPs and PS-NPs can disrupt soil invertebrate microbiomes (Ma, Sheng, et al., 2020; Zhu, Zheng, et al., 2018). Zhu et al. (2018) studied the effects of a 28-day exposure to Ag-NPs on the *F. candida* microbiome in Petri dishes. At 200 mg/kg food, Ag-NPs disrupted the *F. candida* microbiome by negatively affecting several core bacterial phyla. In both *F. candida* and *E. crypticus*, seven-day long exposures to PS-NPs in an agar medium are associated to alterations of the microbiome of these animals; however, effects only

occur at very high concentrations (≥ 1% w/w food) (Ma, Sheng, et al., 2020; Zhu et al 2018). In *E. fetida* earthworms, a 28-day exposure to Ag-NPs (up to at 400 mg/kg) in soil does not significantly alter the resident gut microbiome of these animals (**Chapter 4**). In the current study, we also found no effects of long-term exposure to aged Ag-NPs (at 20 mg/kg soil) and aged PS-NPs (at 100 mg/kg soil) on the earthworm microbiome structure and diversity. Most of the abovementioned studies differ fundamentally in their experimental design. Exposure complexity, NP state as well as exposure duration and invertebrate species are likely factors driving the effects of NPs on soil invertebrate microbiomes, which makes it difficult to compare the effects of NPs on microbiomes between different studies. In this study, the concentrations of Ag-NP were equivalent to the concentrations that may be found in agricultural soils under application of sludges containing relative high concentrations of Ag-NP for at least eight years. The PS-NP test concentration was not based on any measured or predicted environmental values but represented a hypothetical high concentration scenario. The absence of any clear effects of the Ag-NPs and PS-NPs on the microbiome of *A. calignosa* and *L. terrestris* indicates that at these test concentrations the microbiome of earthworms are not at risk due to Ag-NP or PS-NP exposure.

6.5 Conclusions

We showed that long-term exposure to two biocidal metal NPs and polymeric NPs in a 6-month long mesocosm experiment had no or little effects on the overall bacterial community structure and diversity in the gut of two ecologically relevant earthworms. However, the earthworm symbiont '*Candidatus* Lumbricincola' was negatively affected by CuO-NP exposure in the faecal microbiome reflecting a potential effect through exposure within the consumed and processed soil. '*Candidatus* Lumbricincola' is likely involved in the anaerobic degradation of structural polysaccharides in the gut of earthworms. The exposure to environmentally relevant concentrations of copper may thus adversely affect the digestive capacity of the gut system to degrade these rigid carbohydrates, with possible effects on the host. Future research should focus on elucidating the specific contribution that '*Candidatus* Lumbricincola' makes to host health under various feeding regimes. Such information will help to assess the further implications for the host of the adverse effects of copper exposure on this symbiont.

7

Final discussion

7.1 The resident gut microbiome of earthworms

Previous research indicated that earthworms house a bacterial community that is tightly associated to the earthworm gut wall (Egert et al., 2004; Jolly et al., 1993; Singleton et al., 2003; Thakuria et al., 2010). Previously, this community had mostly been studied using microscopy or DNA fingerprinting approaches. Except for the earthworm *L. rubellus* (Pass, 2015), a detailed description of the composition and structure of the resident gut microbiome of earthworms was missing. This thesis provides an in-depth analysis of the resident midgut bacterial microbiome of three ecologically relevant earthworms: *E. fetida*, *A. calignosa* and *L. terrestris*.

7.1.1 On the origin of the resident gut bacteria

Comparative assessment indicates that only eleven taxa were detected in the core resident gut microbiome of at least two of the three earthworm species (**Table 7.1**). Taxa detected in all three earthworm species included *Verminephrobacter*, '*Candidatus* Lumbricincola', *Aeromonas* and *Delftia*. The three earthworm species each inhabit a different ecological niche. *E. fetida* are mostly surface dwelling animals (epigeic) that that live in and feed on manure or leaf litter. *A. calignosa* are endogeic earthworms that inhabit the top mineral layer of the soil and form horizontal burrow structure through soil consumption. *L. terrestris* on the other hand make deep vertical burrows that can extent to depth into the mineral soil, while feeding primarily on leaf litter obtained from the surface (anecic). These three taxa thus rely on different food sources and, therefore, are likely exposed to different microbial communities, which is a known driver for the resident gut bacterial community of earthworms (Thakuria et al., 2010). The detection of these four bacterial taxa in each of these earthworm species indicate that these bacterial taxa may be part of earthworm resident gut microbiome that is independent from local soil conditions, species and habitat. A global survey of the nephridial bacterial communities of various Lumbricidae earthworms, indicated a significant overlap also in the composition of the nephridial bacterial community of different earthworm species (Davidson et al., 2013). However, the earthworms that were sampled in the current study were collected during laboratory or mesocosm exposures, which may not fully reflect the environmental conditions of earthworms in the field. To further identify a common set of earthworm resident gut taxa, earthworms collected from field populations will need to be investigated to create a microbiome inventory across different earthworm species, soil types and habitats.

Table 7.1: Bacterial taxa detected in the core resident gut microbiome of at least two of the three earthworm species analysed in this study. '%' indicates the mean relative abundance of taxa in the respective microbiome. Blue colour spectrum indicates the magnitude of the relative abundance (light blue: <1%, medium blue: 1–10% and dark blue: >10%). The relative abundance of ASVs belonging to the same genus are summed. 'Factor' indicates the factor between the relative abundance of a taxa in the earthworm gut vs. that of the soil bacterial community. Orange colour spectrum indicates the magnitude of the factor (light orange: 1–10, medium orange: 10–100 and dark orange: >100). When a taxa was composed of multiple ASVs, a factor range is given representing the factor range for those ASVs.

			E. fetida[a]		*A. calignosa*[b]		*L. terrestris*[b]	
Phylum	**Family**	**Genus + species**	*%*	*Factor*	*%*	*Factor*	*%*	*Factor*
Actinobacteria	Micrococcaceae	Pseudarthrobacter			8.9	1	7.5	1
Bacteroidetes	Flavobacteriaceae	Flavobacterium	0.8	1–10			0.6	1
Firmicutes	Bacillaceae	Bacillus			2.8	5	0.4	1
Proteobacteria	Aeromonadaceae	Aeromonas	32	10–560	11	22–292	0.4	8
	Burkholderiaceae	Verminephrobacter	33	>1000	12	>1000	7.2	>1000
	Burkholderiaceae	Delftia	0.1	>100	1.5	>1000	2.4	>1000
	Enterobacteriaceae	(Unknown taxa)	<0.1	1–10	21	>1000		
	Pseudomonadaceae	Pseudomonas	3.7	3–45	3.8	11–65		
	Xanthobacteraceae	Bradyrhizobium	0.3	1–10	0.8	1		
Spirochaetes	Spirochaetaceae	(Unknown taxa)	6.7	>1000			0.5	>1000
Tenericutes	Mycoplasmataceae	'Candidatus Lumbricincola'	9.0	>1000	6.0	432	3.0	127

[a] For the *E. fetida* data, the relative abundance represents the mean relative abundance of the taxa in five different soil types in the soil transfer experiment (**Chapter 3**), except for '*Candidatus* Lumbricincola' for which values represent the mean relative abundance of the 'Rinsed 2x' samples in **Chapter 3.** [b] Values are taken from supplemental tables in **Chapter 6.**

The ability of some earthworm bacteria to be vertically transferred from parent to offspring has so far been described for only nephridial associated bacteria: *Verminephrobacter*, '*Candidatus* Nephrothrix' and *'Candidatus Lumbricidophila'* (Davidson et al., 2010; Lund et al., 2018). In this study, all three of these taxa were also associated to the gut tissue of *E. fetida* (**Chapter 3**). This could indicate translocation between different earthworm tissues. During the cocoon phase of earthworm development, the developing embryos consume albumin as food. Albumin typically contains a variety of bacteria, including these nephridial symbionts. Through consumption of the albumin, the nephridial symbionts are potentially also able to colonize the embryonic gut lumen (Davidson & Stahl, 2008). With the approach and techniques used in this study, it was is not possible to distinguish bacteria that are present inside the gut from bacteria that are attached to the exterior of the gut. To confirm the presence of these earthworm symbionts inside the gut, studies using fluorescent *in-situ* staining hybridization (FISH) are required to confirm their exact spatial localisation. For those bacteria that are also described as nephridial symbionts (this may also include *Spirochaetaceae* family members which are known nephridial symbionts of *E. fetida* [Davidson et al., 2013], but in this study were also found in the *E. fetida* and the *L. terrestris* resident gut microbiome), the colonization of the gut lumen during embryonic development is a possible route of entry into the gut system. All other bacteria that are part of the resident gut microbiome

in adults, for which no evidence for vertical transmission exists (unless there is another undescribed vertical transmission route), are likely taken up from the environment and from there may become integrated within the resident gut microbiome community.

A key question that remains regarding the earthworm microbiome structure is whether the 'environment derived bacteria' are merely casual associates to the gut wall (for example as associated to remaining faecal particles) or form prolonged associations with the gut epithelium. In the case of *E. fetida*, *A. calignosa* and *L. terrestris*, there was a strong disconnect between the resident gut communities and the bulk soil communities. For example, some bacterial taxa that were among the most abundant in the resident gut microbiome were detected in the soil but at 100−1000 fold lower levels. This disconnect may be partly driven by the selection and activation of specific ingested bacteria during gut passage (Schönholzer et al., 1999; Wüst et al., 2011), which is driven by the specific conditions in the earthworm intestinal microenvironment (Horn et al., 2003), as discussed in **1.3.2.** The significant correlation between the faecal microbiome and the resident gut microbiome of *L. terrestris* (as reported in **Chapter 6**), suggests that the gut wall-associated microbiome is, at least, partly a reflection of the faecal microbiome. However, for the sample preparation for these *L. terrestris* 'resident gut samples', the earthworms were not depurated. Instead, gut contents were first removed using a spatula and the gut tissue was subsequently rinsed twice in a saline solution. This procedure may not have been as efficient at removing soil/faecal particles as the depuration method is meaning that a significant signature of the bulk soil community remains.

