Abstract

Biosolids (treated sewage sludge) are used as agricultural fertilizer but are frequently contaminated with macrolide antibiotics, to which resistance is rising among historically susceptible bacteria. To determine if the land-application of macrolides carried in biosolids could promote antibiotic resistance in soil bacteria, soil plots were exposed annually to environmentally realistic or high doses of macrolides for ten years. I sequenced the bacterial 16S ribosomal DNA, metagenomic DNA, and integron gene cassettes within the treated and antibiotic-free soil to compare the compositions and diversities of the bacterial communities, antibiotic resistance genes, and mobile genetic elements. I determined that the high dose treatment of macrolides, but not the realistic dose, increased the diversity of clinically relevant antibiotic resistance genes and mobile genetic elements and decreased the abundance of soil cyanobacteria. Overall, typical concentrations of macrolides found in biosolids are unlikely to promote antibiotic resistance of concern to human health within soil bacteria.

Keywords: agriculture, antibiotic resistance, bioinformatics, biosolids, ecotoxicology, integrons, metagenomics, microbial ecology, mobile genetic elements, soil

Summary for Lay Audience

Antibiotics — chemicals that are used to treat bacterial infections — are widespread pollutants in the environment due to their mass production and use in human and veterinary medicine. Antibiotics that are consumed by people enter wastewater treatment plants where most treatment processes are unable to effectively eliminate these antibiotics. The persistent antibiotics may then contaminate the environment and promote antibiotic resistance in environmental bacteria.

Macrolide antibiotics are a group of antibiotics that are frequently detected in wastewater due to their use in human medicine and are important to human health. Unfortunately, bacterial pathogens (which are acquired from the environment) are becoming increasingly resistant to macrolide antibiotics. Therefore, there is an urgent need to understand the effects of macrolide antibiotic pollution on environmental bacteria to reduce the increase of antibiotic resistance in human pathogens.

One pathway for macrolides to enter the environment and possibly promote antibiotic resistance is through the use of treated sewage sludge (biosolids) as agricultural fertilizer. Macrolide antibiotics carry-over from wastewater into biosolids and then enter agricultural soil upon land-application. Macrolide antibiotics are known to promote antibiotic resistance in soil bacteria when present at high levels, but their effect on soil bacteria at environmentally realistic levels remains unclear.

I investigated if an environmentally realistic dose of macrolides for a

biosolids exposure event may promote antibiotic resistance in soil bacteria. In 2010, 2 m^2 soil field plots were established in London, ON and were annually exposed to a mixture of macrolides at an environmentally realistic dose or an effect-inducing unrealistically high dose, or were left antibiotic-free. After ten years, DNA from each of these soil plots was isolated, sequenced, and then analyzed to investigate changes in the soil bacterial community that may have occurred due to antibiotic exposure.

I determined that an environmentally realistic dose of macrolides is unlikely to harm soil bacterial diversity or promote clinically relevant antibiotic resistance. However, at an unrealistically high dose, genetic elements which encode and promote antibiotic resistance were increased. Further studies should investigate intermediate macrolide doses which may pose a risk to human health by promoting antibiotic resistance in soil bacteria.

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List of Abbreviations

3'-CS	3' conserved sequence
ANCOM-BC	Analysis of composition of microbiomes with bias correction
ANOVA	Analysis of variance
attC	Cassette-associated recombination site
attI	Integron-associated recombination site
CARD	Comprehensive antibiotic resistance database
CARSS	Canadian antimicrobial resistance surveillance system
CLR	Center log ratio
COG	Cluster of orthologous groups
dw	Dry weight
intI	Integron integrase gene
Meta-MARC	Metagenomic markhov models for antimicrobial resistance characterization
MIC	Minimum inhibitory concentration
NOEC	No-observed-effect concentration
PCA	Principal component analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
PNEC	Predicted no-effect concentation
rRNA	Ribosomal ribonucleic acid

1 Introduction and Literature Review

Antibiotics are chemicals that are used to treat and prevent bacterial infections. The first antibiotics were isolated in the early 20th century from environmental bacteria and fungi and were quickly adopted into human medicine (Hutchings et al., 2019). At the same time, antibiotics were used for chemotherapy, prophylaxis, and growth promotion in animal agriculture (Kirchhelle, 2018). From a year to a couple of decades after each antibiotic reached the drug market, however, resistance was acquired in a bacterium which was historically susceptible (Ventola, 2015). To make matters worse, we are in an antibiotic discovery void (Silver, 2011): the most recent antibiotic drug class to be discovered, the acid lipopeptides, was reported in 1987, and novel antibiotics that have been reported since then are members of existing drug classes (Debono et al., 1987).

Acquired antibiotic resistance is estimated to have caused 5,400 Canadian fatalities in 2018 — a number which is expected to rise to 13,700 deaths per year by 2050, and with increased rates of hospitalization, will likely result in a cumulative gross domestic product decline of $388 billion (Finlay et al., 2019). By 2050, the number of deaths globally due to multidrug-resistant microbial infections is estimated to overtake those caused by road traffic accidents and cancer combined (O'Neill, 2016). Despite the relatively recent industrialized use of antibiotics in healthcare and agriculture, antibiotic resistance is a modern

crisis of ancient origin (D'Costa et al., 2011). To ensure the continued efficacy of our existing antibiotics, we must understand the origins of antibiotic resistance and the factors which contribute to increased antibiotic resistance in clinically relevant bacteria.

1.1 Antibiotics as a global pollutant

The mass consumption of antibiotics beginning in the mid-20th century coincides with rising antibiotic resistance in zoonotic pathogens (Kirchhelle, 2018; Ventola, 2015) and environmental bacteria (Madueño et al., 2018). In addition to their critical role in human medicine, antibiotics are used for chemotherapy and prophylaxis in farm animals and were historically fed *en* masse to food-producing animals as growth promotion agents (Kirchhelle, 2018; Witte, 1998). The use of antibiotics as growth promotion agents has recently been banned in several countries such as the United States in 2017 (Scott et al., 2019), Canada in 2018 (Finlay et al., 2019), and China in 2020 (Hu & Cowling, 2020), but this practice still continues in many countries with few restrictions on usage (Chuanchuen et al., 2014). The industrialized use of antibiotics in healthcare and agriculture continues to require mass production, which allows antibiotics to enter the environment through many pathways, including discharge from antibiotic manufacturing facilities and hospitals (Bielen et al., 2017; Marathe et al., 2019), municipal sewage (Pärnänen et al., 2019), aquaculture (Reverter et al., 2020), and animal agriculture (Kirchhelle, 2018). Antibiotic pollution in the environment selects for antibiotic resistance genes (Bielen et al., 2017; Jechalke et al., 2014; Lau et al., 2020; Yi et al., 2019) which could be transferred to the human microbiome through interactions between humans, animals, and the environment (Berendonk et al., 2015; Hernando-Amado et al., 2019; Robinson et al., 2016; Tiedje et al., 2019).

1.2 Antibiotic resistance: A modern crisis of ancient origin

Antibiotic resistance is ancient and ubiquitous in the environment (D'Costa et al., 2011; Dunivin et al., 2019). For as long as bacteria and fungi have produced antibiotics, antibiotic resistance mechanisms were necessary as a defence against these toxins (Cundliffe, 1989). Soil is one of the largest known reservoirs of environmental antibiotic resistance (Dunivin et al., 2019). Soil bacteria are in a state of perpetual chemical warfare and use antibiotics to compete for valuable nutrients such as carbon and nitrogen but may also use them for cellular signalling (Fajardo & Martínez, 2008; Traxler & Kolter, 2015). The saturation of antibiotics in soil has led to an impressive arsenal of antibiotic resistance genes which are currently known to encode resistance to over a dozen antibiotic drug classes (Dunivin et al., 2019; Wright, 2007). Antibiotic resistance genes have been sequenced from 30,000 year-old permafrost, and some extant resistance gene families, such as serine beta-lactamases, have been predicted to share the same function as their ancestral sequences from two billion years ago (D'Costa et al., 2011; Hall & Barlow, 2004). Because of this conservation of function and continued selection due to antibiotic production, the totality of antibiotic resistance genes in soil — the soil "resistome" — is incredibly diverse, and antibiotic resistance genes can be selected for by anthropogenic antibiotic pollution (Jechalke et al., 2014; Lau et al., 2020).

1.3 One Health as a way forward

"One Health" is a framework that describes the interconnectedness of human, animal, and environmental health, and has been adopted by global health organizations, nations, and researchers to help understand and mitigate the

crisis of acquired antibiotic resistance (Tiedje et al., 2019). In 2015, the World Health Organization released their Global Action Plan on Antimicrobial Resistance which identified an important knowledge gap of "understanding how resistance develops and spreads, including how resistance circulates within and between humans and animals and through food, water and the environment" (World Health Organization, 2015). In this Action Plan, the World Health Organization recommended that individual member nations establish national action plans on antimicrobial resistance by adopting the One Health approach to mitigate resistance. Canada's Federal Framework for Action established the Canadian Antimicrobial Resistance Surveillance System (CARSS) to expand antimicrobial resistance surveillance to a national level, and in the CARSS 2020 report, the federal government acknowledged that "there is limited data regarding environmental surveillance — a necessary component of any One Health framework" (Public Health Agency of Canada, 2014, 2020). Of the three pillars of the One Health framework, the role of the environment in clinically relevant antibiotic resistance continues to be the least understood (Robinson et al., 2016).

1.3.1 The shared human-soil resistome

As described by the One Health framework, anthropogenically-driven increases of antibiotic resistance in soil bacteria may pose a threat to human health due to the shared human-soil resistome. Because of the ability of bacteria to exchange genetic material, the human microbiome and human bacterial pathogens share antibiotic resistance genes with environmental bacteria (Forsberg et al., 2012; Pal et al., 2016; Smillie et al., 2011), but the frequency and context of this exchange is poorly understood due to the challenges associated with source attribution (Berendonk et al., 2015; Huijbers et al., 2015) — i.e. determining the exact pathway of a resistance gene from environment to the human microbiome (Li et al., 2018; Tiedje et al., 2019). In a

bioinformatics analysis involving 200 soil and 100 human gut metagenomes, 25% of gut-associated antibiotic resistance genes (*n* = 12) were shared with resistance genes found in soil (Pal et al., 2016). This *in silico* work is also supported by functional metagenomics studies which have recovered resistance genes in soil bacteria that are identical or very similar to those detected in clinical isolates (Allen et al., 2009; Forsberg et al., 2012; Lau et al., 2017).

Antibiotic resistance genes may be transmitted from soil bacteria to the human microbiome through the consumption of produce (Blau et al., 2018; Maeusli et al., 2020). The transmission of antibiotic resistance from an environmental bacterium on a leafy vegetable to *Escherichia coli*, and then to a commensal gut bacterium has recently been demonstrated in the mouse microbiome which is a useful model for the human microbiome (Krych et al., 2013; Maeusli et al., 2020). Multi-drug resistance plasmids containing tetracycline, beta-lactam, sulfonamide, aminoglycoside, and fluoroquinolone resistance genes have also been captured in *E. coli* from bacteria in cilantro and mixed salad, indicating that this process could occur in the human gut (Blau et al., 2018). In addition, vegetables grown in soil enriched with antibiotic resistant bacteria can themselves be enriched with the same antibiotic resistance genes (Murray et al., 2019; Rahube et al., 2016; Rahube et al., 2014). Overall, transmission of antibiotic resistance genes from soil bacteria to the human microbiome is plausible, but more research is needed to determine the frequency and mechanisms of this transmission.

1.4 The interaction of the soil bacterial resistome and mobilome

The soil bacterial resistome is generally considered to be structured by bacterial community composition as most antibiotic resistance genes in soil

bacteria are encoded within the bacterial chromosome and are therefore inherited vertically (parent to progeny) (Dunivin et al., 2019; Forsberg et al., 2014). Additionally, species diversity has been proposed to "act as a biological barrier" for antibiotic resistance — a loss of soil bacterial diversity is correlated with increased antibiotic resistance gene abundance, suggesting that antibiotic resistant taxa tend to dominate less diverse communities (Chen et al., 2019; van Goethem et al., 2018; Vivant et al., 2013). When a selective pressure (e.g. antibiotics) is strong enough, however, the soil resistome could become 'decoupled' from bacterial community composition and diversity as antibiotic resistance genes can be exchanged and re-arranged horizontally (between parents, between progeny) (Johnson et al., 2016).

1.4.1 Mobile genetic elements and horizontal gene transfer

Mobile genetic elements are entities that promote the mobility of DNA sequences within (chromosome–plasmid, plasmid–plasmid, chromosome–chromosome) and between bacterial genomes, and the totality of all mobile genetic elements in an environment is referred to as the "mobilome" (Partridge et al., 2018; Perry & Wright, 2013). The mobilome facilitates the horizontal gene transfer of antibiotic resistance genes between bacteria, and includes elements such as plasmids, transposons, and integrons (Partridge et al., 2018), bacteriophages (Colomer-Lluch et al., 2011; Subirats et al., 2016), and membrane vesicles (Chattopadhyay & Jaganandham, 2015). Horizontal gene transfer occurs via three main mechanisms: conjugation (physical interaction between bacteria), transformation (intake of extracellular DNA), and transduction (phage-mediated) (Partridge et al., 2018). While all three of these mechanisms are known to occur in soil, conjugation has been studied the most extensively and is the most frequent mechanism of horizontal transfer of antibiotic resistance in soil (Perry & Wright, 2013), though transformation and transduction likely also play important roles (Aminov, 2011;

Perry & Wright, 2013). Of all of the known non-plasmid mobile genetic elements to mobilize antibiotic resistance in soil bacteria, integrons may be the most genetically diverse (Ghaly et al., 2019).

1.4.2 Integrons

Integrons are mobile genetic elements that are capable of acquiring, expressing, and re-arranging antibiotic resistance genes within their environments, but notably lack the capability to move themselves, relying upon other mobile genetic elements such as plasmids and transposons for mobility (Gillings, 2014). Integrons sample their environment for gene cassettes (Ghaly et al., 2020) — pieces of DNA that usually contain one open reading frame followed by a cassette-associated recombination site (*attC*). Gene cassettes are known to carry a diverse repertoire of antibiotic resistance genes, putative virulence genes, and many other genes of unknown function that have been proposed as a discovery platform for potentially novel natural products (Ghaly et al., 2019; Ghaly et al., 2020; Ma et al., 2017). In a recent sequencing study of the diversity of integron gene cassettes (gene cassette metagenomes) within soil samples from Antarctica and Australia, it was estimated that there are 4,000 to 18,000 unique gene cassettes per 0.3 g of soil (Ghaly et al., 2019).

Integrons are characterized by i) an integron-integrase gene (*intI*) encoding a site-specific tyrosine recombinase, ii) an integron-associated recombination site (*attI*), where incoming gene cassettes are inserted with the help of IntI, and iii) an integron-associated promoter (P_C) which expresses downstream gene cassettes (Figure 1) (Gillings, 2014). IntI catalyzes the recombination of *attC* with *attI* to insert an incoming gene cassette downstream of P_C and can also reversibly excise an integrated gene cassette from the integron structure. The recombination event produces two daughter molecules: a duplicate of the original integron structure, and the other with the integrated gene cassette

(Ghaly et al., 2020). This phenomenon allows the host bacterium to sample each gene cassette for fitness tradeoffs prior to stable integration, and in the context of a cassette-encoded antibiotic resistance gene, maintain the antibiotic resistance phenotype if it confers a selective advantage (Ghaly et al., 2020).

1.4.3 Class 1 integrons

Of the hundreds of different classes of integrons (Abella et al., 2015), the class 1 integron is the most prolific in human pathogens and is also abundant in soil (Dawes et al., 2010; Gillings, 2018; Ruiz-Martínez et al., 2011) (Figure 1). Class 1 integrons typically carry less than six and no more than eight gene cassettes (Gillings, 2014; Naas et al., 2001), and are distinguished from other classes of integrons by their *intI1* gene known as the class 1 integron-integrase, which are 98% identical in amino acid sequence (Roy et al., 2021). In addition to their ability to form multi-drug resistance gene cassette arrays, class 1 integrons form 'linkage clusters' of antibiotic resistance in soil, as they frequently co-occur with other mobile genetic elements and with antibiotic resistance genes that are not embedded within gene cassettes (Johnson et al., 2016; Pal et al., 2015). The "clinical" or "*sul1*-type" variant of class 1 integrons has a 3' conserved segment (3'-CS) with a partially deleted but semi-functional disinfectant resistance gene *qacE*Δ*1*, followed by the sulfonamide antibiotic resistance gene *sul1* (Figure 1) (Partridge et al., 2018).

1.4.4 Co-selection

Mobile genetic elements, especially class 1 integrons, facilitate the co-selection of antibiotic resistance genes in soil (Pal et al., 2015). Co-selection occurs when antibiotic exposure selects for resistance to the present antibiotic drug class and to one or more environmentally absent antibiotic drug classes. Co-selection can be explained through two main processes: i) cross-resistance, when an

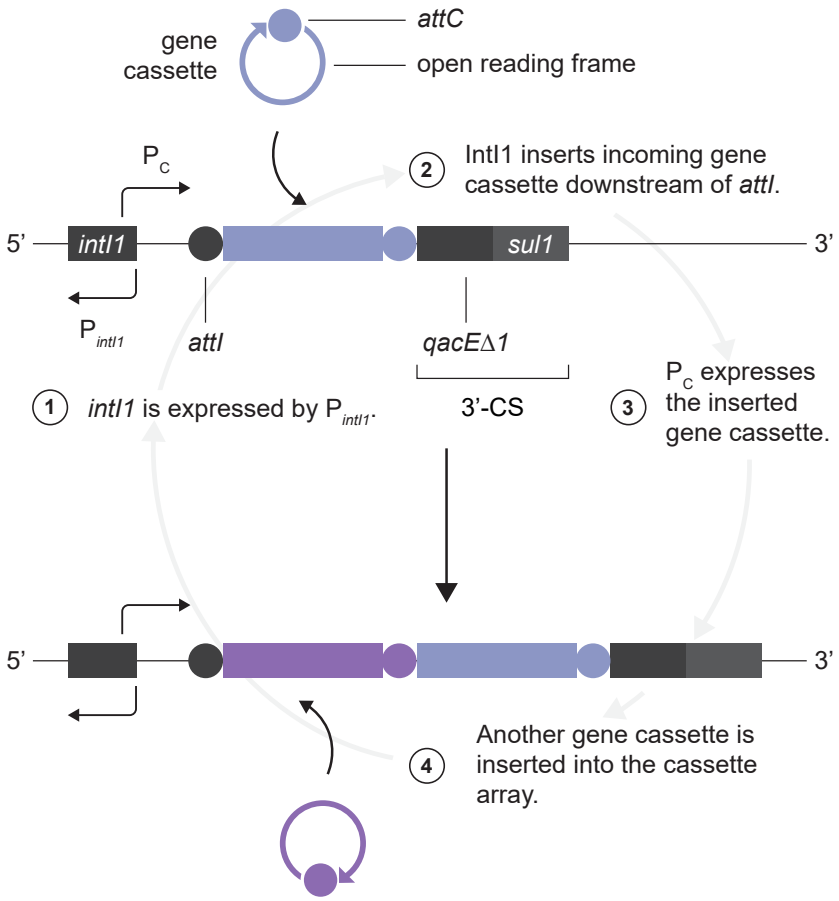


Figure 1 Structure of a class 1 integron. Class 1 integrons are clinically-relevant integrons that are ubiquitous in human-impacted environments, and are characterized by their class 1 integron-integrase gene (*intI1*) and 3' conserved sequence (3'-CS) which contains a partially deleted biocide resistance gene (*qacEΔ1*) and a sulfonamide antibiotic resistance gene (*sul1*). Cassette-associated recombination is similar for all classes of integrons: **1**) *intI1* is expressed by the integron-integrase promoter P_{intI1}, **2**) the tyrosine recombinase IntI1 catalyzes the recombination of the incoming gene cassette's attachment site (*attC*) with the integron-associated attachment site (*attI*), **3**) the integron-associated promoter (P_C) expresses the inserted gene cassette, and **4**) another gene cassette may be inserted to form a gene cassette array of the two cassettes.

antibiotic resistance gene is selected by an environmentally present drug class and, due to its mechanism of action, also confers resistance to an absent drug class; and ii) co-resistance, when an antibiotic resistance gene is selected and is physically linked to a different resistance gene which confers resistance to an absent drug class (Wales & Davies, 2015). Class 1 integrons, which are known to possess gene cassettes that are heavily biased towards conferring antibiotic resistance phenotypes (Ghaly et al., 2020; Yang et al., 2021), facilitate the co-selection of antibiotic resistance genes in the environment by forming multi-drug resistance gene cassette arrays (Naas et al., 2001).

From a One Health perspective, class 1 integrons are of particular concern because i) they have become endemic to human and environmental microbiomes (Gillings, 2017), ii) they are increased in the presence of antibiotic pollution (Gillings, 2017; Stalder et al., 2014; Wright et al., 2008), iii) their gene cassette content is biased towards conferring antibiotic resistance phenotypes (Yang et al., 2021), iv) some antibiotics indirectly increase the transcription of *intI1*, thereby increasing gene cassette recombination (Baharoglu et al., 2010; Guerin et al., 2009), and v) they form co-occurrence linkage clusters with other mobile genetic elements and antibiotic resistance genes (Pal et al., 2015). Class 1 integrons are known to be enriched in soils that have been polluted with macrolide antibiotics (Lau et al., 2020).

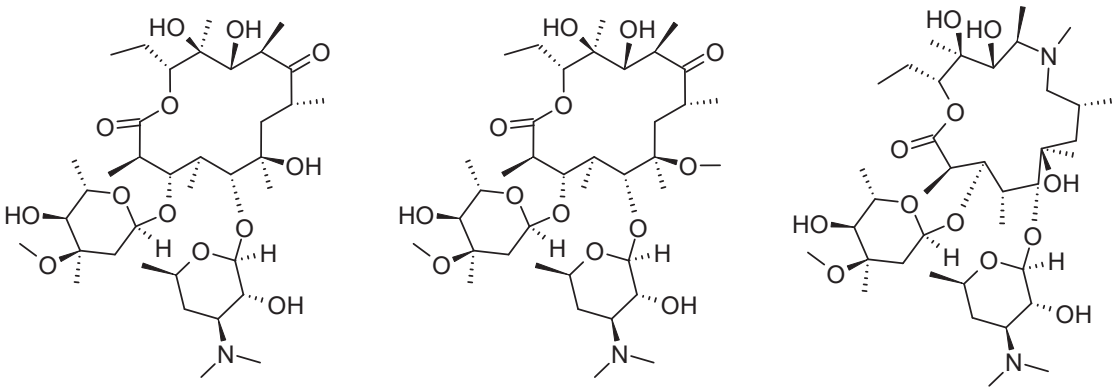
1.5 Macrolide antibiotics

1.5.1 Importance to human and animal medicine

Macrolide antibiotics are the third most-consumed antibiotics in Canada and are used as first-line treatments for serious diseases such as community acquired pneumonia (caused by *Streptococcus* and *Mycoplasma*), campylobacteriosis (*Campylobacter jejuni*, *Campylobacter coli*), and as alternatives for individuals allergic to beta-lactam antibiotics (Lohsen &

Stephens, 2019; Public Health Agency of Canada, 2020). Despite their prolific use in human medicine, most macrolide antibiotics that are sold are consumed by food-producing animals for chemotherapy and prophylaxis (Lohsen & Stephens, 2019). In 2018 alone, 87,221 kg of macrolide antibiotics were sold for consumption in Canadian agriculture (Public Health Agency of Canada, 2020). These antibiotics have been deemed “critically important” for human medicine by the World Health Organization and resistance to these drugs is rising (Public Health Agency of Canada, 2020; World Health Organization, 2017). Risk management strategies that focus on reducing macrolide presence in the environment will mitigate future risks to human health.

1.5.2 Structure



Erythromycin A ($C_{37}H_{67}NO_{13}$)

Clarithromycin ($C_{38}H_{69}NO_{13}$)

Azithromycin ($C_{38}H_{72}N_2O_{12}$)

Figure 2 Chemical structures and molecular formulas of the macrolide antibiotics erythromycin A, clarithromycin, and azithromycin.

Erythromycin A was first isolated from the soil bacterium *Saccharopolyspora erythraea* in 1952 and most other macrolide antibiotics are chemically modified derivatives of erythromycin A, which is the primary active compound in the antibiotic medicine erythromycin (Haight & Finland, 1952). Erythromycin, clarithromycin, and azithromycin are the most consumed

macrolides in human medicine, as reflected by their prevalence in wastewater (Miao et al., 2004; Rodriguez-Mozaz et al., 2020). Macrolide antibiotics are characterized by a 14-, 15-, or 16-membered macrocyclic lactone ring bound to at least one deoxy sugar (erythromycin A, clarithromycin, and azithromycin are bound to desosamine and cladinose; Figure 2) (Lohsen & Stephens, 2019). Clarithromycin is identical to the 14-membered erythromycin A but with a methylated C6-hydroxy group, resulting in a more acid-labile molecule. Azithromycin is a 15-membered macrolide created from the insertion of a nitrogen atom into the lactone ring of erythromycin A, resulting in more potent antibacterial activity against many gram-negative pathogens such as *Haemophilus influenzae* (bacterial flu) and *Neisseria gonorrhoeae* (gonorrhea) (Yanagihara et al., 2009).

1.5.3 Mechanisms of action and resistance

Macrolides inhibit protein synthesis in gram-positive (and some gram-negative) bacteria by reversibly binding to the 23S ribosomal RNA (rRNA) within the bacterial 50S ribosomal subunit at the entrance of the peptide exit tunnel, which imperfectly prevents assembly and elongation of the peptide (Fyfe et al., 2016; Lohsen & Stephens, 2019). This mechanism is usually bacteriostatic — the macrolides alone do not kill all of the bacterial cells but they prevent cellular replication, and the host's immune system must clear the remainder of the infection (Pankey & Sabath, 2004). Resistance mechanisms for macrolide antibiotics are diverse (Fyfe et al., 2016). Resistance can be evolved through target site mutation in the bacterial ribosome or can be horizontally acquired. Antibiotic resistance genes may encode a methyltransferase which methylates the ribosome and prevents binding of the antibiotic (*erm* gene family), or an efflux pump to remove the antibiotic from the cell (*msr* and *mef* gene families), or a phosphotransferase to inactivate the antibiotic (*mph* gene family) (Fyfe et al., 2016). Many of these antibiotic resistance genes are mobile as

demonstrated by the *erm* gene family, as over 40 *erm* genes have been identified and most of them are plasmid-encoded (Alcock et al., 2020; Leclercq, 2002). Some macrolide antibiotic resistance genes (e.g. *ereA1*, *ereA2*) are known to be encoded in integron gene cassettes (Partridge et al., 2009).

1.5.4 Effects of long-term macrolide antibiotic pollution in agricultural soil

Macrolide antibiotic pollution of soil is known to promote antibiotic resistance (Lau et al., 2020). Over an eight-year period, soil field plots were annually exposed to the macrolide antibiotics erythromycin, clarithromycin, and azithromycin which resulted in increased abundances of antibiotic resistance genes and mobile genetic elements, including class 1 integrons. Interestingly, most of the increased antibiotic resistance genes were predicted to confer resistance to non-macrolide antibiotic drug classes, indicating that macrolide antibiotic exposure of soil co-selects for resistance to aminoglycosides, sulfonamides, and trimethoprim. Several of these antibiotic resistance genes are known to be associated with class 1 integrons, suggesting a role for class 1 integrons in this co-selection process (Lau et al., 2020). Macrolide antibiotics are also more rapidly degraded in soil with a previous exposure history to macrolides, indicating that macrolides may affect the diversity and composition of the soil microbial community (Topp et al., 2016). This effect could be detrimental to some of the important ecosystem services provided by soil microorganisms.

Soil microorganisms perform many important ecosystem services, including organic carbon cycling, carbon and nitrogen fixation, and macronutrient solubilization — all of which are essential for plant growth (Waldrop & Creamer, 2019). The protection of soil microbial communities is increasingly important as human activities, such as antibiotic pollution,

threaten to disrupt these important ecosystem services, thereby threatening agricultural productivity (French et al., 2009). Limits for antibiotic pollution in aquatic environments have been proposed based upon toxicity to the microbial community: No-observed-effect concentrations (NOECs) for cyanobacteria (i.e. the concentrations above which growth is inhibited) have been proposed as baseline limits in aqueous environments due to the susceptibility of cyanobacteria to several antibiotic drug classes, but similar biological indicators have not yet been proposed for terrestrial environments (Committee for Medicinal Products for Human Use, 2015; Tell et al., 2019). Further research is needed to identify limits and biological indicators for antibiotic pollution in soil.

1.6 Biosolids as a vector for macrolide antibiotic pollution of soil

Macrolide antibiotics, among various other pharmaceuticals, are discharged into the environment through human waste and are inefficiently removed by most wastewater treatment processes (Le-Minh et al., 2010; Luo et al., 2014). In Canada, only 28% of the population is served by tertiary wastewater treatment, which is the most effective treatment stage for removing antibiotics; additionally, the purpose of this treatment is to remove pathogens rather than to remove pharmaceuticals (Environment and Climate Change Canada, 2020; Le-Minh et al., 2010). Abundances of antibiotics and antibiotic resistance genes are currently unregulated in Canadian wastewater effluent and many other countries, and as a result, wastewater effluent is a large reservoir of antibiotic resistance genes and mobile genetic elements (Che et al., 2019; Rizzo et al., 2013). Macrolide antibiotics from wastewater effluent can contaminate soil through the agricultural use of treated sewage sludge (McClellan & Halden, 2010; Sabourin et al., 2012).

1.6.1 Agricultural use of biosolids

Biosolids (treated sewage sludge) are recycled material from wastewater treatment plants that can be used as an agricultural fertilizer and soil amendment (Sharma et al., 2017). The solid portion of biosolids is comprised of approximately 50% organic matter and 50% mineral material (Ontario Ministry of Agriculture, Food and Rural Affairs, 2010). Unfortunately, antibiotics that survive the wastewater treatment process can carry over into biosolids, including those of the macrolide antibiotics drug class such as erythromycin, clarithromycin, and azithromycin (Chenxi et al., 2008; McClellan & Halden, 2010; Sabourin et al., 2012).

Biosolids are produced from the separation of wastewater into water and solids, followed by treatment of the solid portion to reduce pathogens and odour using a combination of chemical, biological, or physical processes (Le-Minh et al., 2010). Biosolids improve soil quality and fertility: soil that is more fertile requires less inorganic fertilizer, which reduces the risk of fertilizer runoff into adjacent water sources, and soil that has more organic matter has increased moisture retention. Biosolids are applied to agricultural soil on every continent except Antarctica, but usage is highly variable: almost all of the biosolids that are produced in the United Kingdom (78%) and Ireland (96%) are land-applied, whereas only 55% are land-applied in the United States (Sharma et al., 2017). There are concerns, however, that the long-term application of biosolids to agricultural soil could introduce macrolide antibiotics into the environment and promote resistance in soil bacteria, which could be transferred to humans via consumption of produce as described by the One Health framework (Lau et al., 2020; Sabourin et al., 2012).

1.6.2 Concentrations of macrolide antibiotics in biosolids and comparison to predicted no-effect concentration (PNEC)

In a survey of 74 locations producing treated biosolids in the United States, the upper 95th percentile concentrations of detected macrolides were 0.1 mg kg^{-1} biosolids (dry weight, dw) erythromycin, 0.2 mg kg^{-1} clarithromycin, and 3.2 mg kg^{-1} azithromycin (U.S. Environmental Protection Agency, 2021). Biosolids are typically applied at a rate of 1–10% dw dw^{-1} soil, meaning that the upper-range of environmentally relevant concentrations for these macrolides in biosolids-applied soil would be at-minimum 0.001 mg kg^{-1} erythromycin, 0.002 mg kg^{-1} clarithromycin, and 0.032 mg kg^{-1} azithromycin. These concentrations are equal to, 8-fold, and 128-fold greater than the Predicted No-Effect Concentrations (PNEC) for erythromycin, clarithromycin, and azithromycin, which were based upon lowest minimum inhibitory concentrations (MICs) for these antibiotics (Bengtsson-Palme & Larsson, 2016).

The MIC is the lowest concentration of an antibiotic required to inhibit all visible growth of a bacterial strain after a 24 h incubation period, and when considering that antibiotic resistance can be selected for at concentrations below the MIC, the lowest MIC observed over a range of bacteria can be divided by 10 to arrive at the PNEC-MIC, which describes a conservative predicted concentration above which antibiotic resistance could be selected for in the environment (Bengtsson-Palme & Larsson, 2016; Tell et al., 2019). The PNEC-MIC has been proposed as one regulatory limit for antibiotics residues in manufacturing facility effluents (Tell et al., 2019). The 95th percentile concentrations of macrolides in biosolids-amended soil are equal to or exceed their PNEC-MICs; therefore, macrolide antibiotics could realistically select for antibiotic resistance in biosolids-amended soil.