For 'resident gut' taxa whose relative abundance is comparable to that in the soil or the faecal microbiome, it seem likely that these taxa are not part of a true 'resident gut microbiome'. However, for other taxa (see, for example, a set of *Proteobacteria* in **Table S6.2**) which are highly abundant in the 'resident gut microbiome' but not detected or are very scarce in the soil or faecal microbiome, it seems plausible that these taxa form a more intimate association with the earthworm gut intestine. Examples of such hypothetical true resident taxa are *Delftia*, *'Candidatus* Lumbricincola' and some *Aeromonas*. However, the identification of a 'true resident' gut bacterial community through the comparison of the relative abundance of a specific taxa in the gut tissue and the soil is problematic (see the discussion in **Chapter 3.4**). Earthworms are selective feeders, and so the bulk soil bacterial community may not reflect the actual microbial communities that earthworms are ingesting. Therefore, a soil transfer approach, as applied in **Chapter 3**, provides a better alternative to study host specificity.

7.1.2 Future studies

The approach and methods applied in this study did not allow the specific localization of bacteria associated with the gut tissue. FISH probes that can differentiate the three of the major *E. fetida* nephridial symbionts have previously been used for the localization of bacterial communities in the nephridia and may also be used to study the gut bacterial communities (Davidson et al., 2010; Davidson & Stahl, 2008). The exact role of bacterial symbionts in earthworm health and functioning is still not fully understood (Møller et al., 2015), although genetic analysis of nephridial symbionts indicates a potential role of the nephridial symbionts in nitrogen retention (Davidson et al., 2013). So far experimental studies have not been able to provide unequivocal evidence for any specific host-microbe interaction (Lund, Holmstrup et al., 2010). Recent studies have indicated that the earthworm gut bacterium *'Candidatus* Lumbricincola' can be stimulated by the supplementation of xylan and chitin (Zeibich et al., 2019a). The degradation products of these structural polysaccharides may be taken up by the earthworm and so these gut symbionts may facilitate the host ability to feed on recalcitrant carbon sources. Indeed the trophic transfer of microbial fermentation products from microbe to earthworms has previously been reported (Sampedro et al., 2006), but it remains unclear whether microbe mediated degradation of, for example, xylan and chitin leads to an increased carbon flow to the host. To test the role of a specific microbe, it is crucial to be able to clear a host from a specific symbiont (J.K. Kim et al., 2015; B.L. Weiss et al., 2012). Although this is possible for vertically transmitted symbionts (Lund, Holmstrup et al., 2010; Viana et al., 2018), the clearance of gut associates may prove more difficult because of the guts' continuous and intimate interaction with a medium highly rich in bacteria (i.e. soil). As symbionts like *'Candidatus* Lumbricincola' are likely taken up from the environment, it may be possible to clear earthworms from this symbiont by rearing developing juveniles in a sterilized culture medium. Alternatively, screening of the gut bacterial populations under different earthworm feeding regimes (e.g. enhanced rigid carbohydrate contents) may help to further identify specific roles of the gut symbionts. Further, metabolic profiling using ^{13}C stable isotope labelled carbon sources in combination with mass-spectrometry or nuclear magnetic resonance techniques, may give further insights in the role of microbial symbionts in the trophic transfer of carbon to the host (Tokuda et al., 2014). Lastly, through genomic sequencing and annotation, the metabolic capacity of bacterial symbionts can be predicted. Such information may help to determine the potential role of a symbiont in host functioning (Agamennone et al., 2019). Over the last years, the availability and accuracy of sequencers that are able to sequence long fragments (>10000 bp) have greatly improved. This has enhanced the ability to sequence and assemble complete genomes of bacteria that are difficult to culture (Moss et al., 2020). The relative abundance of *'Candidatus* Lumbricincola' in the gut contents of *L. terrestris* is relatively high (mean relative abundance of 4.5%

in **Chapter 6**) and so in-depth sequencing of earthworm gut contents using long-read sequences (i.e. Oxford Nanopore Technologies or PacBio SMRT Sequencing) may be sufficient to assemble the complete chromosome of this earthworm symbiont to support the identification of genes involved in metabolic functions. A disadvantage of shotgun sequencing approach is the loss of species-specific information of plasmids, which often carry crucial functionality. An alternative approach that preserves species information of plasmids, may be targeted single-cell sorting combined with single cell genomics. This approach was recently applied on sewage sludge samples and helped to genetically unravel microbes that were previously undetectable (Dam et al., 2020). This thesis mostly focussed on describing the bacterial resident microbiome composition and structure. Future studies should focus on improving our understanding of the functionality of the earthworm microbial communities. Latest technologies (e.g. single-cell genomics, metabolomics, etc.) may help to functionally characterize the earthworm microbial symbionts and their interaction with their host.

7.2 The microbiome as new endpoint in ecotoxicology

Environmental pollutants may adversely affect organisms via direct toxicity (e.g. cytotoxicity, neurotoxicity, etc.), however, owing to the interdependence of microbial symbionts and their hosts, chemicals may also adversely affect organisms (e.g. plants, soil invertebrates, fish, etc.) through the disruption of their microbial associates and the associated symbiont-host relationships. The level of dependence of hosts on their microbial symbionts can vary. In some animals the loss of a symbiont may result in host death (see for example, the *Buchnera*-aphid symbiosis [Shigenobu & Wilson, 2011]), whereas in other species symbionts provide a more supportive role which can boosts host fitness but symbiont loss does not result in the inability of the host to survive (for example, the nephridial symbionts in earthworms, see [Lund, Holmstrup et al., 2010]). Conventional ecotoxicological tests do not capture these indirect symbiont mediated effects, especially when interactions are facultative. The herbicide glyphosate, for example, has low direct toxicity towards honey bees (Y.C. Zhu et al., 2015). However, at environmentally relevant concentrations, glyphosate can disrupt the microbiome of honey bees (Blot et al., 2019; Motta et al., 2018). The glyphosate-mediated disruption itself does not result in mortality of honey bees, only when honey bees are also exposed to a bee pathogen, the honey bees with a glyphosate disrupted microbiome are much more likely to die of the infection (Motta et al., 2018). Increasingly, the microbiome as a route for adverse effects on ecologically relevant organisms is recognized (see for example (Evariste et al., 2019), for a review on ecotoxicological studies assessing the effects of chemical pollutants on fish microbiomes).

7.2.1 Methodological recommendations for future ecotoxicological microbiome studies

Increasingly the effects of environmental pollutants on the microbiome of soil invertebrates are studied (**Table 1.1**). So far, most experimental studies have focussed on the effects of metals (in nanoparticulate or salt form), antibiotics (e.g. tetracycline) or polymeric particles (e.g. micro- or nanoplastics). In contrast, the impact of organic pesticides on soil invertebrate microbiomes is less well studied. With the costs of next-generation sequencing continuously decreasing, it is likely that the number of ecotoxicological microbiome studies will increase in the future. With this increase in focus on microbiome effects of pollutants, there is a need to define the prerequisites for such research to guarantee quality and reproducibility of studies. Based on the studies listed in **Table 1.1**, a non-exhaustive list of methodological recommendations for future ecotoxicological microbiome studies is defined below:

1. Replication, replication, replication

   Replication is crucial for microbiome analysis (Prosser, 2010). Of the fourteen studied listed in **Table 1.1**, ten used four or fewer replicates per treatment, with five studies using as few as three replicates. Microbial communities are often characterized by large spatial and temporal variation (Falony et al., 2016; Thompson et al., 2017; Wong et al., 2013). The higher the between-individual variation, the more replication will be needed to find significant patterns of effect. Especially when relying on non-parametric tests, the chance of finding significant effects are very low when only a few replicates are included and so with low replication, there is an increased chance for finding false negatives. Characterization of the microbiome variability before an ecotoxicological experiment may help to estimate the appropriate sample size. The greatly diminishing costs of sequencing means that at least for community structure assessments, sufficient replication within any reasonable size experiment will generally be affordable at the relevant scale needed.

2. Consider a concentration series

   The basis for ecological hazard assessment of chemicals is the estimation of EC_{50} values, the calculation of which requires testing of effects of chemicals at multiple concentrations. Currently, in ecotoxicological microbiome studies testing at multiple concentrations is not the norm. In nine of the fourteen studies listed in **Table 1.1**, only one exposure concentration is tested. This in combination with often small sample sizes means that in some cases conclusions are derived from a total of six samples, including the negative control samples. This, in combination with the often high between-individual variation of microbiomes, is likely to increase the number of false positives. The usage of a limited number of test concentrations in ecotoxicological microbiome studies has previously been

debated (van Gestel & Selonen, 2018). In order to establish a link between a pollutant and effects, determination of effects across a concentrations series is required. This principle should also be applied for microbiome research. Further development of methods to assess the effects over a concentration series on microbiomes (such as the SSD approach applied in Chapter 4 and previous studies including Doolette et al. [2016] and Yang et al. [2018]) will be required.

3. Characterize the medium

Soil invertebrates are in continuous contact with microbes in the soil. Not surprisingly, the gut microbiome of soil invertebrates is often driven by the microbial communities of the substrates that the animals live on (Johnke et al., 2020; Thakuria et al., 2010). Effects on soil invertebrate microbiome may be driven by the direct effects of pollutants on the invertebrate microbiomes, but also through pollutant mediated changes to the microbial communities of the medium itself. In a recent study, Zhang and colleagues investigated the effects of a concentration series of the fungicide azoxystrobin on the gut bacterial microbiome of *E. crypticus* and the bacterial communities of the surrounding soils (Q. Zhang et al., 2019). Concentration-dependent effects of this fungicides were reported on *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* in the gut microbiome but not in the surrounding soil bacterial communities. This could indicate that the gut microbial communities, which may provide functions to the host, are particular sensitive to pollution. The concurrent characterization of invertebrate microbiome and surrounding microbial communities may thus help to better interpret the pattern of effects and therefore should be considered as a prerequisite for future ecotoxicological microbiome studies.