1.6.3 Challenges of studying the effects of biosolids-introduced antibiotics on the soil resistome and mobilome

There are a few challenges associated with studying the effects of macrolide antibiotic exposure on soil bacteria from biosolids land-application. First, biosolids are known to carry enteric bacteria (Sidhu & Toze, 2009), which could compete with the native soil bacterial community to alter bacterial composition and/or diversity, leading to changes in the abundances of native antibiotic resistance genes. Second, these enteric bacteria may also carry antibiotic resistance genes (Chen et al., 2016; Rahube et al., 2014), making it difficult to determine the source (native or non-native) of any increased antibiotic resistance gene in response to biosolids exposure, especially as organic matter creates areas of increased horizontal gene transfer in soil (Heuer & Smalla, 2007). Third, because biosolids often contain dozens of pharmaceuticals (U.S. Environmental Protection Agency, 2021), determining which of these substances select for antibiotic resistance in soil bacteria would be very difficult without further experimentation. The direct exposure of soil to macrolide antibiotics is a less-realistic approximation of biosolids land-application, but avoids challenges associated wtih the introduction of non-native bacteria, antibiotic resistance genes, organic matter, and other pharmaceuticals into soil.

1.6.4 Critical knowledge gaps

In summary, the land-application of biosolids introduces pharmaceuticals into agricultural soil, including antibiotics, that have carried over from the wastewater treatment process (McClellan & Halden, 2010; Sabourin et al., 2012). These antibiotics are present at concentrations that are predicted to select for resistance in the soil bacterial community (Bengtsson-Palme &

Larsson, 2016; U.S. Environmental Protection Agency, 2021), and the exposure of soil to macrolide antibiotics increases the abundance of antibiotic resistance genes and mobile genetic elements in soil bacteria, including class 1 integrons (Lau et al., 2020). This antibiotic exposure is also known to co-select for resistance to anthropogenically absent drug classes of antibiotics, and for resistance genes that are known to be associated with class 1 integron gene cassettes (Lau et al., 2020).

Because the health of humans and soil are interconnected as described by the One Health framework (Tiedje et al., 2019), and because biosolids are a vector for the introduction of macrolide antibiotics into the environment (McClellan & Halden, 2010; Sabourin et al., 2012), the long-term repeated exposure of soil to biosolids may pose a risk to human health by promoting antibiotic resistance. The effect of macrolide antibiotic exposure on the soil bacterial community and the integron gene cassette metagenome remains to be determined, as does the potential for macrolides to select for antibiotic resistance in soil bacteria at concentrations that are environmentally relevant to a biosolids exposure scenario. The purpose of this thesis was to address these critical knowledge gaps by investigating the response of the soil bacterial community, resistome, and mobilome to a decade of macrolide antibiotic contamination using sequencing-based methods.

1.7 Review of sequencing-based methods

1.7.1 16S rDNA amplicon sequencing

Most soil bacteria are difficult to cultivate: of the approximately 10^8 cells of bacteria that can be found in a single gram of bulk soil, less than 1% are estimated to be cultivable using standard growth techniques (Raynaud & Nunan, 2014; van Pham & Kim, 2012). The selectivity of nutrient media, competition in media by faster growing organisms, and low abundance in the

environment relative to other species all contribute to the difficulty in culturing most soil bacteria (da Rocha, 2019). Sequencing-based approaches to investigate the bacterial community have shone a light on the incredible diversity of uncultivable soil bacteria (Hug et al., 2016) and have allowed researchers to investigate the responses of the soil bacterial community to environmental perturbations (Isobe et al., 2019; Isobe et al., 2020). Of the different sequencing-based approaches available to investigate soil bacterial community composition, 16S rDNA amplicon sequencing and metagenomic sequencing are presently the most common.

High-throughput 16S rDNA amplicon sequencing (i.e. metabarcoding) involves the targeted polymerase chain reaction (PCR) amplification, then sequencing of the complete or partial 16S rRNA gene, whereas metagenomic sequencing involves the direct sequencing of environmental DNA without prior PCR amplification of a specific gene target. Unlike with Sanger sequencing, the high-throughput sequencing of 16S rDNA amplicons allows researchers to assay an entire environment rather than single organisms or sequences. The bacterial 16S rRNA gene is used as a taxonomic marker because of i) its universal presence in all prokaryotes, ii) regions of highly conserved sequences between bacterial species, and iii) hypervariable regions which allow for species-specific classification (van Pham & Kim, 2012). Typically, only a subset of the hypervariable regions are sequenced (usually some combination of the V3, V4, V5, V6 regions) to classify bacterial taxa based upon sequence variation, though advances in long-read technologies have made full-length high-throughput 16S rDNA sequencing an attractive alternative (Numberger et al., 2019; Shin et al., 2016; Yang et al., 2016).

Software such as DADA2 can attempt to infer biological sequences of 16S rRNA prior to PCR and sequencing by constructing amplicon sequence variants (Bolyen et al., 2019; Callahan et al., 2016). Amplicon sequence variants are a reflection of DNA that was actually sequenced in an

environment (Callahan et al., 2017); therefore, sequence variants that are derived from 16S rDNA amplicons can be compared to a database of sequences with established taxonomic classifications to predict the identities of bacteria in an environment. The resulting identities and abundances of classified amplicon sequence variants can then be analyzed to determine the diversity and composition of the bacterial community.

1.7.2 Metagenomic sequencing

Metagenomic sequencing, the non-selective sequencing of the total genomic DNA in an environment, is another popular approach for determining bacterial community composition in soil, along with genetic composition and functional potential. Unlike 16S rDNA sequencing, metagenomic sequencing generates data from all DNA within a sample, allowing for functional and diversity analyses of non-ribosomal gene targets (Li et al., 2015). At present, the greatest downside to metagenomic sequencing is the higher financial cost associated with metagenomic sequencing compared to 16S rDNA sequencing, as a greater sequencing depth is required to detect low-abundant taxa (Scholz et al., 2012).

In addition, metagenomic sequencing can be used as a cultivation-independent method to identify antibiotic resistance genes and mobile genetic elements within a bacterial community (Boolchandani et al., 2019). Metagenomic sequencing confers many advantages over other methods for studying antibiotic resistance in soil bacteria. Antibiotic resistance genes and mobile genetic elements are distributed among diverse soil bacterial taxa — many of which are difficult to cultivate under normal laboratory conditions (Dunivin et al., 2019). Metagenomic sequencing, compared to PCR-based methods, also allows for the discovery of novel antibiotic resistance genes and mobile genetic elements for which PCR primers have not been developed or are not available (Boolchandani et al.,

2019). Antibiotic resistance genes and mobile genetic elements can be identified in metagenomic sequence data by aligning sequence reads to databases of known antibiotic resistance genes and mobile genetic elements, such as the Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al., 2020). Other bioinformatics software, such as Metagenomic Markov models for Antimicrobial Resistance Characterization (Meta-MARC), use machine learning principles to identify novel antibiotic resistance genes from metagenomic sequence data (Lakin et al., 2019).

1.7.3 Integron gene cassette amplicon sequencing

Integrons can be identified in metagenomic datasets using software that scans for *intI1* and *attC* sites (Cury et al., 2016). Such software could theoretically be fine-tuned to only target specific classes of integrons or could be made more sensitive to detect novel classes of integrons. However, the analysis of metagenomic data alone is unlikely to capture the full diversity of integron gene cassettes in a soil sample due to the complexity of the microbiome. In addition, using sequence alignment software such as BLAST or DIAMOND to search for class 1 integrons by identifying *intI1* wouldn't capture the diversity of the hundreds of other known classes of integrons (Altschul et al., 1990; Buchfink et al., 2015).

Another approach to identifying integron gene cassettes within an environment is the targeted amplicon sequencing of integron gene cassettes, which involves the PCR amplification and sequencing of integron gene cassettes to characterize the diversity of integron gene cassettes in any environment. PCR primers can be designed to target the *attC* or *attI* sites and/or the integron-integrase gene to amplify gene cassettes within a specific integron class or within diverse environmental integrons, and similar to 16S rDNA amplicons, these amplicons can then be sequenced and analyzed using bioinformatics software for antibiotic resistance gene identification (Ghaly

et al., 2019; Yang et al., 2021). Cassette-encoded genes could also be assigned more general functions by matching them to orthologous genes with characterized functional categories, using databases such as eggNOG (Huerta-Cepas et al., 2019).

1.8 Objectives and hypotheses

Following the observed increased abundances of antibiotic resistance genes and mobile genetic elements in agricultural soil that had been annually exposed to macrolide antibiotics for eight years, the contributions of co-selection and bacterial community composition to these increases have not been investigated — nor has the potential for these effects to occur at an environmentally realistic dose for a biosolids land-application scenario (Lau et al., 2020). To further investigate if macrolide antibiotic exposure of soil promotes resistance at an environmentally realistic dose, and to elucidate the mechanisms of increased resistance at an effect-inducing unrealistically high dose, soil DNA was obtained from these same field plots that were either treated with macrolide antibiotics for ten years or were left untreated. The 16S rDNA and class 1 integron gene cassettes were PCR amplified and sequenced, and the total soil metagenome was sequenced.

I hypothesize that long-term macrolide antibiotic exposure of agricultural soil, at both a realistic dose (0.1 mg kg^{-1} soil) and an unrealistically high dose (10 mg kg^{-1}) for biosolids carryover, will affect the composition and diversity of the soil bacterial community, resistome, and mobilome.

I predict that:

1. Antibiotic resistance genes and mobile genetic elements will have increased in response to antibiotic exposure,

2. Bacterial community composition and diversity will differ between antibiotic-exposed and -unexposed soil, and

3. Integron gene cassette composition and diversity will differ between antibiotic-exposed and -unexposed soil.

2 Methods

2.1 Field experiment

Soil microplots were established at Agriculture and Agri-Food Canada in London, Ontario as described by Topp et al., 2016. Briefly, twelve 2 m^2 fibreglass frames were placed into the ground in 2010 and filled with a silt grey loam soil commonly used in Canadian agriculture. Each summer for 10 years, these microplots were exposed to a dose of mixed macrolide antibiotics (erythromycin, clarithromycin, azithromycin) at concentrations of 0.1 mg kg^{-1} soil (low, $n = 4$), 10 mg kg^{-1} (high, $n = 4$), or were left unexposed ($n = 4$). Stock solutions of erythromycin, clarithromycin, and azithromycin were prepared to 1 mg mL^{-1} in 100% ethanol and stored at -20°C until used. Each June, antibiotics were mixed into 1 kg of soil obtained from each plot and soil was re-incorporated into the source microplots to a depth of 10 cm using a mechanized rototiller. Soybean seeds were planted immediately after adding the antibiotics and plots were maintained throughout the growing season by manual weeding only. In 2019, six 20 cm soil core samples were obtained 30 days post-application, pooled, then sieved to a maximum particle size of 2 mm. Soil was stored at -20°C prior to DNA isolation.

2.2 DNA isolation, PCR, and library preparation

Total genomic DNA was isolated from 250 mg of soil from each microplot (and 10 g of soil for metagenomic sequencing) using the DNeasy PowerSoil Kit (Qiagen) and eluted in 100 μL (or 2 mL for metagenomic sequencing) of 10 mM Tris-HCl following the manufacturer's protocol. Spectrophotometric readings of the eluted DNA were taken using a NanoDrop ND1000 microspectrophotometer (NanoDrop Technologies) to assess DNA quality (A260/A280) and a Qubit™ dsDNA HS Assay Kit was used to determine DNA concentration with a Qubit™ 4 Fluorometer (Invitrogen). DNA was stored at -20°C.

2.3 Next-generation sequencing

16S rDNA amplicons were sequenced on a MiSeq instrument (Illumina), total metagenomic DNA was sequenced on a NovaSeq instrument (Illumina), and integron gene cassette amplicons were sequenced on a HiSeq instrument (Illumina). The assignment of each experiment to a sequencing instrument and the number of lanes per flow cell (if applicable) was decided based upon the estimated number of bases sequenced by each instrument, which are approximately 15 Gb per MiSeq flow cell using 300 cycles, 200–250 Gb per NovaSeq 6000 SP flow cell using 150 cycles, and 450–500 Gb per HiSeq 2500 high throughput flow cell using 125 cycles.

2.3.1 16S rDNA amplicon sequencing

For 16S rDNA sequencing, the total genomic DNA was diluted 10-fold in Tris-EDTA buffer and then used as template for single-step PCR amplification and adapter ligation of the V3 and V4 regions of the bacterial 16S rRNA gene to generate 16S rDNA amplicon libraries (600 cycle; Illumina) (Supplementary

Table A.1). To label each amplicon library with a unique DNA sequence tag, index sequences were ligated onto the amplicons using the Nextera® XT Index Kit Set A (Illumina) following PCR. The indexed DNA libraries were purified using AMPure XP (Beckman Coulter) magnetic beads at a 1.8X bead-supernatant ratio, quantified using the Qubit™ dsDNA HS Assay Kit (Invitrogen), and sized using the Agilent High Sensitivity DNA Kit on a Bioanalyzer 2100 (Agilent). The indexed libraries were then normalized and pooled to a final concentration of 4 nM for 2 x 300 bp sequencing on a MiSeq instrument (Illumina) using the MiSeq Reagent Kit v3 (600-cycle; Illumina).

2.3.2 Metagenomic sequencing

Total genomic DNA was sent to The Hospital for Sick Children in Toronto, ON for library preparation and sequencing. Only three of four biological replicates of total genomic DNA (nine samples in total, $n = 3$) were used to prepare DNA libraries. The Nextera® XT DNA Library Preparation Kit was used to prepare DNA libraries of the total genomic DNA by following the manufacturer's protocol. The DNA libraries were then indexed using the Nextera® XT Index Kit v2 (Illumina) following the tagmentation step. Finally, the indexed libraries were normalized and pooled to a final concentration of 2.15 nM for 2 x 150 bp sequencing across two lanes (half of the library in each lane) on a NovaSeq 6000 instrument (Illumina) using an SP flow cell.

2.3.3 Integron gene cassette amplicon sequencing

Integron gene cassettes were PCR amplified using primers described by Stokes et al., 2001 with 33 and 34 bp Illumina adapter overhang sequences ligated onto the 5' ends (Supplementary Table A.1). The purpose of these 5' ends was to extend the distance between the tagmentation site and the desired gene cassette sequence, as 50 bp from each distal end of the amplicon was expected to be lost during preparation of the sequencing library

due to transposome activity. The gene cassette PCR primers anneal to highly conserved GTTRRRY motifs within the *attC* sites of gene cassettes and the *attI* site of integrons (Figure 3).

Total genomic DNA was isolated in technical duplicates (3 treatments $\times$ 4 biological replicates $\times$ 2 technical replicates = 24 samples total) from the microplot soils, diluted 10-fold in Tris-EDTA buffer, and used as template DNA for five technical replicates of 25 μL PCR reactions (125 μL total), and amplified under the following thermocycler conditions: 94°C for 3 min; 35 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 2 min 30 s; 72°C for 5 min. Each PCR reaction was comprised of 2 μL of diluted template DNA, 0.25 μL of Q5® High-Fidelity DNA Polymerase (New England BioLabs), 0.2 μL of 25 mM dNTPs, 5 μL of 5X Q5® Reaction Buffer, and 1.13 μL of each 10 μM forward and reverse primer. Technical replicates were pooled together and PCR product was purified using the GenepHlow PCR Cleanup Kit (Geneaid), and eluted in 25 μL of nuclease-free water. DNA concentrations of the cleaned PCR products were determined using the Qubit™ dsDNA HS Assay Kit (Invitrogen).

The Nextera® XT DNA Library Preparation Kit was used to prepare DNA libraries of the gene cassette amplicons by following the manufacturer's protocol. The DNA libraries were indexed using the Nextera® XT Index Kit v2 (Illumina) following the tagmentation step. The indexed DNA libraries were purified, quantified, and sized as described in 2.3.1 except using a 0.8X bead-supernatant ratio for purification. The indexed libraries were pooled and sent to The Hospital for Sick Children in Toronto, ON for sequencing: the pooled library was denatured and diluted to achieve a final concentration of 15 pM prior to 2 x 125 bp sequencing in one lane (shared with other samples) on a HiSeq 2500 instrument (Illumina) using a high throughput flow cell.