4. Functionality is the key

The main reason for including microbiomes in ecotoxicological research is that when pollutants disrupt the microbiome, adverse effects on the host can be expected. However, so far most studies have only assessed the impact of pollutants on microbiome composition and structure. As it's the functions that microbial associates provide to hosts that makes microbiome assessment relevant for ecotoxicological research, it is also the effects on functionality that will need to be the major future focal point. Information on the functionality of microbiome is largely missing for most soil invertebrates. Future studies should therefore aim to improve our understanding of the composition and functionality of microbes associated to soil invertebrates. The development of new sequencing and

bioinformatics approaches to identify functional traits in biomedical research have primed the field for greater functional assessment in host-microbiome ecotoxicological studies.

7.2.2 Future studies

In this study, the main focus of research was on the resident gut microbiome of earthworms. It's this community tightly associated to the earthworm intestinal tract that may play a role in host health, for example by the provision of crucial metabolism or by stimulating host immune responses that may help to prevent infection from pathogens. Previous studies have shown that the faecal (or gut content) microbiome is involved in the anaerobic digestion of carbon sources as well as nitrogen cycling (Ihssen et al., 2003; Wüst et al., 2011; Zeibich et al., 2019a). Recently Wu and colleagues, investigated the effects of $AgSO_4$ and Ag_2S-NPs on the denitrification and the denitrifying bacterial communities in the earthworm gut and surrounding soils. The addition of Ag in the absence of earthworms increased denitrification in the soil. However, in soils with earthworms Ag_2S-NP addition reduced denitrification of the soil by 14-33% and negatively affected earthworm gut denitrifiers (Wu et al., 2020). Transient earthworm communities are, thus, important not only for the earthworm health but also for the ecosystem functioning and so future studies should also focus on the effects of pollutants on the functions provided by these communities.

Generally speaking, the resident microbiome appears to be more resilient towards exposure to metal NPs than the faecal microbiome (see **Chapter 4 and 6**). For example, the earthworm gut symbiont *'Candidatus* Lumbricincola' in the gut contents of *L. terrestris* was negatively affected by exposure to CuO-NPs. However, the relative abundance of the same symbiont was unaffected in the *L. terrestris* resident gut community (**Chapter 6**). Recently, the impact of gut structure and gut lumen physiology on the uptake of metal NPs was reviewed by (van der Zande et al., 2020). In vertebrate animals, there is evidence that metal NPs may accumulate in the mucus liquid that forms a sheet over the gut epithelium (Clark et al., 2019). This could hypothetically increase the metal NP exposure by microbes that are attached to the gut epithelium. However, the specific physiochemical conditions in this layer and the interactions of NPs with bio-molecules could theoretically also increase NP aggregation and reduce dissolution, both behaviours that have been associated to reduce nanoparticle toxicity. The different behaviour of NPs in different compartment of the intestine (i.e. gut lumen vs. gut epithelial surface) could explain the different impacts that NPs have on the microbial communities of these distinct compartments. However, the impact of intestinal physiochemical conditions on NP behaviour and uptake has not been well-studied in invertebrate animals (van der Zande et al., 2020). The earthworm gut is anoxic and has a pH that is typically higher than that of soils (Horn et al., 2003). These specific conditions are likely drivers of transformation of metal NPs in the earthworm gut system such as the sulphidisation of silver and

copper nanomaterials. The transformations of metals in the gut and specifically the interaction of NPs with the sulphated metabolites drilodefensins (which can make up ~1.3% of the total dry weight biomass of earthworms [Liebeke et al., 2015]) will need to be considered in future research and may help to explain the specific impacts of NPs on the distinct gut communities.

Up to date, most authors that studied the effects of pollution on the microbiome of soil invertebrates (**Table 1.2**) used metals or antibiotics as model toxicant and few authors tested the effects of organic pesticides (Xiang et al 2019). In a recent meta-analysis, Ullah and Dijkstra assessed the impact of 38 fungicides and bactericides on soil microbial biomass and soil carbon and nitrogen cycling (Ullah & Dijkstra, 2019). The study showed that nearly all pesticides had a negative impact on at least one of the measured endpoints. With organic pesticides being the major group of pest-control agents in agriculture, there is need to increase the focus towards effects of organic pesticides on microbial communities. The herbicide glyphosate is among the most widely applied herbicide in the world (Benbrook, 2016) and derives its herbicidal activity to its interaction with the shikimate pathway in plants. However, some bacteria also have this amino acid pathway and thereby are potentially sensitive to exposure to this herbicide. Recently, several papers have shown that at environmentally relevant concentrations, this herbicide disrupts the interaction of bacterial symbionts with plants as well as insects (Blot et al., 2019; Motta et al., 2018; Ramirez-villacis et al., 2020). Especially for organic pesticides with a known possible antimicrobial action, assessments of the effects of exposure to these pesticides on non-target microbes that provide functions to hosts and ecosystems (for example, denitrifiers in the earthworm gut [Drake & Horn, 2007], root-associated microbes that protect crops against fungal pathogens [Yin et al., 2013], bacteria that prevent pathogen infection in pollinating insects [Motta et al., 2018]) have to be part of the ecological hazard assessment of chemicals.

7.3 The immune-microbiome interface under chemical stress

The immuno-safety of NMs is increasingly a focal point in environmental and health risk assessments of NMs (Boraschi et al., 2020; Dobrovolskaia & McNeil, 2007). Owing to their particulate nature NMs have an intrinsic potential to induce immune responses in immune cells (Bhattacharya et al., 2017). The latter has been demonstrated in many *in-vitro* systems including human, bivalve, sea urchin and earthworm models (Alijagic et al., 2020; Auguste et al., 2019; Hayashi et al., 2013). However, an immune response towards a foreign object (such as a NP) does not necessarily indicate that there is an immune risk. The interaction of NMs with the immune system may be problematic when exposure to NMs leads to an excessive immune response leading to host damage or when NMs suppress the immune system such that it compromises the host immune system to deal with a disease threat. NMs can further modulate host immunity via their

interaction with host microbiota. One of the main roles of microbial symbionts in host health is the provision of resistance against invading pathogens. This can be achieved through direct competition of symbionts with invaders for nutrients and niches or alternatively symbionts can also boost host immunity through the direct stimulation of host immune responses (**Section 1.2.2**). NMs that affect the microbiome structure and composition may, therefore, also alter host immunity by disrupting the functions provided by microbiota and by disrupting the direct interactions of microbes with the host. Thereby, NMs have the potential to modulate animal immunity through direct interactions with immune cells and also indirectly through the disruption of microbiome-host interactions.

7.3.1 Earthworms as in vivo model for NM-immune safety assessments

So far most research on NM-immune interactions has been based on *in vitro* models. However, NMs are likely environmentally transformed before they reach a non-target organisms. Thus, *in vitro* exposures are unlikely to reflect the exposure by organisms' immune systems in the field and, therefore, there is a need for additional *in vivo* studies. Invertebrates, including earthworms, may provide a good model for such research (Alijagic & Pinsino, 2017; Auguste et al., 2019). As a possible defence against predators, some earthworm species can excrete their coelomic fluid through dorsal pores upon physical or chemical stimulation. The immune cells can subsequently be collected from the coelomic fluid and used for various assays (Svendsen et al., 2004). Due to their constant exposure to microbes in the soil, earthworms have evolved a resilient immune system that can to control microbial infections under most circumstances (Bilej et al., 2010). Environmental pollutants can however negatively affect immune cells viability (Olchawa et al., 2006; Patricia et al., 2017) which in turn may adversely affect the ability of earthworms to control infectious microbes. To test whether pollution compromises immune ability, co-exposure with infectious microbes are necessary (Boraschi et al., 2011; Swart et al., 2020). The approach outlined in **Chapter 4** may provide a useful framework for future immuno-safety assessment of NMs as well as other chemicals.

7.3.2 Future studies

Chapter 4 showed that although a 28 day exposure to copper altered the gut microbiome, this pre-exposure did not alter the immune gene reactivity nor the survival of the earthworms when challenged with a high dose of bacteria. The lack of an immune response at a dose of bacteria that inflicts mortality in a large proportion of the exposed earthworms was unexpected. However, the expression responses of immune genes are dependent on earthworm species, bacterium, time as well as the test gene. Therefore, the immune markers that were tested in this study may have responded but at a different time point than was measured in this study. Further, there are currently only a few earthworm immune markers described, some of which the exact mode of action remains largely unknown (Bruhn et al., 2006; Lassegues et al., 1997). Most of the known

earthworm immune markers are homologs of well-known vertebrate immune genes (Škanta et al., 2016). However, lysenins and fetidins are unique to earthworms and have no similarity to any other described protein. The possible involvement of these unique molecules in earthworm immunity indicates that the earthworm immune system differs from what it is currently understood and established for vertebrate systems. This means that earthworm immunity is provided through as yet unknown and earthworm specific molecular pathways. Future studies should therefore aim to improve the time resolution of known earthworm immune markers as well as focus on the discovery of new immune genes and immune pathways. Full-transcriptomic analysis over a time-course may help to unravel how earthworm maintain immunity in an environment that is dominated by microbial life.