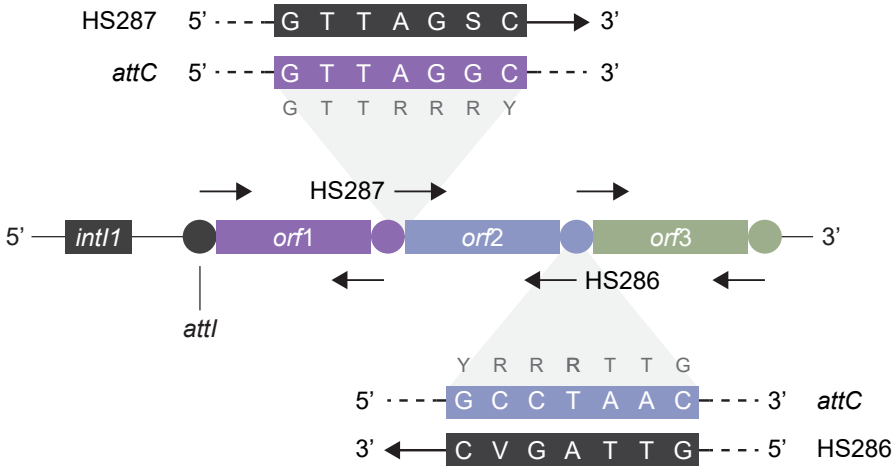


Figure 3 Primer-binding sites for integron gene cassette amplicon sequencing. The class 1 integron in this example contains three gene cassettes, each with one open reading frame (*orf1*, *orf2*, *orf3*). The HS286 and HS287 primers anneal to highly conserved GTTRRRY motifs (shown in grey text) within the cassette-associated recombination sites (*attC*) of integrated gene cassettes and within the integron-associated recombination site (*attI*) (Stokes et al., 2001). Example sequences of *attC* sites and the annealing of HS286 and HS287 to these sequences are shown for *orf1* and *orf2*. In this reaction, there would be six possible amplicon products: *orf1*, *orf2*, *orf3*, *orf1-orf2*, *orf2-orf3*, and *orf1-orf2-orf3*. The 3' conserved sequence of the class 1 integron is not shown.

2.4 Sequence data analysis

For all sequence datasets, the quality of the demultiplexed reads was assessed using FastQC (v0.11.8) and MultiQC (v1.7) and then re-assessed after adapter removal and quality-based trimming (when applicable) (Andrews, 2010; Ewels et al., 2016).

2.4.1 16S rDNA sequence analysis

To remove low-quality bases from the 16S rDNA amplicon reads, Trimmomatic (v0.36) was run in paired-end mode: The first 25 bases of each read were dropped; sliding window trimming was performed where a window of 4 bp would be trimmed if the average quality of the window had a quality score (Q-score) $<$ 15; remaining reads with a length $<$ 25 bp were discarded (Bolger et al., 2014). To correct sequencing errors in the trimmed 16S rDNA amplicon reads, remove chimeric sequences, merge reads, and then establish a set of unique amplicon sequence variants and obtain their counts, the DADA2 denoise-paired plugin within QIIME 2 (v2019.10) was run with default options and without further truncation of the 5' and 3' ends (Bolyen et al., 2019; Callahan et al., 2016). To assign taxonomy to the amplicon sequence variants, the QIIME 2 feature-classifier plugin was first trained with SILVA (v132) 16S rRNA reference sequences using Naïve Bayes classification and was then used to classify the sequence variants (Bokulich et al., 2018; Quast et al., 2013).

2.4.2 Metagenomic sequence analysis

Cutadapt (v2.8) was used to remove adapter sequences from the 3' ends of metagenomic sequence reads (Martin, 2011). Trimmomatic was run in paired-end mode to remove low-quality leading and trailing bases from the adapter-trimmed reads (Q-score $<$ 20), and remaining reads with a length $<$

100 bp were discarded. MetaPhlAn3 (v3.0.7) was used to assign taxonomy to metagenomic reads using the 'very-sensitive' algorithm of Bowtie2, ignoring eukaryotes and archaea, and profiling the metagenomes as relative abundances with estimation of the number of reads coming from each bacterial taxon (Beghini et al., 2020).

To identify metagenomic reads that corresponded to antibiotic resistance genes, the metagenomic reads were mapped to two CARD databases: The CARD 'canonical' database (v3.0.8) of phenotypically confirmed antibiotic resistance genes, and the CARD Prevalence, Resistomes, & Variants (v3.0.7) database of in silico predicted resistance genes, derived from genomic data of 82 human pathogens. The metagenomic reads were mapped to these databases using Bowtie2 implemented by the CARD Resistance Gene Identifier in metagenomics mode (v5.1.0) (Alcock et al., 2020). To identify metagenomics reads that corresponded to mobile genetic elements, the metagenomic reads were mapped to a database of known mobile genetic elements created by Pärnänen et al., 2018 (downloaded from https://github.com/KatariinaParnanen/MobileGeneticElementDatabase on 2021-05-24). The metagenomic reads were mapped to this database using Bowtie2 (v2.4.2) with end-to-end searching and using the pre-defined 'very-sensitive' search algorithm, except for allowing a maximum of one mismatch in the seed alignment (Langmead & Salzberg, 2012). Fold-coverages of antibiotic resistance genes and mobile genetic elements were used as abundances for downstream analysis.

2.4.3 Integron gene cassette sequence analysis

No quality-based trimming was performed for the integron gene cassette reads to preserve primer-binding sites for downstream filtering, but Cutadapt was used to remove adapter sequences from the 3' ends of gene cassette reads. Integron gene cassette sequence reads were assembled into contigs using

MEGAHIT (v1.2.9) with default options (Li et al., 2015). Assembly quality was assessed using BBTools' Stats (v38.90) (Bushnell, 2016). Individual sample assemblies were combined into a master assembly for downstream filtering of gene cassettes.

The highly conserved motifs within integron gene cassette *attC* sites were used to identify the boundaries of gene cassettes from assembled contigs; if an assembled contig did not contain the terminal 9 bp of both of these motifs, it was identified using BBTools' BBDuk (v38.90) and discarded from further analysis (Bushnell, 2016). Prokka (v1.14.6) was used to identify open reading frames — putative genes that may encode a protein — within the curated gene cassette contigs, which were then clustered at 97% identity using CD-HIT (v4.8.1) to obtain a set of unique open reading frames (Fu et al., 2012; Seemann, 2014).

To identify integron gene cassette open reading frames that could correspond to antibiotic resistance genes, the open reading frames were aligned against CARD's 'canonical' protein homolog database using an implementation of BLAST within CARD-RGI, including all 'loose' hits and running in low-quality mode. To further potentiate the discovery of novel antibiotic resistance genes, the translated protein sequences were also scanned using Meta-MARC and hmmer (v3.1b2) with Group I models only (downloaded from https://github.com/lakinsm/meta-marc on 2021-02-13) (Lakin et al., 2019; Wheeler & Eddy, 2013).

The positive identification of integron gene cassette open reading frames as antibiotic resistance genes was considered at three different levels of confidence (high, moderate, low), which were determined by visual inspection of alignment statistics distributions:

- High confidence: CARD-RGI 'strict' hits; Meta-MARC hits with E-value $\leq$ 1E-10.
- Moderate confidence: All high confidence hits; CARD-RGI 'loose' hits with percent identity $>$ 60 and percent length of reference sequence $>$ 60;

Meta-MARC hits with E-value $\leq$ 1E-1.

- Low confidence: All high and moderate confidence hits; CARD-RGI 'loose' hits with percent identity $>$ 40 and percent length of reference sequence $>$ 40; Meta-MARC hits with E-value $\leq$ 1.

To predict general functions for integron gene cassette open reading frames, the open reading frames were scanned for similarity to orthologous groups in the eggNOG database (v5.0) and assigned one or more Cluster of Orthologous Groups (COG) functional categories using the web implementation of eggNOG-mapper (v2.0) (Huerta-Cepas et al., 2019). These COGs were derived from orthologous groups of proteins in microbial genomes and are believed to correspond to ancient conserved domains, which can be grouped by one or more general functions (Galperin et al., 2019).

To obtain fold-coverages for integron gene cassette open reading frames, BBTools' BBMap (v38.90) was used to map gene cassette sequence reads back onto unique open reading frames (Bushnell, 2016). These fold-coverages were used as abundances for downstream analysis.

2.5 Statistical analyses and data visualization

All statistical tests were performed using statistical packages for Python (v3.9.2) unless otherwise stated (van Rossum & Drake, 2009). Data visualizations were generated using plotly (v4.14.3) and matplotlib (v3.4.1) packages (Hunter, 2007; Plotly Technologies Inc., 2015).

2.5.1 Alpha diversity

Alpha diversity (within-group diversity using the Chao1 richness estimator) was computed using the scikit-bio package (v0.5.6). A Shapiro-Wilk test was used to assess the normality of Chao1 richness, followed by a one-way analysis of

variance (ANOVA) for parametric data or a Kruskal-Wallis test for non-parametric data to test if differences in the Chao1 richness between treatment groups were statistically significant, as implemented by the SciPy package (Virtanen et al., 2020).

2.5.2 Beta diversity

Beta diversity (between-group diversity) was analyzed using a principal component analysis (PCA). A pseudocount of 0.5 was added to each table of samples versus abundances (taxa, genes, open reading frames) prior to center log ratio (CLR) transformation to obtain samplewise Aitchison distances — or CLR-transformed relative abundances. This pseudocount was added to avoid an error when obtaining the logarithm of zero. A PCA was performed on the resulting table of CLR-transformed relative abundances to investigate differences in the compositions of antibiotic resistance genes, mobile genetic elements, integron gene cassette open reading frames, and bacterial communities between treatment groups using the scikit-learn package (v0.24.1). Permutational multivariate ANOVA (PERMANOVA) was used to determine if differences in the dispersion between treatment groups within the PCA were statistically significant using the sci-kit bio package.

2.5.3 Differential abundance

Differential abundance analysis was performed to determine if differences in the abundances of bacterial taxa, antibiotic resistance genes, mobile genetic elements, or gene cassette COG functional categories between groups were statistically significant using ANCOM-BC (Analysis of Composition of Microbiomes with Bias Correction, v1.2.0) with Holm-Bonferroni correction as implemented in R (v4.1.0) (Lin & Peddada, 2020; R Core Team, 2021). The results of the differential abundance analyses were reported as effect sizes (fold-changes , W) $\pm$ Bonferroni-adjusted 95% confidence intervals. A

one-way analysis of variance (ANOVA) was used to test if differences in the numbers of merged 16S rDNA amplicon reads between treatment groups were statistically significant as implemented by the SciPy package (Virtanen et al., 2020).

3 Results

DNA from untreated control soil and soil exposed to a low (0.1 mg kg^{-1}) or high (10 mg kg^{-1}) dose of macrolide antibiotics was used to generate three sequence datasets: 16S rDNA was sequenced to investigate the diversity and composition of the soil bacterial community, total metagenomic DNA was sequenced to investigate the diversity and composition of the resistome and mobilome, and integron gene cassettes were sequenced to investigate the diversity and composition of integron gene cassette open reading frames.

3.1 Sequencing statistics

Bacterial 16S rDNA amplicons were sequenced to investigate differences in soil bacterial community composition and diversity in response to antibiotic exposure. 16S rDNA MiSeq sequencing generated 6.21 M reads with an average of 50.7 $\pm$ 12.8 K unique reads per sample over 12 samples (n = 4 for each treatment group) (Supplementary Table A.2). Following quality control, 6,837 to 72,287 merged reads were used for amplicon sequence variant assignment which resulted in 2,587 amplicon sequence variants. One control sample was excluded from the 16S rDNA amplicon analysis due to a low number of merged reads resulting from the DADA2 workflow (n = 2,444). The mean numbers of merged reads were not significantly different between treatment groups after removing this sample (one-way ANOVA, F = 2.3, p = 0.16). The resulting 16S rDNA sequence reads were used to establish amplicon sequence variants which were taxonomically classified, and the

number of reads for each bacterial taxon were obtained.

Next, metagenomic DNA was sequenced to investigate differences in the composition and diversity of the soil bacterial community, antibiotic resistance genes, and mobile genetic elements in response to antibiotic exposure. Metagenomic NovaSeq sequencing generated 1.49 B reads with an average of 153 $\pm$ 18.7 M unique reads per sample over nine samples (n = 3 for each treatment group) (Supplementary Table A.3). The resulting metagenomic sequence reads were i) taxonomically classified to obtain a second dataset of bacterial taxa abundances, and ii) mapped to antibiotic resistance gene and mobile genetic elements to obtain abundances for these antibiotic resistance determinants.

Finally, integron gene cassette amplicons were sequenced to investigate differences in the composition and diversity of gene cassette open reading frames in response to antibiotic exposure. Integron gene cassette HiSeq sequencing generated 9.83 Gb of reads with an average of 0.94 $\pm$ 0.18 M unique reads per sample over 24 samples (n = 4 biological replicates, n = 2 technical replicates) (Supplementary Table A.4). These reads were assembled into 270,368 contigs which were then filtered to retain only 75,850 high-confidence gene cassettes (28%). These gene cassettes were predicted to contain 72,628 open reading frames of which 36,050 (48%) were considered unique. Integron gene cassette open reading frames were analyzed for antibiotic resistance genes at three different levels of confidence (low, moderate, high), and to assign COG functional categories. Integron gene amplicons were mapped onto the unique open reading frames to obtain abundances for these putative protein-coding genes.

The abundances that were obtained from each of these sequencing datasets allowed us to investigate differences in soil bacterial community, antibiotic resistance gene, mobile genetic element, and integron gene cassette open reading frame composition and diversity in response to macrolide

antibiotic exposure.

3.2 Bacterial community composition and diversity

The richness (one-way ANOVA F = 0.78, p = 0.50) and composition (PERMANOVA pseudo-F = 1.0, p = 0.41) of soil bacterial taxa were not significantly affected by antibiotic exposure (Supplementary Figure A.2; Supplementary Figure A.3). However, at the phylum level, the relative abundances of *Cyanobacteria* sequence variants were decreased in the high-dosed soil (Holm-Bonferroni-adjusted p-value $<$ 0.001, W = -5.7 $\pm$ 0.7) but not in the low-dosed soil, relative to the control. This result was observed only in the metagenomic analysis (Figure 4) and not in the 16S rDNA analysis (Supplementary Figure A.1). This difference was driven by the cyanobacteria *Microcoleus vaginatus* (p $<$ 0.001, W = -5.2 $\pm$ 0.8) and *Oscillatoria nigro-viridis* (p $<$ 0.001, W = -5.3 $\pm$ 0.3) in the high-dosed soil.

Only three taxa were differentially abundant by over 10-fold relative to the control: an unknown *Acidobacteria Subgroup 6* sp. was increased by approximately 46-fold in the low-dosed soil (W = 45.8 $\pm$ 3.8), and two unknown *Chloroflexi Gitt-GS-136* spp. were increased by approximately 23-fold (W = 23.0 $\pm$ 0.8) and 59-fold (W = 58.6 $\pm$ 0.4) in the high-dosed soil (Table 1). These differences were observed only in the 16S rDNA analysis.

Overall, five bacterial species were increased in response to antibiotic exposure (three in the low dose, two in the high dose) and six species were decreased (one in the low dose, five in the high dose) (Table 1). The taxa that were differentially abundant in the low- and high-dosed soil did not overlap (were not in-common) between the 16S rDNA amplicon and metagenomic taxonomic datasets.

Table 1 Effect sizes (fold-changes) of differentially abundant soil bacterial taxa in response to macrolide antibiotic exposure at low (0.1 mg kg^{-1}) and high (10 mg kg^{-1}) doses. Effect sizes are stated with 95% Bonferonni-adjusted confidence intervals (CIs). Differential abundance analysis was performed using ANCOM-BC for the 16S rDNA analysis (16S) or metagenomic analysis (M). All p-values are Holm-Bonferroni-adjusted. No taxa were identified as differentially abundant by both analyses.

Differentially abundant bacterial taxon	Effect size ± 95% CI	Adjusted *p*-value	Analysis (M, 16S)
0.1 mg kg^{-1}			
Mycolicibacterium tusciae	-7.09 ± 0.09	1.59E-10	M
Sphingomonas sp. Leaf20	4.51 ± 0.51	7.17E-04	M
Unknown *Gaiella* sp.	4.40 ± 0.92	2.82E-02	16S
Unknown *Acidobacteria Subgroup 6* sp.	45.79 ± 0.41	0	16S
10 mg kg^{-1}			
Arthrobacter globiformi	-5.23 ± 0.31	1.87E-05	M
Arthrobacter sp. Leaf69	-5.56 ± 0.31	2.99E-06	M
Microcoleus vaginatus	-5.22 ± 0.76	2.01E-05	M
Oscillatoria nigro-viridis	-5.29 ± 0.28	1.34E-05	M
Ramlibacter sp. Leaf400	-7.82 ± 0.18	5.89E-13	M
Unknown *Chloroflexi Gitt-GS-136* sp.	23.02 ± 0.80	6.95E-114	16S
Unknown *Chloroflexi Gitt-GS-136* sp.	58.58 ± 0.37	0	16S

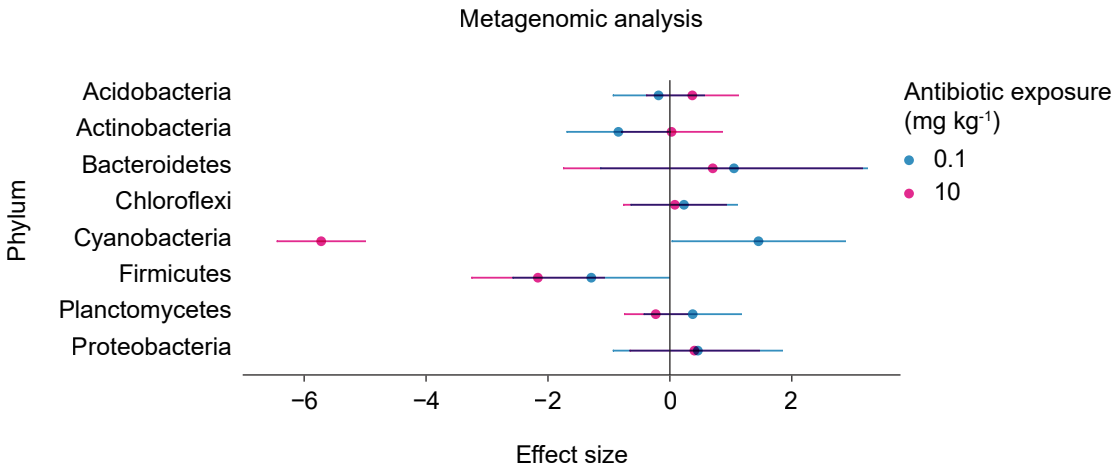


Figure 4 Effect sizes (fold-changes) of differences in the relative abundances of bacterial phyla classified in the metagenomic analysis relative to the untreated control soil. Horizontal lines intersecting with circles are error bars, indicating the extent of Bonferroni-adjusted 95% confidence intervals of effect sizes (n = 3).

3.3 Resistome and mobilome composition and diversity

3.3.1 Resistome

A total of 583 unique antibiotic resistance genes were detected across the soil metagenomes. High macrolide antibiotic exposure significantly increased the richness of total antibiotic resistance genes in agricultural soil (Tukey's all-pairs test, $p < 0.05$) but no effect was observed for the low dose (Figure 5a; Supplementary Figure A.4). Similarly, high exposure but not low exposure changed the composition of antibiotic resistance genes (PERMANOVA pseudo-F = 1.49, $p < 0.05$, 999 permutations) (Figure 6a). These differences in composition were largely driven by 21 increased antibiotic resistance genes in the high dosed soil ($p < 0.05$) (Figure 7). Only five antibiotic resistance genes were differentially abundant (two decreased, three increased) in the

low-dosed soil and no resistance gene was differentially abundant in both treatment groups.

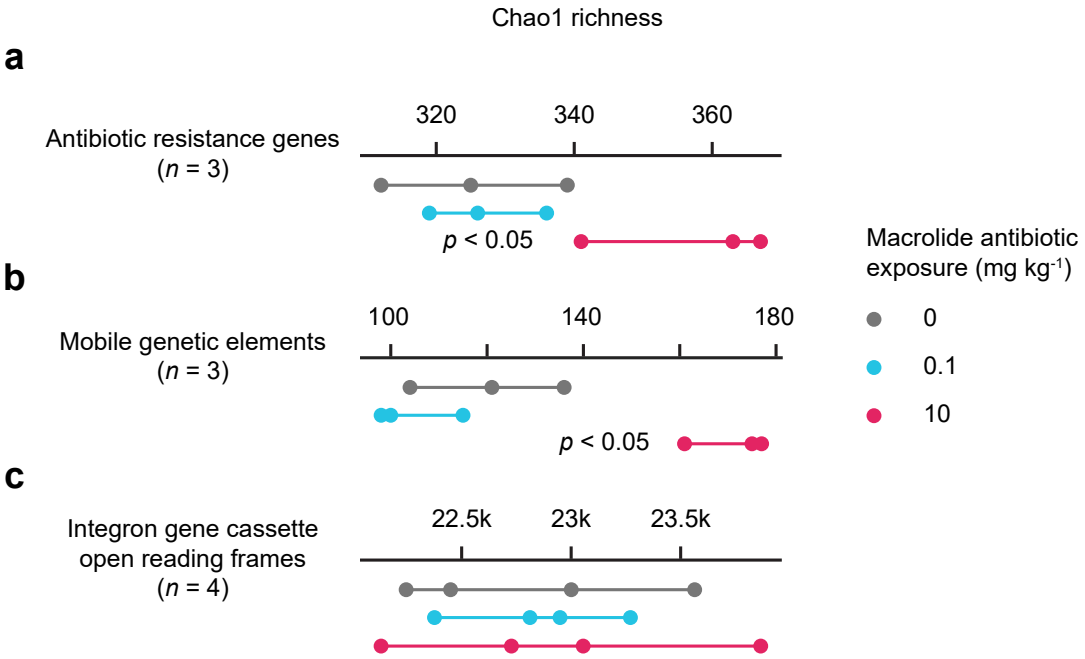


Figure 5 Richness (Chao1) of antibiotic resistance genes, mobile genetic elements, and integron gene cassette open reading frames. Richness was determined for **a**) metagenomic antibiotic resistance genes, **b**) metagenomic mobile genetic elements, and **c**) integron gene cassette open reading frames of antibiotic-exposed and -unexposed soil bacteria. Horizontal lines connect samples within the same treatment group for visual clarity. Statistically significant comparisons between the antibiotic-exposed and untreated control soil are displayed (Kruskal-Wallis test, $p < 0.05$).

The 21 antibiotic resistance genes that had increased relative abundances in the high dosed soil were predicted to confer resistance to 11 different drug classes of antibiotics and triclosan (a biocide), especially aminoglycosides (*n* = 10) and diaminopyrimidines (*n* = 4) (Figure 8). Sixteen of these antibiotic resistance genes were predicted to confer resistance to classes of antibiotics which, like macrolides, target the ribosome. Only two of these increased antibiotic resistance genes were predicted to encode resistance to macrolides (*mphE*, *mexQ*). The gene that had the greatest increase in relative abundance

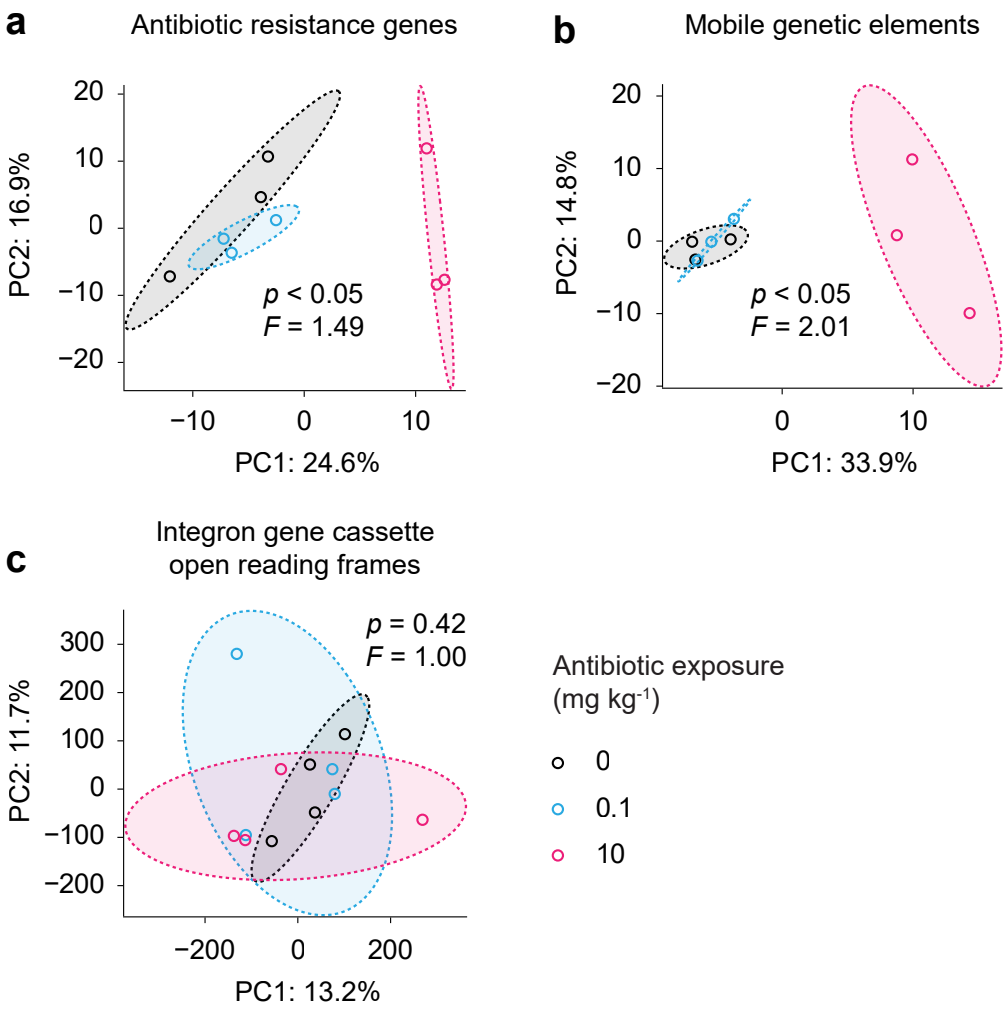


Figure 6 PCA ordination plots (PC1, PC2) of the CLR-transformed relative abundances of **a**) metagenomic antibiotic resistance genes, **b**) metagenomic mobile genetic elements, and **c**) integron gene cassette open reading frames in antibiotic-exposed and -unexposed soil bacteria. PERMANOVA pseudo-F and p-values with 999 permutations are displayed. Shaded areas correspond to 95% confidence ellipses of treatment groups. Percentages of variance explained by each axis are displayed in the axis titles.

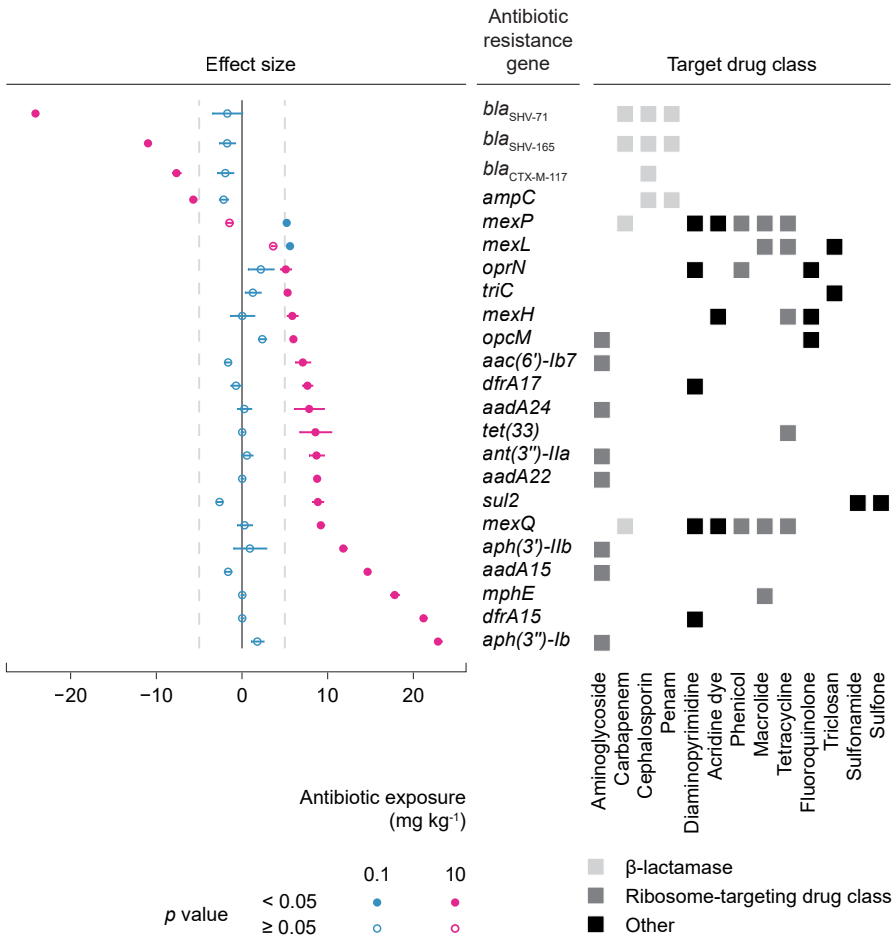


Figure 7 **a**) Effect sizes (fold-changes) of differences in the relative abundances of antibiotic resistance genes in antibiotic-exposed soil metagenomes and **b**) their target drug classes relative to the untreated control soil (n = 3). **a**. Only the genes that were differentially abundant ($p < 0.05$) with an absolute effect size of at least 5 (vertical dashed bars), for either treatment group, are shown. Shaded circles represent genes whose abundances were significantly different from the untreated control soil and open circles represent abundances that were not significantly different. Horizontal lines intersecting with circles are error bars, indicating the extent of Bonferroni-adjusted 95% confidence intervals of effect sizes. **b**. Light grey squares indicate that resistance to a beta-lactam antibiotic is predicted; dark grey squares indicate that resistance to a ribosome-targeting drug class is predicted; black squares indicate that resistance to a non-beta-lactam and non-ribosome-targeting drug class is predicted (other).

in response to high antibiotic exposure was the aminoglycoside resistance gene *aph(3")-Ib* (W = 22.9 $\pm$ 0.5, $p < 0.05$).

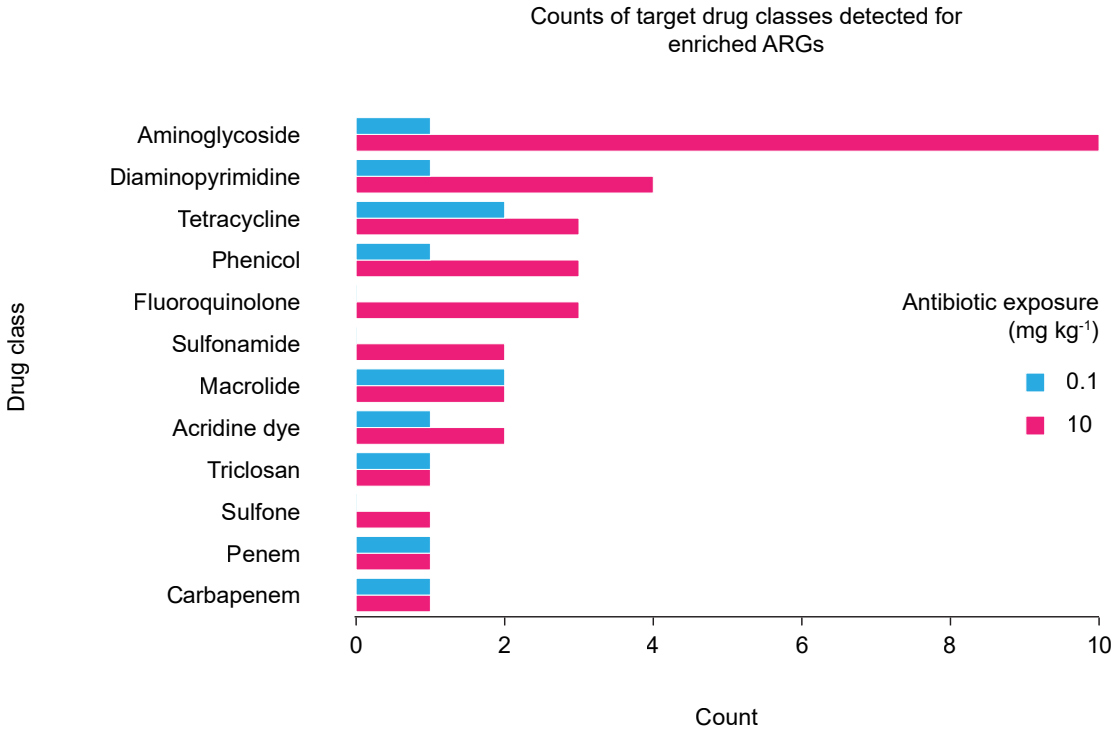


Figure 8 Counts of target drug classes for which resistance was predicted to be encoded by antibiotic resistance genes within the soil metagenome. Only counts for antibiotic resistance genes that were enriched in response to macrolide antibiotic exposure are displayed ($p < 0.05$, n = 3).