A major limitation for earthworm immuno-research is the lack of knowledge on earthworm bacterial pathogens. To induce immune response in earthworms, so far most studies exposed earthworms to a high dose of a soil bacterium (Dvořák et al., 2013, 2016; Josková et al., 2009). Although at these high doses soil bacteria can induce immune responses, there is no evidence that at environmentally relevant doses these bacteria can infect earthworms. Other studies used bacteria that are pathogenic in other animals (e.g. humans and fish) but these are unlikely to occur in the soil (Dales & Kalaç, 1992). It is, therefore, questionable whether the immune response of earthworms upon exposure to such bacteria is reflective of the earthworm's interaction with a true pathogen. Earthworms, and in particular their seminal vesicles, can be parasitized by eukaryotic unicellular parasites called gregarines (Prochazkova et al., 2019; Velavan et al., 2010). Some studies indicate that in natural earthworm populations there is a positive relationship between the level of parasite infection and chemical pollution (Pižl, 1985) and (Pižl & Sterzynska, 1991), as cited by Edwards & Bohlen (1996). Parasite load also negatively relates to earthworm growth (S. G. Field & Michiels, 2005). However, so far immuno-activity of coelomocytes is not associated to parasite load (Stuart G. Field et al., 2004). Under some conditions, lab-reared earthworm populations can completely collapse through infection of yet unknown parasite (personal observation). This could indicate the earthworm's main pathogen are not prokaryotic but may include a significant number of eukaryotic species that have high importance for disease phenotype. Future studies should focus on further describing these earthworm eukaryotic parasites and investigating how infection affect the earthworm immune systems. The isolation and culturing of these parasites would facilitate the opportunity to test the contribution of host bacterial microbiome to earthworm immunity.

7.4 Final conclusions

This thesis used a metabarcoding approach to describe the resident gut microbiome of *E. fetida* and to assess the impact of biocidal nanoparticles on the structure, composition and the provided

functions of this microbiome. It showcases the relevance of including microbiome analysis in ecotoxicological research. Specifically, the following is concluded:

- The structure and composition of the resident gut bacterial microbiome of the earthworm *E. fetida* is largely independent from the soil bacterial community (**Chapter 3**)
- Short and long-term exposure to metal nanoparticles alter the gut microbiomes of earthworms and the bacterial community of the surrounding soils (**Chapter 4 and 6**)
- Effects on bacterial communities are both community and metal specific (**Chapter 4 and 6**)
- CuO-NPs specifically negatively affect the earthworm bacterial symbiont '*Candidatus* Lumbricincola' (**Chapter 4, 5 and 6**)
- CuO-NP mediated changes to the resident gut microbiome do not alter the immune responses or the susceptibility to a bacterial infection in earthworms (**Chapter 5**)
- Identification and culturing of a true earthworm pathogen will allow testing of the contribution of the earthworm microbiome to host immunity (**Chapter 7**).
- To understand the implications of symbiont loss for host and ecosystem functioning, the functionality of earthworm symbionts needs further investigation (**Chapter 7**).
- Future studies should focus on the effects of organic pesticides with known antimicrobial action on microbiomes that provide host or ecosystem services (**Chapter 7**)

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Supplementary materials

Appendix 1: supplementary tables and figure

Chapter 3

Supplementary tables

Table 3.1: Mean (±SD) relative abundance (%) of the core midgut ASVs in the soil bacterial communities. Limit of detection in relative abundance is 0.009%.

			Mean (±SD) relative abundance (%)					
ASV id	**Phylum**	**Genus/species**	**Pre-transfer**	**$^{6.5}$ Culture $_{20}$**	**$^{5.6}$ Lufa $_{4}$**	**$^{5.6}$ Woburn $_{3}$**	**$^{5.5}$ Chiltern $_{19}$**	**$^{7.2}$ N. Wales $_{18}$**
1	*Proteobacteria*	*Verminephrobacter*	<0.1 ±0.0	<0.1 ±0.0	<0.1 ±0.0	<0.1 ±0.0	<0.1 ±0.0	<0.1 ±0.0
2	*Proteobacteria*	*Aeromonas*	0.1 ±0.1	<0.1 ±0.0	0.2 ±0.2	0.2 ±0.4	<0.1 ±0.1	0.2 ±0.3
4	*Proteobacteria*	*Aeromonas rivuli*	n.d.	n.d.	n.d.	n.d.	<0.1 ±0.1	<0.1 ±0.0
6	*Spirochaetes*		<0.1 ±0.0	<0.1 ±0.0	n.d.	<0.1 ±0.0	n.d.	<0.1 ±0.0
7	*Proteobacteria*	*Pseudomonas*	<0.1 ±0.0	0.4 ±0.5	0.2 ±0.3	1.4 ±1.2	<0.1 ±0.0	<0.1 ±0.0
9‡	*Actinobacteria*		n.d.	n.d.	<0.1 ±0.1	n.d.	n.d.	n.d.
11	*Proteobacteria*	*Aeromonas*	n.d.	0.1 ±0.2	0.3 ±0.5	0.2 ±0.2	<0.1 ±0.0	n.d.
13	*Proteobacteria*	*Aeromonas*	0.1 ±0.2	<0.01 ±0.1	0.2 ±0.3	0.2 ±0.2	n.d.	n.d.
17‖	*Bacteroidetes*		n.d.	n.d.	n.d.	n.d.	n.d.	<0.1 ±0.0
48	*Proteobacteria*	*Aeromonas*	n.d.	n.d.	n.d.	<0.1 ±0.0	n.d.	n.d.
64	*Proteobacteria*	*Bradyrhizobium*	0.2 ±0.3	0.3 ±0.1	0.3 ±0.2	n.d.	0.3 ±0.1	0.5 ±0.0
224	*Actinobacteria*	*Cutibacterium*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
438	*Proteobacteria*	*Delftia*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. indicates 'not detected'; ‡ NCBI blastn showed that the 16S rRNA sequence of *Microbacteriaceae* (ASV 9) aligned with 100% identity to a Agromyces-like symbionts which has the proposed name '*Candidatus* Lumbricidophila' (NCBI accession KX078350.1; Lund et al., 2018); ‖ NCBI blastn showed 16S rRNA sequence of *Microscillaceae* (ASV 17) aligned with 99.66% identity to '*Candidatus* Nephrothrix' (NCBI accession KP420702.1; Møller et al., 2015)

Supplementary figures

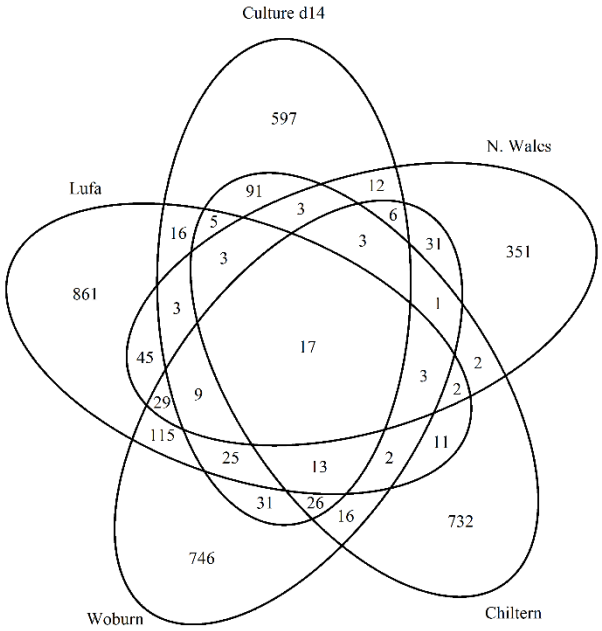

Figure S3.1. Venn diagram showing overlap in soil communities per sample group. For comparisons, only ASV with a relative abundance of >0.1% of the soil community in any of the samples are included.

Chapter 4

Supplementary tables

Table S4.1: Outcomes of models testing the relationship between number of reads per phylum and the measured copper concentrations using data from negative controls and CuO-NP treatments only.

Phylum	Intercept	Slope	Df 1	Df2	F-value	p-value
Verrucomicrobia	1148	-1.68	1	46	25.13	0.00001 ***
Cyanobacteria	33	-0.09	1	46	21.31	0.00003 ***
Patescibacteria	174	-0.44	1	46	16.86	0.00016 ***
Acidobacteria	757	-1.15	1	46	10.32	0.00240 **
BRC1	3	-0.01	1	46	8.61	0.00520 **
Actinobacteria	1215	1.54	1	46	6.71	0.01280 *
Proteobacteria	5510	2.16	1	46	6.54	0.01388 *
Firmicutes	804	-0.83	1	46	6.44	0.01463 *
Spirochaetes	45	-0.14	1	46	5.43	0.02424 *
Chlamydiae	2	0.02	1	46	4.85	0.03267 *
Entotheonellaeota	0	0.00	1	46	4.74	0.03461 *
Gemmatimonadetes	136	-0.15	1	46	4.18	0.04669 *
Fibrobacteres	130	-0.21	1	46	3.70	0.06046
Rokubacteria	5	0.01	1	46	1.06	0.30845
Tenericutes	2	0.00	1	46	1.05	0.31124
Hydrogenedentes	4	-0.01	1	46	0.87	0.35568
Lentisphaerae	0	0.00	1	46	0.80	0.37638
WS2	0	0.00	1	46	0.77	0.38543
Bacteroidetes	2764	0.84	1	46	0.66	0.42075
Dependentiae	2	0.00	1	46	0.56	0.45926
Elusimicrobia	1	0.00	1	46	0.38	0.54130
WS4	0	0.00	1	46	0.28	0.59836
FBP	2	0.00	1	46	0.28	0.60046
Chloroflexi	366	0.11	1	46	0.22	0.64373
Planctomycetes	124	0.04	1	46	0.18	0.66979
Armatimonadetes	2	0.00	1	46	0.09	0.76946
Deinococcus-Thermus	0	0.00	1	46	0.08	0.77748
WPS-2	0	0.00	1	46	0.08	0.77748
Cloacimonetes	0	0.00	1	46	0.08	0.77748
Latescibacteria	5	0.00	1	46	0.01	0.93373
Nitrospirae	10	0.00	1	46	0.00	0.96784

* p-value < 0.05 ** p-value < 0.01 *** p-value < 0.001

Table S4.2: Mean relative abundance (%) (±SD) of the most dominant bacterial phyla in soils and the earthworm midguts in the negative controls from both experiment, *p*-values derived from Mann-Witney-U tests testing the difference between the read abundances of the controls from both experiments per phylum and Bonferroni corrected *p*-values ('Adj. *p*-values'). Bold values indicate *p*-values <0.05.

Bacterial community	Phylum	Relative abundance (%) (±SD) in negative controls		*P*-values	Adj. *p*-values
		Copper experiment	Silver experiment		
Earthworm midgut	*Proteobacteria*	66.5%±20.6	69.8%±26.6	0.867	1.000
	Tenericutes	10.4±16.4%	10.2%±14.9	0.955	1.000
	Bacteroidetes	9.5%±8.9%	7.6% ±16.1	0.054	0.378
	Firmicutes	3.7±3.5%	5.7%±4.2	0.281	1.000
	Spriochaetes	2.9±3.8%	2.9% ±1.7	0.397	1.000
	Actinobacteria	3.7±3.5%	2.1%±1.8	0.224	1.000
	Verrucomicrobia	2.0±2.7%	1.5%±2.5	0.336	1.000
Soil	*Proteobacteria*	41.8±3.1	40.1±2.6%	0.878	1.000
	Bacteroidetes	20.7±5.4%	23.2±4.7	0.505	1.000
	Actinobacteria	8.7±2.4%	7.2±1.6	0.328	1.000
	Verrucomicrobia	8.2±1.4%	6.5±1.9%	0.052	0.363
	Firmicutes	7.2±1.9%	13.6±2.6%	**0.001 ****	**0.008 ****
	Acidobacteria	5.7±2.1%	2.3±0.6%	**<0.001 *****	**0.007 ****
	Patescibacteria	1.3±0.6%	2.6±1.5%	**0.028 ***	0.197

***** p<0.05, ** *p*<0.01, *** *p*<0.001

Table S4.3 Outcomes of models testing the relationship between number of reads per phylum and the measured silver concentrations using data from negative controls and Ag-NP treatments only.

Phylum	Intercept	Slope	Df 1	Df 2	F-value	p-value
Bacteroidetes	1918	3.03	1	45	36.21	<0.00001 ***
Patescibacteria	137	-0.57	1	45	32.73	<0.00001 ***
Gemmatimonadetes	35	-0.11	1	45	31.81	<0.00001 ***
Dependentiae	1	0.02	1	45	26.09	0.00001 ***
Firmicutes	861	-0.86	1	45	14.64	0.00040 ***
Hydrogenedentes	2	-0.01	1	45	11.52	0.00144 **
Cyanobacteria	6	-0.02	1	45	10.04	0.00275 **
FBP	2	-0.01	1	45	7.53	0.00868 **
Spirochaetes	31	-0.12	1	45	7.16	0.01035 *
Planctomycetes	54	-0.11	1	45	6.87	0.01193 *
BRC1	3	-0.01	1	45	5.07	0.02927 *
Acidobacteria	165	-0.18	1	45	3.61	0.06375
Chloroflexi	94	-0.09	1	45	2.18	0.14663
Actinobacteria	567	-0.31	1	45	1.93	0.17196
Lentisphaerae	0	0.00	1	45	1.89	0.17613
Proteobacteria	3099	-0.42	1	45	1.70	0.19917
Verrucomicrobia	430	-0.20	1	45	1.43	0.23821
Tenericutes	1	0.00	1	45	1.15	0.28937
Rokubacteria	1	0.00	1	45	0.79	0.37975
Nitrospirae	2	0.00	1	45	0.68	0.41451
Armatimonadetes	0	0.00	1	45	0.46	0.50068
Latescibacteria	1	0.00	1	45	0.13	0.72274
Fibrobacteres	46	-0.01	1	45	0.10	0.75469
Elusimicrobia	0	0.00	1	45	0.02	0.88352
Chlamydiae	2	0.00	1	45	0.00	0.97340

* p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001

Supplementary figures

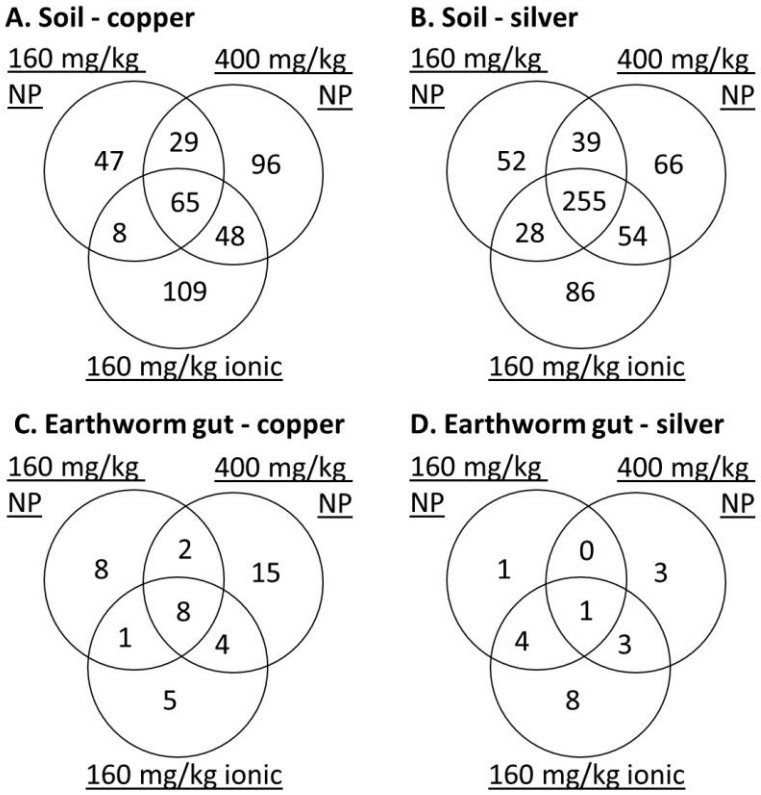

Figure S4.1 Venn diagram comparison of the differentially abundant ASVs in the two highest NP treatments and the ionic treatment for **A**) soil bacterial community from the copper experiment, **B**) soil bacterial community from the silver experiment, **C**) earthworm midgut microbiome from the copper experiment, and **D**) earthworm midgut microbiome from the silver experiment. For each comparison, the number of unique and shared ASVs are presented.

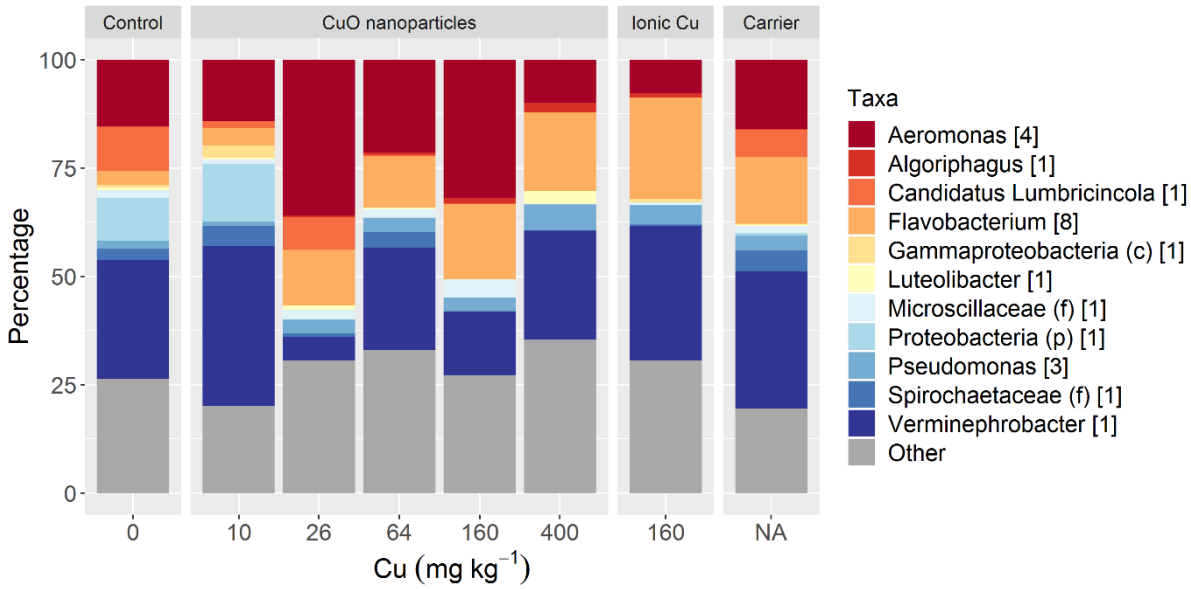

Figure S4.2: Average relative abundance of the bacterial genera per treatment from earthworm gut samples derived from the copper experiment. When genus of an ASV is unknown, next taxonomical level is given. Numbers between square brackets indicate the number of ASVs within the respective taxa. Taxa with a mean relative abundance of <2% are grouped under 'Other'.