Analysis of antibiotic resistance genes grouped by their target drug class indicated that the compositions of aminoglycoside, diaminopyrimidine, phenicol, tetracycline, lincosamide, and streptogramin resistance genes, but not macrolide resistance genes, were significantly altered in the high-dosed soil ($p < 0.05$, 999 permutations) (Figure 9). Of the three antibiotic resistance genes that had increased relative abundances in the low-dosed soil ($p < 0.05$), two were predicted to encode resistance to macrolide antibiotics (*mexL*, W = 5.6 $\pm$ 0.2; *mexP*, W = 5.2 $\pm$ 0.2) and one was predicted to encode resistance to aminoglycosides (*aac(6')-IIa*, W = 4.0 $\pm$ 0.4). No antibiotic resistance genes had increased relative abundances in both doses.

Seven antibiotic resistance genes had significantly decreased relative

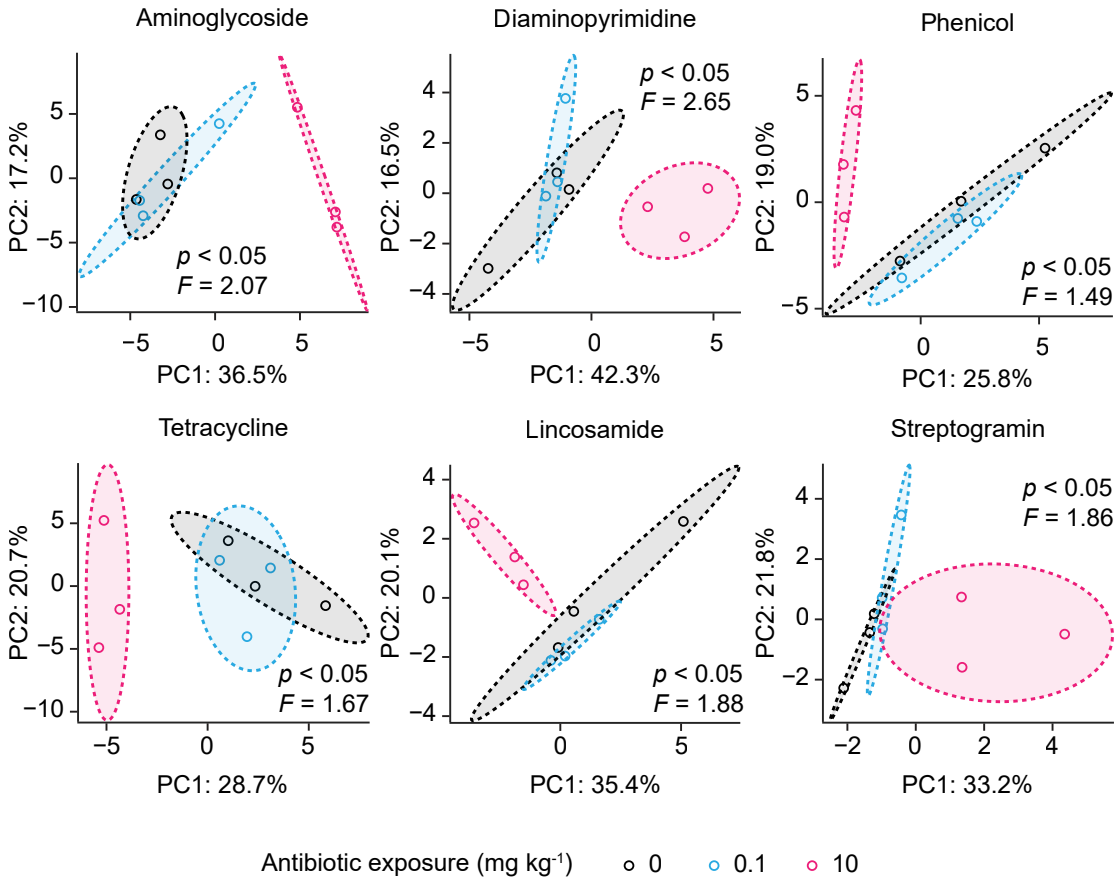


Figure 9 PCA ordination plots (PC1, PC2) of CLR-transformed relative abundances of antibiotic resistance genes in the untreated control soil and in the low- and high-dosed soil, grouped by their target drug class. PERMANOVA pseudo-F and p-values with 999 permutations are displayed. Shaded areas correspond to 95% confidence ellipses of treatment groups. Percentages of variance explained by each axis are displayed in the axis titles.

abundances relative to the control soil: five in the high dose and two in the low dose ($p < 0.05$). Interestingly, all seven of these resistance genes were predicted to encode beta-lactamases (Figure 7). *bla*$_{\text{SHV-71}}$ (W = -24.1 $\pm$ 0.3), *bla*$_{\text{SHV-165}}$ (W = -11.0 $\pm$ 0.3), *bla*$_{\text{CTX-M-117}}$ (W = -7.6 $\pm$ 0.5), *E. coli ampC* (W = -5.7 $\pm$ 0.4), and *bla*$_{\text{PEDO-1}}$ (W = -4.4 $\pm$ 0.2) were decreased in the high dose, and *bla*$_{\text{TEM-1}}$ (W = -4.9 $\pm$ 0.3) and *bla*$_{\text{TEM-22}}$ (W = -4.1 $\pm$ 0.8) were decreased in the low dose.

3.3.2 Mobilome

In addition to antibiotic resistance genes, the composition and diversity of mobile genetic elements within the soil metagenome was investigated. Overall, 398 unique mobile genetic element variants were detected across the soil metagenomes, including several transposases and insertion sequence elements (e.g IS91, IS26). As observed with antibiotic resistance genes, the richness of mobile genetic elements was significantly increased in the soil metagenome (Tukey's all-pairs test, $p < 0.05$) (Figure 5b; Supplementary Figure A.4), and the composition of mobile genetic elements was significantly affected by the high dose of macrolides (PERMANOVA pseudo-F = 2.01, $p <$ 0.05, 999 permutations) (Figure 6b).

This altered composition of mobile genetic elements in the high-dosed soil was largely driven by 23 mobile genetic element variants with increased relative abundances ($p < 0.05$) (Figure 10). Of these 23 increased mobile genetic elements, 15 were identified as *tnpA*, three as *intI1*, three as *qacE*Δ*1*, one as IS91, and one as *tnpAN* variants. The maximum effect size of the mobile genetic element variants that were increased in the high dose was W = 23.8 $\pm$ 0.1 for *intI1* ($p < 0.05$).

The only mobile genetic element variant with an increased relative abundance in the low-dosed soil was identified as *tnpA* (W = 6.0 $\pm$ 0.3, $p <$ 0.05) (Figure 10). Of the three mobile genetic element variants that were

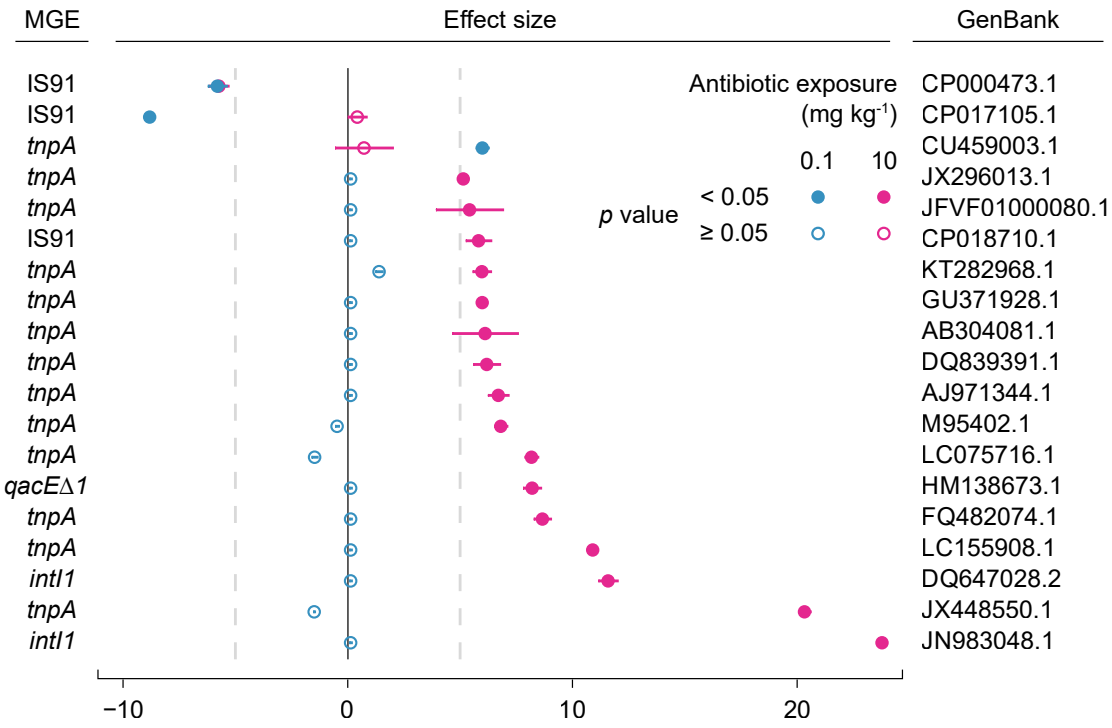


Figure 10 Effect sizes (fold-changes) of differences in the relative abundances of mobile genetic elements (MGEs) in antibiotic-exposed soil metagenomes relative to the untreated control soil (n = 3). Only the mobile genetic elements that were differentially abundant ($p < 0.05$) with an absolute effect size of at least 5 (vertical dashed bars), for either treatment group, are shown. The name of the moobile genetic element is shown on the left, and the GenBank accession number of the reference sequence's genome is shown on the right. Shaded circles represent mobile genetic elements whose abundances were significantly different from the untreated control soil and open circles represent abundances that were not significantly different. Horizontal lines intersecting with circles are error bars, indicating the extent of Bonferroni-adjusted 95% confidence intervals of effect sizes.

decreased in the low-dosed soil (IS91, *n* = 2; *tnpA*, *n* = 1), one IS91 variant was similarly decreased in the high-dosed soil (low dose, W = -5.8 $\pm$ 0.4; high dose, W = -5.7 $\pm$ 0.4). No other mobile genetic element variants were differentially abundant in both doses.

3.4 Composition and diversity of open reading frames from integron gene cassettes

Both the richness (one-way ANOVA F = 0.05, p = 0.95) and composition (PERMANOVA pseudo-F = 1.0, p = 0.42) of integron gene cassette open reading frames were unaffected by antibiotic exposure (Figure 5c; Figure 6c). Overall, 370 open reading frames (1%) were identified as differentially abundant relative to the untreated control soil ($p < 0.05$), and of these 370 open reading frames, more were differentially abundant in the high-dosed soil (*n* = 246, 67%) than the low-dosed soil (*n* = 144, 39%).

In total, 60 to 2,997 unique open reading frames (0.2 to 8.4%) were predicted to encode antibiotic resistance depending on the confidence level used (see Chapter 2.4.3). For the antibiotic resistance genes predicted at each confidence level, the most frequently detected target drug class of antibiotic resistance genes was aminoglycoside and the most frequently detected drug resistance mechanism was antibiotic inactivation. Depending on the confidence level, 1 to 17 putative antibiotic resistance genes had increased relative abundances in response to antibiotic exposure and 1 to 13 putative antibiotic resistance genes decreased ($p < 0.05$) (Supplementary Table A.5). However, no putative antibiotic resistance genes were increased or decreased in both treatment groups at any confidence level.

Integron gene cassette open reading frames were also assigned COG functional categories to investigate if macrolide exposure changed the overall function of the cassette metagenome. Only 5,206 (15%) unique open reading

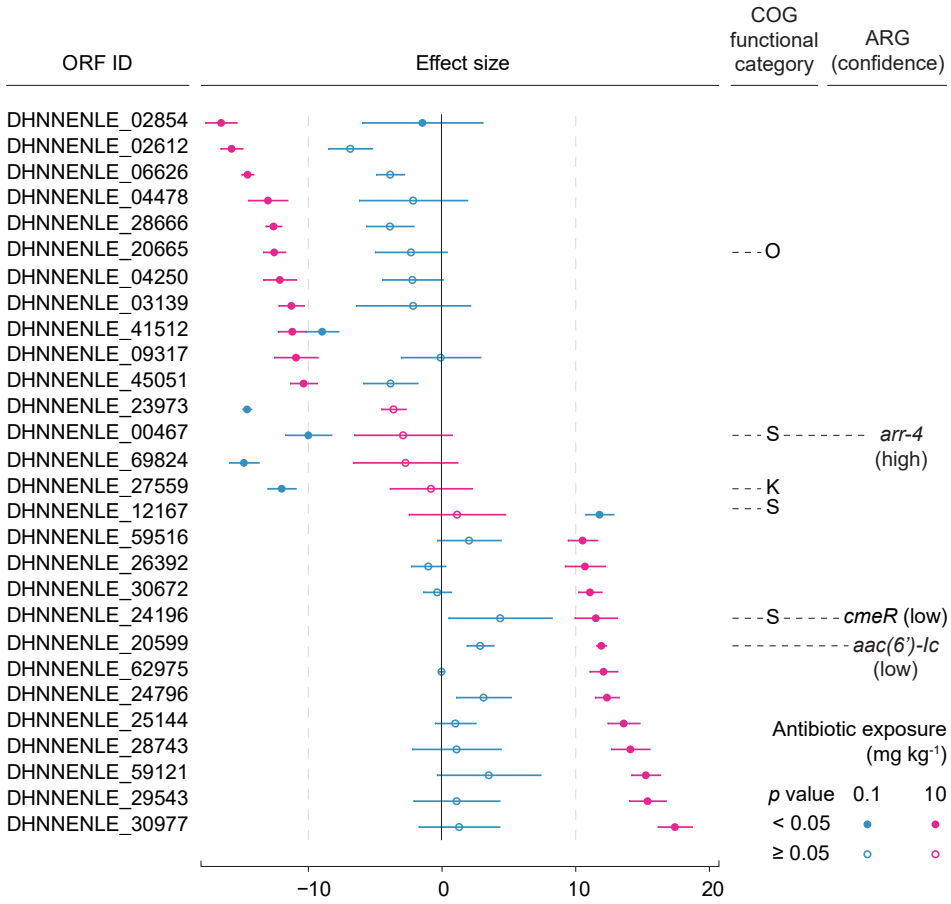


Figure 11 Effect sizes (fold-changes) of differences in the relative abundances of integron gene cassette open reading frames in antibiotic-exposed soil bacteria relative to the untreated control soil. Only the open reading frames that were differentially abundant ($p < 0.05$, $n = 3$) with an absolute effect size ≥ 10 (vertical dashed bars), for either treatment group, are shown. The open reading frame (ORF) ID is shown on the left and the assigned COG functional category on the inner-right (if available). The name and highest confidence level (low, moderate, high) of predicted antibiotic resistance genes are shown on the outer-right. Shaded circles represent open reading frames whose abundances were significantly different from the untreated control soil and open circles represent abundances that were not significantly different. Horizontal lines intersecting with circles are error bars, indicating the extent of Bonferroni-adjusted 95% confidence intervals of effect sizes. COG functional categories are as follows: K = Transcription, O = Post-translational modification, protein turnoover, chaperones; S = Function unknown.

frames could be assigned a functional category, and of those, 2,053 (39%) were assigned a functional category other than 'function unknown' (S) (Supplementary Figure A.5). The open reading frames that were assigned to functional category EK (E: amino acid transport and metabolism; K: transcription) had slightly increased relative abundances (W = 3.4 $\pm$ 1.1, $p <$ 0.05), and those assigned to category DJ (D: cell cycle control, mitosis and meiosis; J: translation, ribosomal structure and biogenesis) had slightly decreased relative abundances (W = -3.6 $\pm$ 0.8, $p <$ 0.05) in the soil bacteria exposed to a high dose of macrolide antibiotics, but only a few open reading frames were assigned to each of these categories (EK, *n* = 3; DJ, *n* = 2).

4 Discussion

The purpose of this project was to investigate the effect of long-term macrolide antibiotic exposure on the soil bacterial community, resistome, and mobilome — and more specifically, to determine if an environmentally realistic dose of antibiotics for a biosolids exposure scenario could promote clinically relevant antibiotic resistance in soil bacteria. Human and environmental health are interconnected as described by the One Health framework, and antibiotic resistance in soil bacteria may affect antibiotic resistance in the human microbiome or in pathogens.

4.1 Realistic antibiotic exposure does not affect the diversity or composition of the soil bacterial community, resistome, or mobilome

Overall, no effect of environmentally realistic antibiotic exposure (low dose, 0.1 mg kg^{-1}) on the diversity or composition of the soil bacterial community, resistome, or mobilome was detected. This dose is similar to what would be expected in soil following the land-application (1–10% dw dw^{-1}) of municipal biosolids containing 95th percentile concentrations of erythromycin, clarithromycin, and azithromycin antibiotics. The absence of a treatment effect for any of these endpoints indicates that repeated annual application of biosolids in agriculture is unlikely to promote antibiotic resistance in agricultural soil at levels that would be of concern to human health.