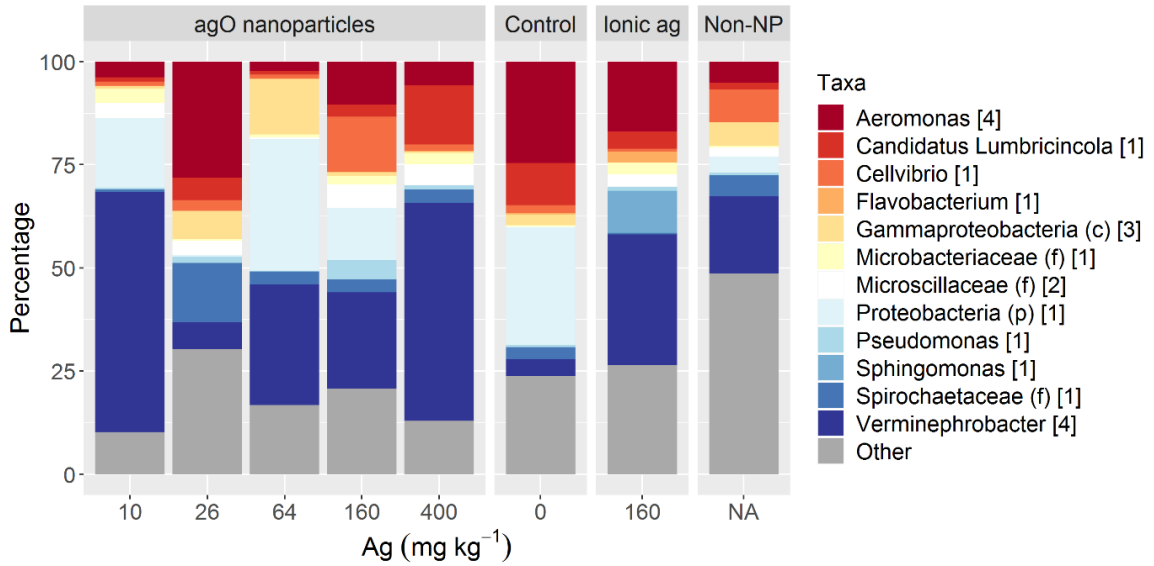

Figure S4.3: Average relative abundance of bacterial genera per treatment from earthworm gut samples derived from the silver experiment. When genus of ASV is unknown, next taxonomical level is given. The numbers between square brackets indicate the number of ASVs within the respective taxa. Taxa with a mean relative abundance of <2% are grouped under 'Other'.

Figure S4.4: Relative phylum abundance per sample from negative control soils, highest NP treatments soils and TiO_2 treated soils. Taxa with a mean relative abundance of <2% are not shown.

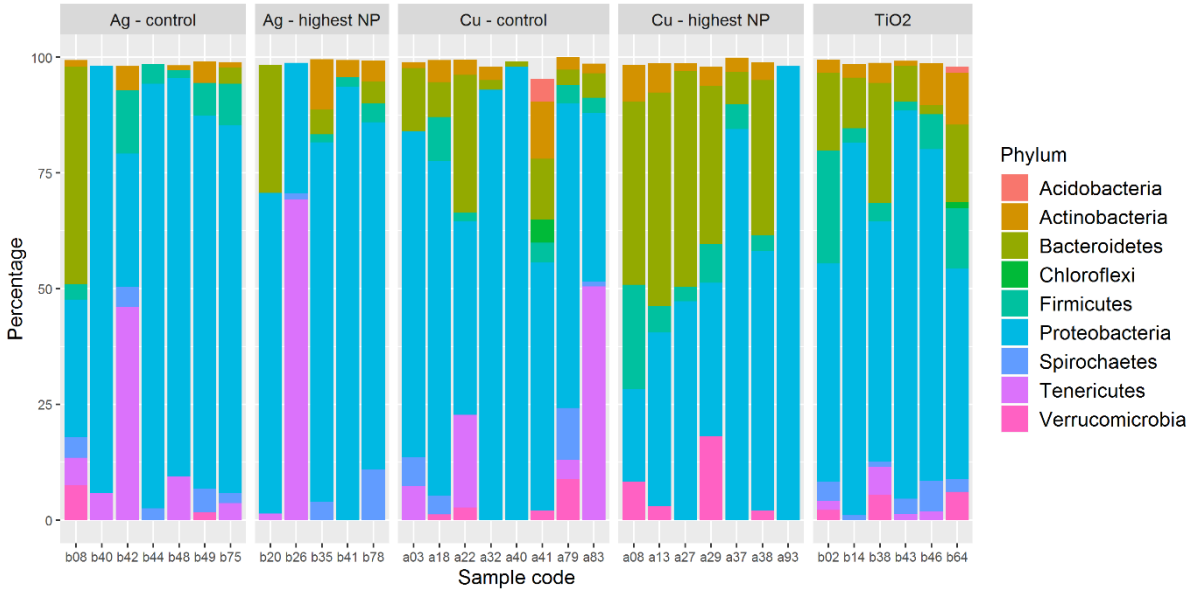

Figure S4.5: Relative phylum abundance per sample in earthworm gut samples taken from negative control soils, highest NP treatments soils and TiO_2 treated soils. Taxa with a mean relative abundance of <2% are not shown.

Chapter 5

Supplementary figures

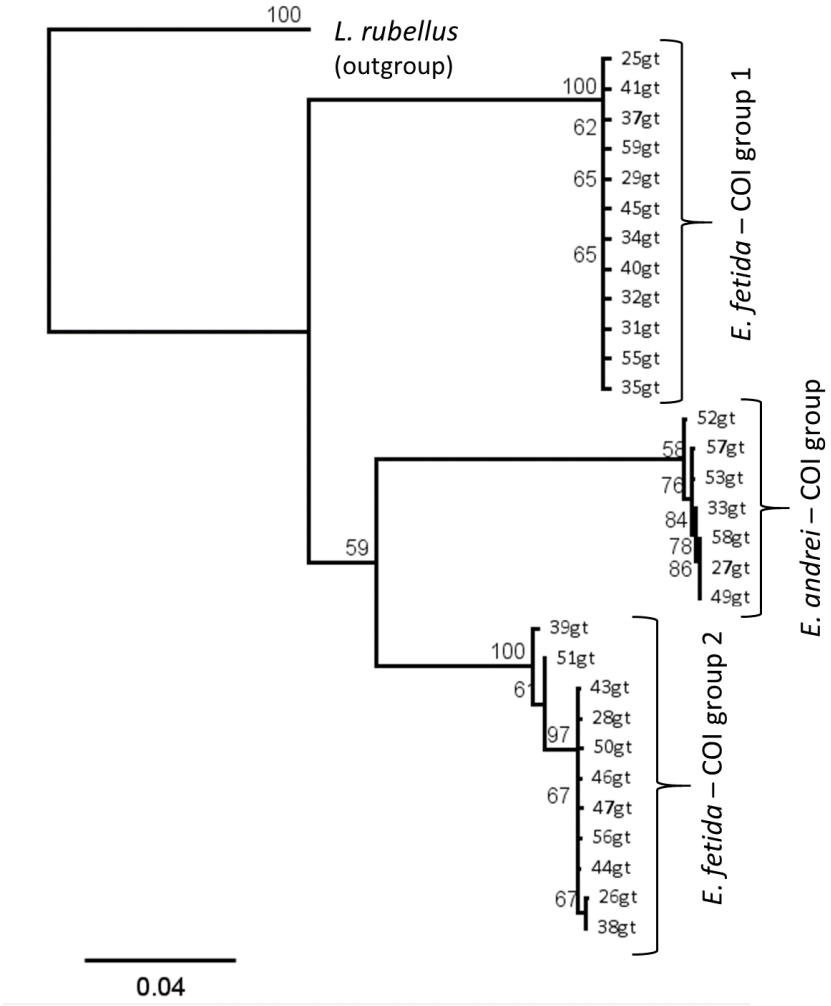

Figure S5.1: Maximum-likelihood phylogenetic tree of showing phylogenetic relation between all samples. Brackets indicate COI clusters. Bootstrap values are derived using 500 bootstraps.

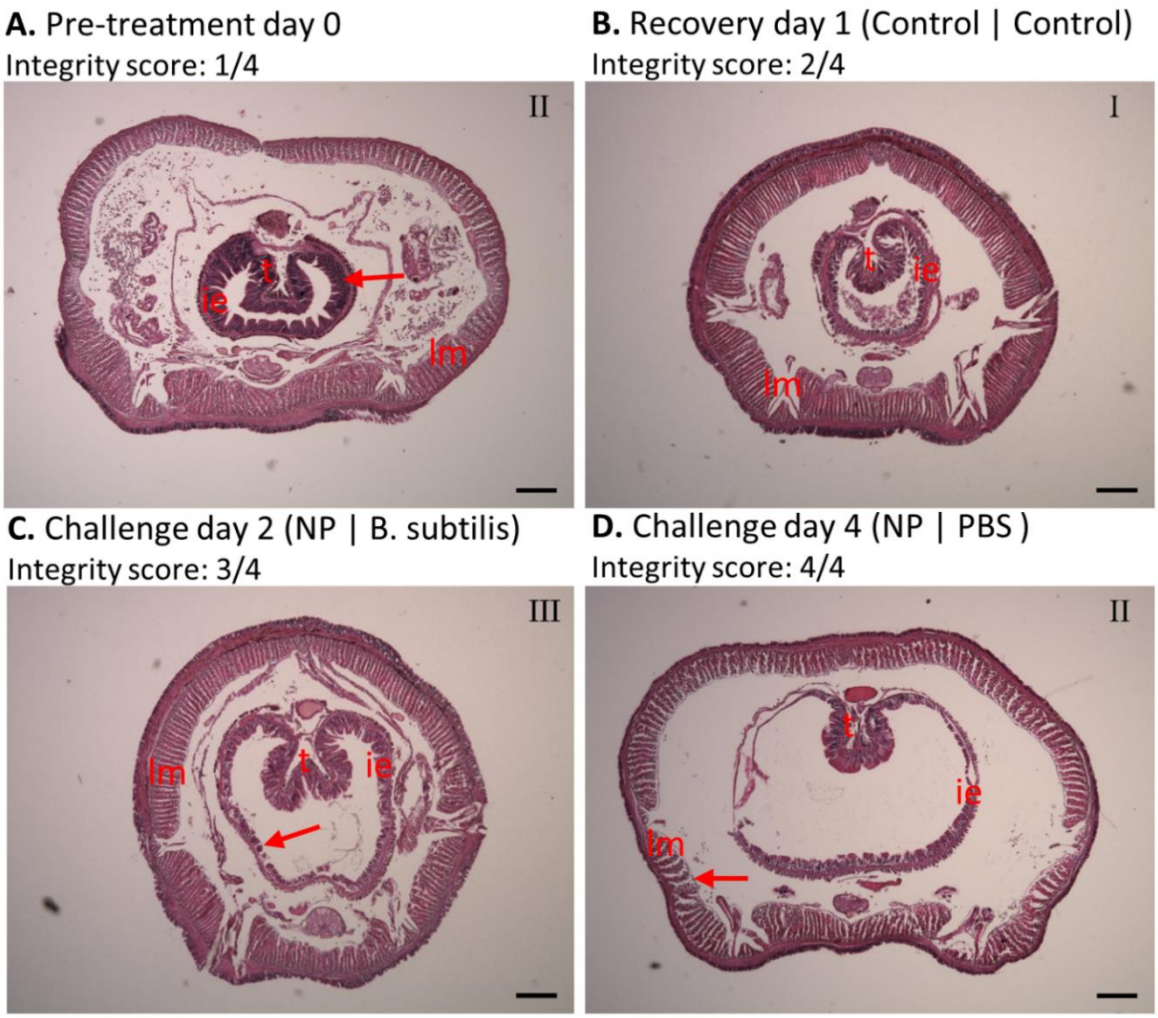

Figure S5.2: Examples of cross-sections of *E. fetida* stained with hematoxylin/eosin representing a range of tissue integrity scores. **A** represents a typical example of an undamaged specimen: a thick and dark colored intestinal lining (arrow). Damage to the intestine is manifested as the thinning the lining surrounding the intestinal epithelia as indicated by the arrow in **C**. A further indicator for tissue damage is the thinning of muscle fibers indicating necrosis of which a mild example is indicated by the arrow in **D.** Abbreviations: **t** typhlosole, **ie** intestinal epithelium and **lm** longitudinal muscles

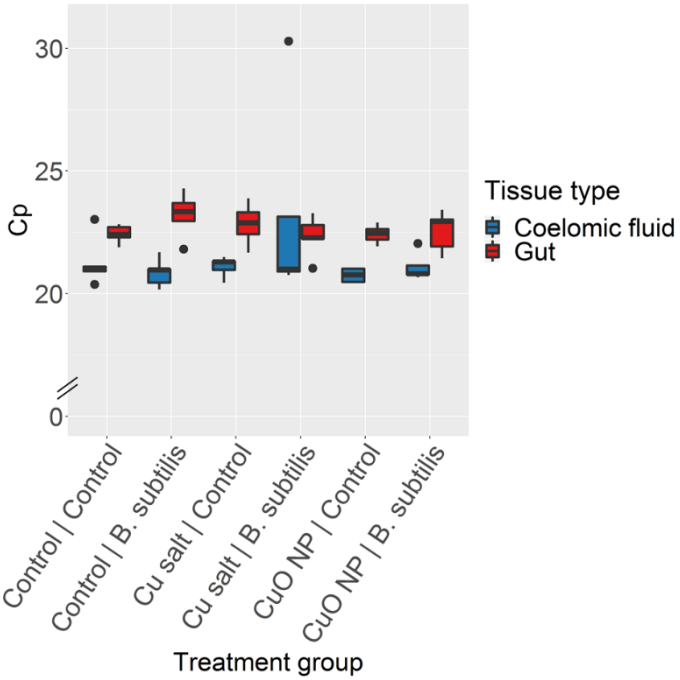

Figure S5.3: Boxplots of C_p values of elongation factor 1 alpha (ef1α) per treatment in coelomic fluid and gut tissue. On the x-axis, text before vertical bar indicates 'pre-treatment, text after vertical bar indicates 'bacterial challenge treatment'. No significant differences between treatment groups in C_p values were found for both coelomic fluid samples ($F(5,54)=1.071$ $p=0.401$) and gut tissue samples ($F(5,24)=1.37$ $p=0.27$). The outlier with C_p value ~30 in the 'Cu salt | B. subtilis' treatment group was removed from all further analysis.

Chapter 6

Supplementary tables

Table S6.1: Mean relative abundance (±SD) of the top 30 most abundant ASVs in the core resident gut bacterial community of *A. calignosa* and the mean relative abundance (±SD) of those core ASVs in top and bottom soils. In the third column, for ASVs for which the bacterial family is unknown the next taxonomical level is shown. 'n.d.' means 'not detected'.

Phylum	ASV id	Family or Order (o)	Genus + species	Resident gut		Top soil		Bottom soil	
				Mean	SD	Mean	SD	Mean	SD
Actino-bacteria	1	Micrococcaceae	Pseudarthrobacter	8.82	13.02	7.91	6.64	3.82	2.15
	13	Micrococcaceae	Pseudarthrobacter	1.41	3.12	0.47	0.51	1.38	0.86
	25	Micrococcaceae	Paeniglutamici-bacter	1.14	2.29	0.53	0.53	0.03	0.06
	35	Microbacteriaceae	Frigoribacterium	1.12	1.42	0.24	0.20	0.06	0.09
	61	Gaiellaceae	Gaiella	0.91	1.17	0.33	0.26	0.50	0.30
Chloro-flexi	22	Thermomicrobiaceae	Sphaerobacter thermophilus	3.59	7.54	0.27	0.26	0.48	0.32
Firmicutes	29	Bacillaceae	Bacillus	2.05	2.98	0.34	0.18	0.33	0.20
	49	Planococcaceae	Sporosarcina globispora	0.95	2.12	0.15	0.10	0.01	0.03
	74	Thermoactinomycetaceae	Planifilum	1.76	3.61	0.01	0.03	0.01	0.02
	92	Bacillaceae	Bacillus gottheilii	0.76	1.06	0.16	0.12	0.14	0.12
	114	Bacillaceae	Geobacillus	1.05	1.83	0.02	0.03	0.02	0.03
	136	Peptostreptococcaceae	Romboutsia sedimentorum	1.23	6.79	n.d		0.01	0.02
	215	Bacillales (o)		0.64	1.00	0.01	0.02	0.01	0.02
Proteobacteria	2	Enterobacteriaceae		20.92	29.41	n.d		n.d	
	10	Burkholderiaceae	Verminephrobacter	12.34	22.78	n.d		n.d	
	14	Moraxellaceae	Acinetobacter	1.32	6.11	0.26	0.47	0.36	0.25
	16	Burkholderiaceae	Delftia	1.47	2.12	n.d		n.d	
	18	Aeromonadaceae	Aeromonas	3.52	8.59	<0.01	0.02	0.14	0.77
	19	Aeromonadaceae	Aeromonas	4.18	9.23	0.01	0.05	0.01	0.06
	30	Aeromonadaceae	Aeromonas	2.13	5.22	0.05	0.10	<0.01	0.01
	34	Pseudomonadaceae	Pseudomonas	1.86	4.23	0.18	0.31	0.04	0.12
	38	Methyloligellaceae		1.88	2.60	0.41	0.28	0.54	0.31
	58	Pseudomonadaceae	Pseudomonas	1.95	6.84	0.01	0.02	0.03	0.09
	72	Xanthobacteraceae	Bradyrhizobium	0.81	1.36	0.18	0.13	0.85	0.43
	80	Burkholderiaceae	Herbaspirillum	3.00	5.47	n.d		n.d	
	85	Burkholderiaceae	Herbaspirillum	2.93	4.96	n.d		n.d	
	89	Enterobacteriaceae	Lelliottia	1.49	8.34	0.01	0.04	0.02	0.09
	97	Aeromonadaceae	Aeromonas	1.11	2.02	0.01	0.03	n.d	
	158	Burkholderiaceae	Acidovorax	1.58	3.03	n.d		n.d	
Teneri-cutes	6	Mycoplasmataceae	'Candidatus Lumbricincola'	5.97	17.53	0.01	0.05	<0.01	<0.01

Table S6.2: Mean relative abundance (±SD) of the top 30 most abundant ASVs in the core resident gut bacterial community of *A. calignosa* and the mean relative abundance (±S) of those core ASVs in top and bottom soils. In the third column, for ASVs for which the bacterial family is unknown the next taxonomical level is shown. 'n.d.' means 'not detected'.

Phylum	ASV id	Family or Order (o)	Genus + species	Resident gut		Top soil		Bottom soil	
				Mean	SD	Mean	SD	Mean	SD
Actinobacteria	1	Micrococcaceae	Pseudarthrobacter	4.57	3.61	7.91	6.64	3.82	2.15
	13	Micrococcaceae	Pseudarthrobacter	0.71	0.79	0.47	0.51	1.38	
	20	Nocardiaceae	Rhodococcus	0.96	1.27	0.22	0.30	0.01	0.02
	26	Intrasporangiaceae	Ornithinibacter	0.69	0.67	0.33	0.38	0.01	0.04
	32	Nocardioidaceae	Nocardioides	0.39	0.32	0.45	0.42	0.11	0.14
	35	Microbacteriaceae	Frigoribacterium	0.48	0.53	0.24	0.20	0.06	0.09
	40	Microbacteriaceae		0.43	0.35	0.29	0.27	0.03	0.05
	55	Microbacteriaceae	Microbacterium	0.39	0.50	0.07	0.11	0.02	0.05
	69	Nocardioidaceae	Nocardioides	0.32	0.33	0.07	0.08	0.01	0.03
Bacter-oidetes	17	Flavobacteriaceae	Flavobacterium	0.60	0.84	0.51	0.69	0.19	0.50
Chloro-flexi	22	Thermomicrobiaceae	Sphaerobacter thermophilus	0.35	0.39	0.27	0.26	0.48	0.32
Firmi-cutes	11	Planococcaceae	Planomicrobium koreense	0.75	0.76	0.69	0.58	0.01	0.02
	29	Bacillaceae	Bacillus	0.38	0.30	0.34	0.18	0.33	0.20
	33	Family_XII	Exiguobacterium	0.75	1.22	0.01	0.03	n.d.	
Proteobacteria	4			7.48	17.35	n.d.		n.d.	
	8	Burkholderiaceae	Verminephrobacter	5.53	15.93	n.d.		n.d.	
	12			1.89	11.86	n.d.		n.d.	
	16	Burkholderiaceae	Delftia	2.36	3.22	n.d.		n.d.	
	21	Gammaproteobacteria (c)		2.08	11.08	n.d.		n.d.	
	24	Cellvibrionaceae	Cellvibrio	0.69	1.42	0.21	0.33	0.02	0.08
	28	Burkholderiaceae	Verminephrobacter	1.74	6.81	n.d.		n.d.	
	30	Aeromonadaceae	Aeromonas	0.42	0.88	0.05	0.10	0.00	0.01
	42	Shewanellaceae	Shewanella	0.57	2.38	0.01	0.03	n.d.	
Spiro-chaetes	98	Spirochaetaceae		0.47	1.09	n.d.		n.d.	
Tenericutes	6	Mycoplasmataceae	'Candidatus Lumbricincola'	1.75	3.33	0.01	0.05	0.00	0.00
	7	Mycoplasmataceae		4.72	7.70	0.00	0.00	n.d.	
	9	Mycoplasmataceae		4.05	7.80	n.d.		n.d.	
	39	Mycoplasmataceae	'Candidatus Lumbricincola'	1.24	3.89	n.d.		0.00	0.01
	44	Mycoplasmataceae		1.15	3.65	0.00	0.01	n.d.	
Verruco-microbia	37	Rubritaleaceae	Luteolibacter	0.55	0.55	0.19	0.22	0.06	0.10