However, three antibiotic resistance genes had increased relative abundances in response to antibiotic exposure at the low dose, and two of them are known to be associated with resistance to macrolide antibiotics (Figure 7). *mexL* and *mexP* are members of the *mex* gene family which are components of chromosomally encoded efflux pumps in *Pseudomonas* spp. (Chuanchuen et al., 2005; Mima et al., 2005). The *mexL* gene encodes a repressor for *mexJK* transcription, which are members of the efflux pump-encoding *mexJK-OpmH* (triclosan resistance) and *mexOprM* (macrolide, tetracycline resistance) operons (Chuanchuen et al., 2005). The *mexP* gene encodes the membrane fusion protein for the multi-drug efflux pump that is encoded by the *MexPQ-OpmE* operon, which is known to confer resistance to several antibiotic drug classes including macrolides (Mima et al., 2005). The other antibiotic resistance gene that was increased in the low dosed-soil was *aac(6')-IIa*, which encodes an aminoglycoside acetyltransferase that is distributed among a variety of gram-negative pathogens, including *Pseudomonas* spp., and is carried by plasmids and integrons (Partridge et al., 2009; Shaw et al., 1989). Only one mobile genetic element variant, *tnpA*, was increased in the low-dosed soil (Figure 10). The *tnpA* gene, of which there are many variants, encodes a transposase which is involved in the mobilization of several bacterial mobile genetic elements, such as transposons and insertion sequences across a broad host range (Partridge et al., 2018). Three bacterial taxa were increased and are discussed in Chapter 4.3.

Despite the increased relative abundances of three antibiotic resistance genes, one mobile genetic element variant, and three bacterial taxa in the low-dosed soil — the overall diversity and composition of the soil bacterial community, resistome, and mobilome was unchanged by the low dose as indicated by richness (Figure 5, Supplementary Figure A.2) and PCA ordination plots (Figure 6, Supplementary Figure A.3). The increased

abundances of a few antibiotic resistance genes in the low-dosed soil and that of *tnpA* could be due to the increased abundance of a particular taxon that is intrinsically resistant to macrolide antibiotics, such as the human pathogen *Pseudomonas aeruginosa*, though no taxa that were both significantly increased and known to carry this assortment of genes were detected. These results are in clear contrast to the high dose of macrolide antibiotics.

4.2 Unrealistically high antibiotic exposure alters the diversity and composition of the soil bacterial resistome and mobilome

At a high dose of macrolide antibiotics (10 mg kg^{-1}) — approximately 100-fold greater than the concentrations of macrolides that would be expected to result from land-application of municipal biosolids — significant effects on the diversity and composition of the soil bacterial resistome and mobilome were detected. While this dose is considered unrealistically high for soil receiving biosolids with macrolide concentrations within an upper-realistic range (upper 95th percentile), the maximum azithromycin concentration detected in the U.S. Environmental Protection Agency, 2009 biosolids survey (5 mg kg^{-1}) was within an order-of-magnitude of the high dose used in this present study. This high dose of macrolide antibiotics has also been detected in sediments surrounding macrolide antibiotic manufacturing facilities (González-Plaza et al., 2019). Therefore, while this dose is described as unrealistically high for a biosolids land-application scenario, comparable doses are certainly seen under other environmental contexts, and the findings of this experiment may be useful for predicting antibiotic resistance in other macrolide-contaminated environments.

The high dose of macrolide antibiotics significantly increased the number of

unique antibiotic resistance genes that were detected within the soil metagenome (Figure 5a). The majority of antibiotic resistance genes that were detected in the metagenome were detected within all three groups (control, low, and high), but approximately twice as many unique antibiotic resistance genes were detected within the high treatment group alone than the control and low groups alone (Supplementary Figure A.4a). The most likely explanation for the increased number of unique antibiotic resistance genes in the high-dosed soil is selection or co-selection for genes that were below the detection limit for the control and low groups, and were raised above the limit of detection by high antibiotic exposure (but not significantly increased based upon the differential abundance analysis).

In addition, a high dose of macrolide antibiotics changed the composition of antibiotic resistance genes within the soil metagenome (Figure 6a), and this effect extended to when antibiotic resistance genes were grouped by several drug classes (Figure 9). These differences in composition indicated that the relative abundances of several antibiotic resistance genes and target drug classes were more similar within the high-dosed soil than in the control and low-dosed soil. The altered composition of antibiotic resistance genes in the high-dosed soil was driven by increased relative abundances of 21 antibiotic resistance genes in the high dose — only two of which are known to confer resistance to macrolide antibiotics, while 11 are known to confer resistance to non-macrolide, ribosome-targeting drug classes (aminoglycoside, phenicol, tetracycline) (Figure 7).

Like macrolides, the aminoglycoside, phenicol, and tetracycline drug classes of antibiotics also target the bacterial ribosome, but bind to different locations within the ribosome than do the macrolides (Lohsen & Stephens, 2019; Pyörälä et al., 2014). Because the precise ribosomal targets of these drug classes are different to that of macrolides, cross-resistance of antibiotic resistance genes to macrolides and drug classes other than lincosamide and

streptogramin B antibiotics (whose targets overlap with that of macrolides) is uncommon, except for antibiotic resistance genes that encode multi-drug efflux pumps such as the *mex* gene family in *Pseudomonas* spp. The increased relative abundances of non-macrolide antibiotic resistance genes in the high-dosed soil strongly suggests co-selection via co-resistance rather than cross-resistance (except for *mexQ*), which could be facilitated by mobile genetic elements.

Co-resistance to different drug classes of antibiotics can occur due to the genetic linkage of antibiotic resistance on mobile genetic elements such as class 1 integrons, or when antibiotic resistance genes are carried within the same host (Pal et al., 2015). Of the 21 increased antibiotic resistance genes in the unrealistically high-dosed soil metagenome, eight are known to be associated with class 1 integrons (*sul1*, *aac(3)-Ib*, *aadA*, *aadA15*, *aadA22*, *aadA24*, *dfrA17*, *dfrA15*), which agrees with the increased relative abundance of *intI1* and *qacE*Δ*1* (a component of the 3' conserved sequence) in the high dose (Herrero et al., 2008; Partridge et al., 2009; Yan et al., 2006), and with previously reported quantitative PCR observations (Lau et al., 2020). All of these antibiotic resistance genes have been detected in gram-negative human pathogens (Alcock et al., 2020).

Despite the increased relative abundances of several metagenomic antibiotic resistance genes that are known to be associated with gene cassettes (except *sul1*, which is a member of the 3' conserved sequence), integron gene cassette richness (Figure 5c) and composition (Figure 6c) were unaffected by antibiotic exposure, as determined by the integron gene cassette sequence analysis. The absence of a treatment effect of macrolide antibiotic exposure on gene cassette richness and composition may be due to how *intI1* gene expression — and therefore, gene cassette recombination — is regulated in bacteria.

The transcription of *intI1* is regulated by the bacterial SOS system which is

”a coordinated response to DNA damage” that is present in most bacteria (Maslowska et al., 2019). Antibiotic drug classes that damage DNA (e.g. fluoroquinolones, nitrofurans) or affect DNA synthesis (e.g. sulfonamides, trimethoprim) induce the bacterial SOS response, which increases the expression of *intI1* and thereby triggers integron gene cassette recombination (Baharoglu et al., 2010; Guerin et al., 2009). Antibiotic drug classes that do not damage DNA, such as macrolides, likely do not induce the bacterial SOS response and therefore do not increase the expression of *intI1*, which would otherwise trigger integron gene cassette recombination (Hastings et al., 2004). Therefore, macrolide antibiotic exposure of soil bacteria may not trigger gene cassette recombination, but antibiotics that induce the SOS response in soil bacteria may alter the richness or composition of the gene cassette metagenome and should be investigated for these effects.

Alternatively, because environmental integron gene cassettes were sequenced and class 1 integrons were not specifically targeted in the integron gene cassette targeted amplicon sequencing, the environmental classes of integrons (of which there are hundreds) may have overwhelmed our gene cassette sequencing dataset, leaving few reads for class 1 integron gene cassettes, whose diversity and composition may have been affected by antibiotic exposure. A future study investigating the response of the gene cassette metagenome of class 1 integrons to macrolide antibiotics could reveal differences that our compositional data analysis was not powered to detect.

Of the remaining non-cassette-associated metagenomic antibiotic resistance genes that were increased in the high-dosed soil, five are known to be carried on plasmids in human pathogens (*sul2*, *aac(6')-Ib7*, *pp-flo*, *mphE*, *ant(3")-IIa*), *tet(33)* is carried by the insertion sequence IS6100, and *aph(3")-Ib* is carried on several mobile genetic elements including plasmids and transposons (Alcock et al., 2020; Tauch et al., 2002). The remaining genes with increased relative abundances are known to be chromosomally-encoded

in *Pseudomonas* spp. (*aph(3')-IIb*, *oprN*, *mexH*, *triC*, *mexQ*) or in *Burkholderia* spp. (*opcM*) (Burns et al., 1996; Hächler et al., 1996; Mesaros et al., 2007; Mima et al., 2007; Mima et al., 2005). All of these remaining genes with the exception of *aph(3')-IIb*, an aminoglycoside phosphotransferase, encode components of antibiotic efflux pumps.

Overall, of the 21 increased antibiotic resistance genes in the high-dosed soil, 15 are known to be carried by mobile genetic elements and all are known to be associated with human pathogens. In considering the threat of these antibiotic resistance genes to human health, future research should be performed to determine if these genes reside within pathogenic bacteria in the soil or have the potential to be mobilized to human pathogens.

The high dose of antibiotics similarly increased the number of unique mobile genetic element variants (Figure 5b) and altered the composition of the mobilome (Figure 6). More mobile genetic element variants were detected in the high-dosed soil group alone (n = 119) than were shared between any combination of the other groups, suggesting that the high dose of macrolides raised many mobile genetic element variants over the limit of detection (Supplementary Figure A.4b). Of the 23 mobile genetic element variants with increased relative abundances in the high-dosed soil (Figure 10), most were *tnpA* variants (n = 15), which suggests that some of the increased antibiotic resistance genes may have been mobilized by transposons or insertion sequences.

The exact mechanism of co-selection of macrolide and non-macrolide antibiotic resistance genes in the high-dosed soil could not be elucidated. However, because antibiotic resistance genes that are known to be associated with several types of mobile genetic elements were increased, and several mobile genetic element variants were increased, it's plausible that multiple co-selection processes were active in the high-dosed soil simultaneously.

4.3 Antibiotic exposure enriches for fastidious taxa

In this study, increased relative abundances of three bacterial taxa in the low-dosed soil and two taxa in high-dosed soil were detected (Table 1). For the low-dosed soil, the effect sizes for two of the three increased taxa were relatively low ($W < 5$), but an unknown *Acidobacteria Subgroup 6* taxon was over 45-fold more abundant in the low-dosed soil than in the control. This taxon was present in both antibiotic-treated groups but not in the control soil.

Acidobacteria are largely uncultivated, highly abundant bacteria in agricultural soil and play an important role in shaping the soil bacterial community through their decomposition of organic carbon (Banerjee, Baah-Acheamfour, et al., 2016; Banerjee, Kirkby, et al., 2016; Solden et al., 2016). Furthermore, acidobacteria are a known reservoir of macrolide antibiotic resistance in urban surface waters through their expression of the *erm* gene family, and have been reported to be increased in macrolide-polluted sediments, suggesting intrinsic macrolide resistance among some taxa (Milaković et al., 2020; Yi et al., 2019). Conservatively, the unknown *Acidobacteria Subgroup 6* taxon may represent a macrolide-resistant decomposer, but more speculatively, could represent an organism that is able to use macrolides as an alternative source of carbon. Further studies would be required to investigate the macrolide biodegradation potential of this taxon.

For the high-dosed soil, the effect sizes for both increased taxa were high ($W > 20$) and both were identified as unknown *Chloroflexi Gitt-GS-136* spp. (Table 1). Chloroflexi are fastidious bacteria with diverse metabolisms and, like acidobacteria, are a known reservoir of macrolide resistance in the environment, though their overall role in environmental antibiotic resistance is still poorly understood (Gupta, 2013; Islam et al., 2019; Yi et al., 2019). To our knowledge, this phylum has not been reported to carry any of the antibiotic

resistance genes that were increased in this study, though the relationship between *Chloroflexi* taxa and antibiotic resistance remains understudied (Razavi et al., 2017).

In the present study, it was assumed that the bacterial hosts of the antibiotic resistance genes that were increased at the high dose would be revealed as differentially abundant in at least one of the taxonomic analyses, but it's possible that the bacterial taxa that hosted these resistance genes were not significantly differentially abundant, yet were still sufficiently increased to enrich for antibiotic resistance. These bacterial taxa could be revealed in a future co-abundance network analysis to identify taxa whose relative abundances are correlated with those of antibiotic resistance genes and mobile genetic elements, thereby allowing us to identify candidate taxa as hosts of these gene targets (Forsberg et al., 2014).

Although there was no significant effect of macrolide antibiotic exposure at either dose on the overall richness or composition of the soil bacterial community, some taxa are known to respond to exposure: in a previous investigation of the persistence of macrolide antibiotics in soils that were annually exposed to a low or high dose of erythromycin, clarithromycin, and azithromycin for five years, or were left untreated, macrolide antibiotics were degraded more rapidly in the soils with an exposure history to macrolides than in the untreated control soil (Topp et al., 2016). It is possible that *Acidobacteria* or *Chloroflexi* taxa may have played a role in the accelerated biodegradation of these macrolide antibiotics.

4.4 Unrealistically high antibiotic exposure decreases relative abundances of cyanobacteria

The only bacterial phylum that was differentially abundant in response to antibiotic exposure was *Cyanobacteria* (Figure 4). The relative abundances of *Cyanobacteria* sequence variants were decreased in the high-dosed soil but not in the low-dosed soil, and this effect was observed only in the metagenomic analysis and not in the 16S rDNA analysis (Supplementary Figure A.1).

Cyanobacteria have recently been considered as indicator species for antibiotic pollution of aquatic ecosystems due to their sensitivity to several drug classes of antibiotics (Committee for Medicinal Products for Human Use, 2015; Le Page et al., 2017), but this response is not uniform across all species and to all antibiotics (Dias et al., 2015; Le Page et al., 2017). For example, the MIC of the cyanotoxin-producing cyanobacterium *Microcystis aeruginosa* to β-lactam antibiotics can be as low as 0.1 mg L^{-1}, while the MICs of β-lactams for the tropical cyanobacteria *Gloeocapsa* sp. and *Chroococcidiopsis* sp. may be 100-fold greater (Dias et al., 2015; Reynaud & Franche, 1986).

The decreased relative abundance of *Cyanobacteria* sequence variants in the high-dosed soil of this present study suggests that the high dose, but not the low dose of macrolides is inhibitory to at least some cyanobacteria. The minimum NOECs of azithromycin and erythromycin for growth inhibition of cyanobacteria were reported to be at-most 0.0015 and 0.0062 mg L^{-1}, which are approximately 20–70-fold lower than the concentration of macrolides in the low-dosed soil, and 1,600–6,700-fold lower than the concentration of macrolides in the high-dosed soil (Le Page et al., 2019). Therefore, the decreased abundance of *Cyanobacteria* sequence variants in the high-dosed

soil of this present study is in agreement with the known NOECs for erythromycin and azithromycin, but it should be noted that these values were determined for an aqueous environment and are likely different for soil. Our inability to detect an effect of macrolide antibiotic exposure at the low dose may be due to a higher MIC for soil cyanobacteria or insufficient sensitivity to detect this effect using metagenomic sequencing.

The detection of this treatment effect in the high-dosed soil for only one of two taxonomic analyses may be due to differences in how abundances are calculated for each approach: for the metagenomic analysis, metagenomic sequence reads were matched to a database of clade-specific marker genes and fold-coverages of these genes were obtained; for the 16S rDNA analysis, amplicon sequence variants of 16S rDNA sequences were constructed and assigned taxonomy using a 16S rRNA gene database, and the number of times each variant was observed was counted. Because 16S rRNA gene copy numbers are variable in bacteria, the relative abundances obtained from the 16S rDNA analysis are biased towards bacterial genomes with high copy numbers of the 16S rRNA gene (Kembel et al., 2012), whereas the metagenomic sequence analysis excluded the use of multicopy marker genes to assign taxonomy for this reason (Segata et al., 2012).