Table S6.3: Mean relative abundance (±SD) of the top 30 most abundant ASVs in the faecal bacterial community of *L. terrestris* and the mean relative abundance (±SD) of those core ASVs in top and bottom soils.

Phylum	ASV id	Family or Order (o)	Genus + species	Resident gut		Top soil		Bottom soil	
				Mean	SD	Mean	SD	Mean	SD
Actinobacteria	1	Micrococcaceae	Pseudarthrobacter	6.76	3.95	7.91	6.64	3.82	2.15
	13	Micrococcaceae	Pseudarthrobacter	1.33	1.11	0.47	0.51	1.38	0.86
	20	Nocardiaceae	Rhodococcus	1.09	0.86	0.22	0.30	0.01	0.02
	26	Intrasporangiaceae	Ornithinibacter	0.99	0.54	0.33	0.38	0.01	0.04
	32	Nocardioidaceae	Nocardioides	0.70	0.36	0.45	0.42	0.11	0.14
	35	Microbacteriaceae	Frigoribacterium	0.70	0.43	0.24	0.20	0.06	0.09
	40	Microbacteriaceae		0.70	0.35	0.29	0.27	0.03	0.05
	54	Intrasporangiaceae	Intrasporangium	0.57	0.26	0.32	0.33	0.01	0.07
	55	Microbacteriaceae	Microbacterium	0.53	0.33	0.07	0.11	0.02	0.05
	62	Microbacteriaceae		0.42	0.20	0.28	0.28	0.11	0.16
	66	Nocardioidaceae	Nocardioides	0.44	0.22	0.12	0.16	0.07	0.09
	69	Nocardioidaceae	Nocardioides	0.51	0.30	0.07	0.08	0.01	0.03
	90	Demequinaceae	Demequina	0.44	0.40	0.06	0.13	n.d.	
Bacteroidetes	17	Flavobacteriaceae	Flavobacterium	1.58	1.29	0.51	0.69	0.19	0.50
	41	Flavobacteriaceae	Flavobacterium	0.72	0.98	0.65	0.91	0.04	0.08
	52	Flavobacteriaceae	Flavobacterium reichenbachii	0.57	0.59	0.11	0.21	0.07	0.17
Chloroflexi	22	Thermomicrobiaceae	Sphaerobacter thermophilus	0.71	0.40	0.27	0.26	0.48	0.32
Firmicutes	11	Planococcaceae	Planomicrobium koreense	0.85	0.85	0.69	0.58	0.01	0.02
	29	Bacillaceae	Bacillus	0.72	0.21	0.34	0.18	0.33	0.20
	33	Family_XII	Exiguobacterium	1.02	1.24	0.01	0.03	n.d.	
	74	Thermoactinomycetaceae	Planifilum	0.44	0.19	0.01	0.03	0.01	0.02
Proteobacteria	24	Cellvibrionaceae	Cellvibrio	1.52	2.20	0.21	0.33	0.02	0.08
	30	Aeromonadaceae	Aeromonas	0.79	1.02	0.05	0.10	0.00	0.01
	34	Pseudomonadaceae	Pseudomonas	0.46	0.68	0.18	0.31	0.04	0.12
	38	Methyloligellaceae		0.57	0.27	0.41	0.28	0.54	0.31
	42	Shewanellaceae	Shewanella	0.98	3.06	0.01	0.03	n.d.	
Tenericutes	6	Mycoplasmataceae	'Candidatus Lumbricincola'	3.38	4.84	0.01	0.05	0.00	0.00
	39	Mycoplasmataceae	'Candidatus Lumbricincola'	0.47	0.88	n.d.		0.00	0.01
	57	Mycoplasmataceae	'Candidatus Lumbricincola'	0.59	0.95	0.00	0.01	n.d.	
Verrucomicrobia	37	Rubritaleaceae	Luteolibacter	1.01	0.63	0.19	0.22	0.06	0.10

Supplementary figures

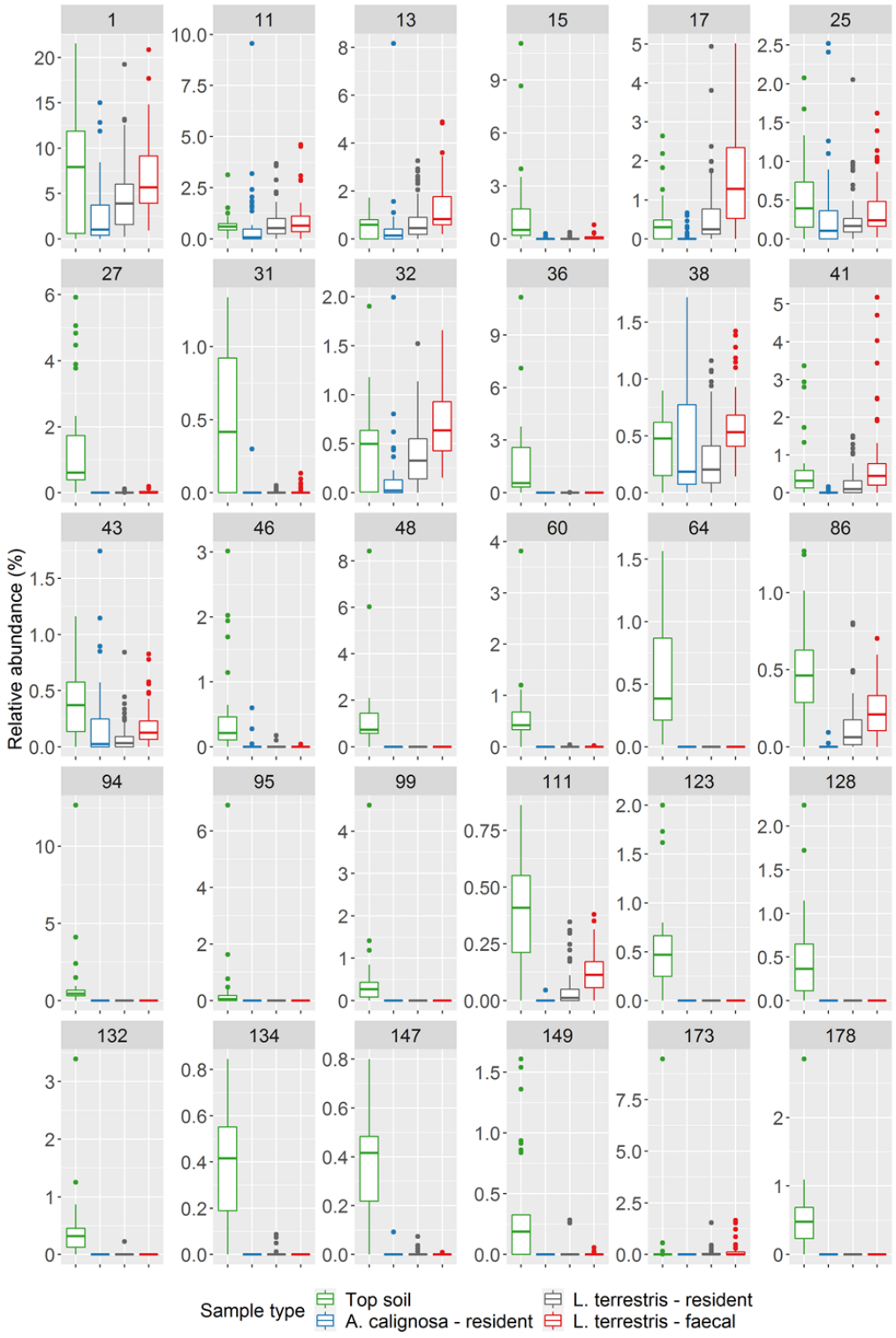

Figure S6.1: boxplots of the relative abundances of the top 30 most abundant top soil ASVs in top soil bacterial community (green boxplots), the resident gut microbiome of *A. calignosa* (blue), the resident gut microbiome of *L. terrestris* (grey) and the faecal microbiome *L. terrestris* (red). Numbers above each graph indicates the ASV id of the respective ASV.

Appendix 2: supplementary datasets

Six additional datasets (**Appendix 2A-F**) (format: .xlsx) part of **Chapter 4** describing log-logistic modelling outcomes, linear modelling outcomes and relative abundance of the core gut ASV in soils are available via the link below or per request via elmerswart@gmail.com.

OneDrive link: https://1drv.ms/x/s!Aq1o5akMDxRZh2c-LBV52OSUFKPf?e=yPHhHM