Another potential concern with the results obtained from the 16S rDNA sequencing experiment was the low number of merged reads resulting from the DADA2 workflow (Supplementary Table A.2). A low number of merged reads can result from poor sequence quality or from excessive trimming of the 3' ends of paired-end reads. Our quality control analysis revealed overall good sequence quality for the 16S rDNA dataset, thus it is likely that the Trimmomatic parameters that were used need to be re-adjusted to optimize the read-merging step while also discarding low-quality bases. This loss of data could explain why *Cyanobacteria* sequence variants were not identified as differentially abundant in the 16S rDNA sequence analysis but were

identified as differentially abundant in the metagenomic sequence analysis.

4.5 High antibiotic exposure decreases relative abundances of β-lactam resistance genes

Of the seven antibiotic resistance genes that were decreased in response to macrolide antibiotic exposure (five in the high dose, two in the low dose), all were predicted to encode resistance to β-lactam antibiotics (Figure 7). β-lactam antibiotics are bactericidal against both gram-negative and gram-positive bacteria by inhibiting synthesis of the cell wall, thereby leading to lysis and cell death (Balsalobre et al., 2019). The β-lactam drug class of antibiotics was among the first to be brought to the drug market with the discovery of penicillin in 1928 by Alexander Fleming (Fleming, 1929). The subsequent industrialized production and mass consumption of penicillins by the mid-1940's has resulted in increased acquired resistance to β-lactams, especially due to methicillin-resistant strains of *Staphylococcus aureus* (Public Health Agency of Canada, 2020).

β-lactam resistance genes are highly abundant in soil bacteria, even in the absence of anthropogenic antibiotic pollution, and over 90% of these genes are encoded chromosomally (Dunivin et al., 2019; Mindlin & Petrova, 2017; van Goethem et al., 2018). Of the β-lactam resistance genes that were decreased in relative abundance, two SHV-family β-lactamase encoding genes (*bla*$_{\text{SHV-71}}$, *bla*$_{\text{SHV-165}}$), one CTX-M β-lactamase (*bla*$_{\text{CTX-M-117}}$), one PEDO-family metallo-β-lactamase (*bla*$_{\text{PEDO-1}}$), and one *ampC*-type β-lactamase (*E. coli ampC*) were decreased in the high-dosed soil, while two TEM-family β-lactamase encoding genes (*bla*$_{\text{TEM-1}}$, *bla*$_{\text{TEM-22}}$) were decreased in the low-dosed soil. *bla*$_{\text{TEM-1}}$ was the first plasmid-associated β-lactam resistance gene to be identified and has since spread throughout gram-negative pathogens (e.g. *Acinetobacter baumanii*, *E. coli*, *Klebsiella pneumoniae*).

Other members of the TEM-family of β-lactamase genes, including *bla*$_{\text{TEM-22}}$, have a more narrow host range but confer resistance to extended-spectrum β-lactams (able to hydrolyze oximino-cephalosporins) (Arlet et al., 1993; Bradford, 2001).

The most likely explanation for the decreased abundances of β-lactam resistance genes in the macrolide antibiotic-exposed soil is the decreased abundance of macrolide-susceptible bacteria carrying these resistance genes. While the co-selection of several non-macrolide antibiotic resistance genes in the high-dosed soil may have been due to genetic linkage between macrolide and non-macrolide antibiotic resistance genes, it is possible that macrolide and β-lactam resistance genes were infrequently genetically linked in the soil bacteria that were sampled, and that the β-lactam-resistant bacteria were outcompeted by macrolide-resistant bacteria in the presence of macrolides. None of the decreased taxa in this study (*Arthrobacter globiformi*, *Arthobacter* sp. Leaf69, *Mycolicibacterium tusciae*, *M. vaginatus*, *O. nigro-viridis*, *Ramlibacter sp. Leaf400*) are known to carry β-lactam resistance genes, although one β-lactam resistance gene *estA* has been identified in *Arthrobacter nitroguajacolicus* Rü61a and several have been identified in the plasmidome of *Mycolicibacterium* spp.

4.6 Policy implications

There are currently no globally accepted standards for setting limits for pollutant levels in biosolids. In Europe, a “precautionary principle”-based approach for managing pollutant concentrations in biosolids and biosolids-applied soils has been used; the absence of toxicity and fate data for these pollutants has led to huge variability in limits for pollutants in biosolids-applied soils between individual European nations — sometimes by 2–3 orders of magnitude (McCarthy & Loyo-Rosales, 2015). In the United

States, the United Kingdom, and Canada, a risk assessment-based approach for managing pollutant concentrations in biosolids has been preferred (McCarthy & Loyo-Rosales, 2015). In the United States, limits for pollutant levels have been established by considering different exposure pathways to humans (e.g. crop consumption, groundwater contamination), and by using the conservative approach of considering risk to the most highly-exposed individuals in society (McCarthy & Loyo-Rosales, 2015).

In Canada, most provinces and territories have set limits for pollutants (mostly inorganic) in biosolids, though this has not always been the case: as more data of pollutant concentrations in biosolids has become available, more pollutants have been added to lists of agents requiring further research — including antibiotics (Sabourin et al., 2012; U.S. Environmental Protection Agency, 2009; Water Environment Association of Ontario, 2010). Today, only two provinces in Canada (Québec and Nova Scotia) have set maximum limits for levels of organic compounds in biosolids, and no provinces or territories have set limits for antibiotics (McCarthy & Loyo-Rosales, 2015).

Due to the absence of experimental data on the effects of antibiotics in biosolids on soil bacteria, risk assessments based upon PNECs have been performed to guide policy decisions for managing antibiotic concentrations in biosolids (Eriksen et al., 2009; Jensen et al., 2012). Unfortunately, these risk assessments have faced many challenges, including the absence of existing toxicity and fate data for antibiotics (especially in biosolids-amended soil), and a failure to consider the simultaneous exposure to other antibiotics (and other pollutants) present in biosolids (McCarthy & Loyo-Rosales, 2015). Generally, environmental risk assessments of antibiotics do not measure the potential for selection of antibiotic resistance (Lee & Choi, 2019). However, a risk assessment by the Norwegian Scientific Committee for Food Safety compared the predicted environmental concentrations of 37 antibiotics in biosolids-applied soil to the MIC values of these antibiotics for *Escherichia coli*

and *E. faecium*; they concluded that antibiotic resistance is unlikely to be promoted at an application rate of 60 tons biosolids ha $^{-1}$ soil for all tested antibiotics except for the fluoroquinolone antibiotic, ciprofloxacin (Eriksen et al., 2009). However, *E. coli* and *E. faecium* are intrinsically resistant to many of the antibiotics that were tested in this risk assessment, including erythromycin, clarithromycin, and azithromycin, and therefore cannot be used to conclude that antibiotic resistance will not be selected for in other biosolids-exposed soil bacteria.

In this thesis, I investigated the effects of long-term, repeated exposure of macrolide antibiotics on the soil bacterial community, resistome, and mobilome using sequencing-based methods, and I determined that an environmentally realistic dose of macrolides for a biosolids exposure scenario is unlikely to change soil bacterial diversity or promote clinically relevant antibiotic resistance. These results suggest that typical concentrations of macrolide antibiotics within Canadian municipal biosolids are unlikely to harm environmental and human health in the context of antibiotic resistance. However, the absence of an intermediate concentration between the low dose (0.1 mg kg^{-1}) and the high dose (10 mg kg^{-1}) means that I was unable to precisely determine the 'threshold concentration' beyond which the soil resistome and mobilome were significantly affected by macrolide antibiotic exposure for the soils used in this study. If this threshold concentration were to be within the range of 0.1 to 1 mg kg^{-1}, there could be cause-for-concern for some biosolids with a high macrolide antibiotic load to promote antibiotic resistance in soil. Furthermore, an intermediate concentration of macrolides (1 mg L^{-1}) is more likely to be observed in other anthropogenically polluted environments than the high dose (Bielen et al., 2017).

Overall, to protect human and environmental health, more data are required to establish acceptable limits for antibiotics in biosolids that are intended for agricultural use. A similar investigation to this present study at

intermediate concentrations of macrolides and in different contaminated environments may reveal similar treatment effects to those observed in the high-dosed bacteria of this study. Future research is needed to elucidate the range of concentrations within which the soil resistome and mobilome are affected by macrolide antibiotic exposure.

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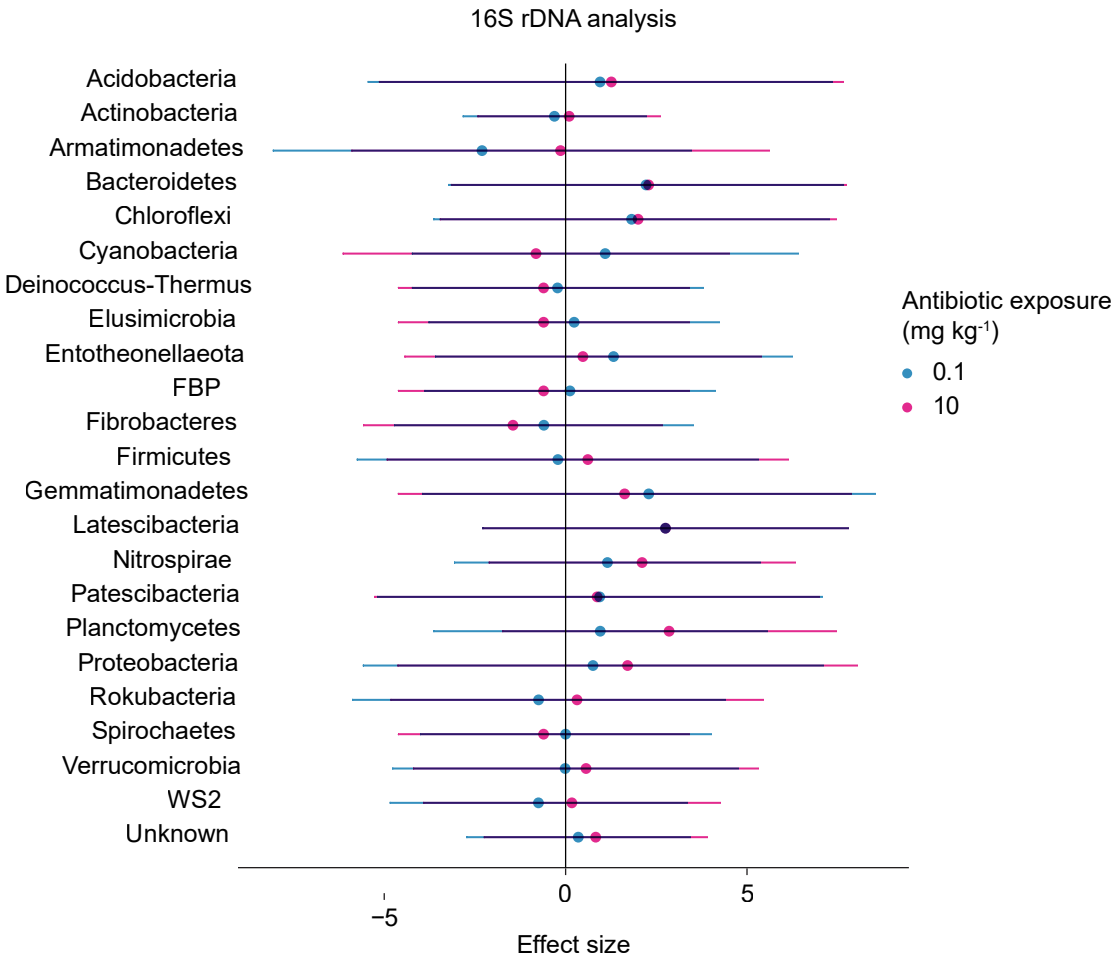
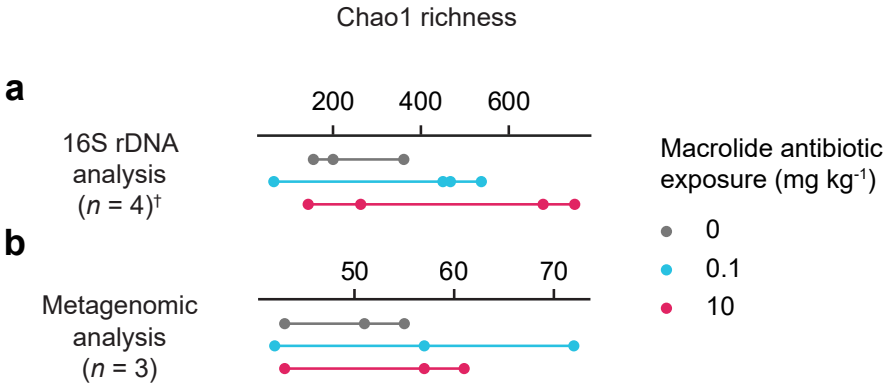
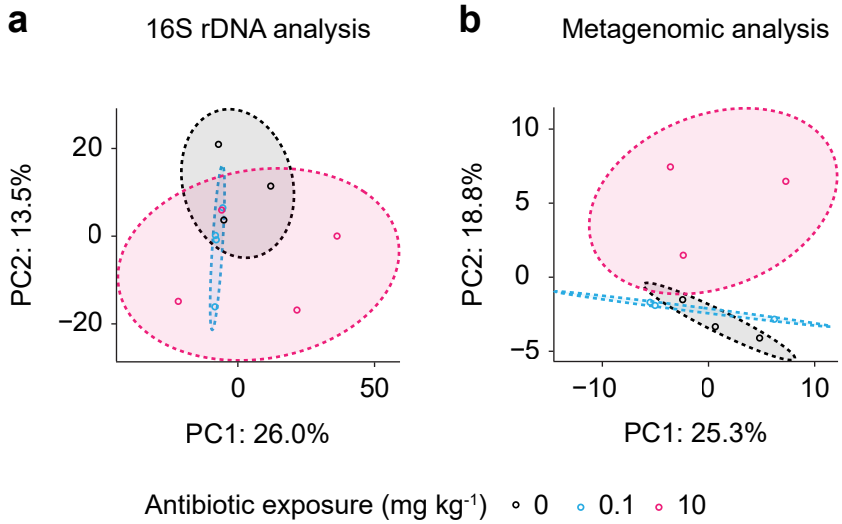
A Supplementary Information



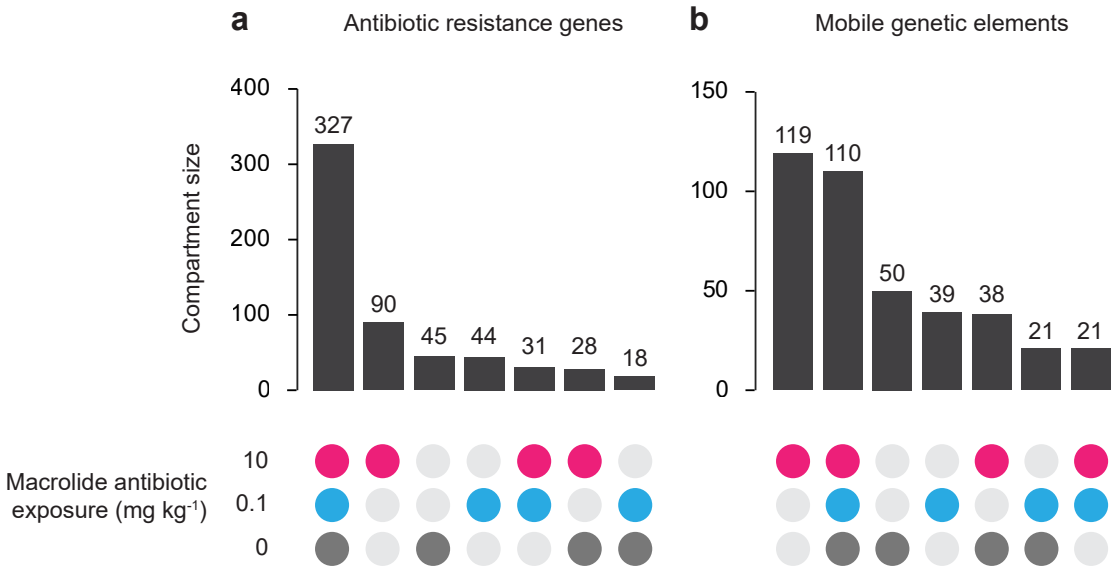
Supplementary Figure A.1 Effect sizes (fold-changes) of differences in the relative abundances of bacterial phyla classified in the 16S rDNA analysis relative to the untreated control soil (n = 4 for antibiotic-exposed groups, n = 3 for untreated control group). Horizontal lines intersecting with circles are error bars, indicating the extent of Bonferroni-adjusted 95% confidence intervals of effect sizes.



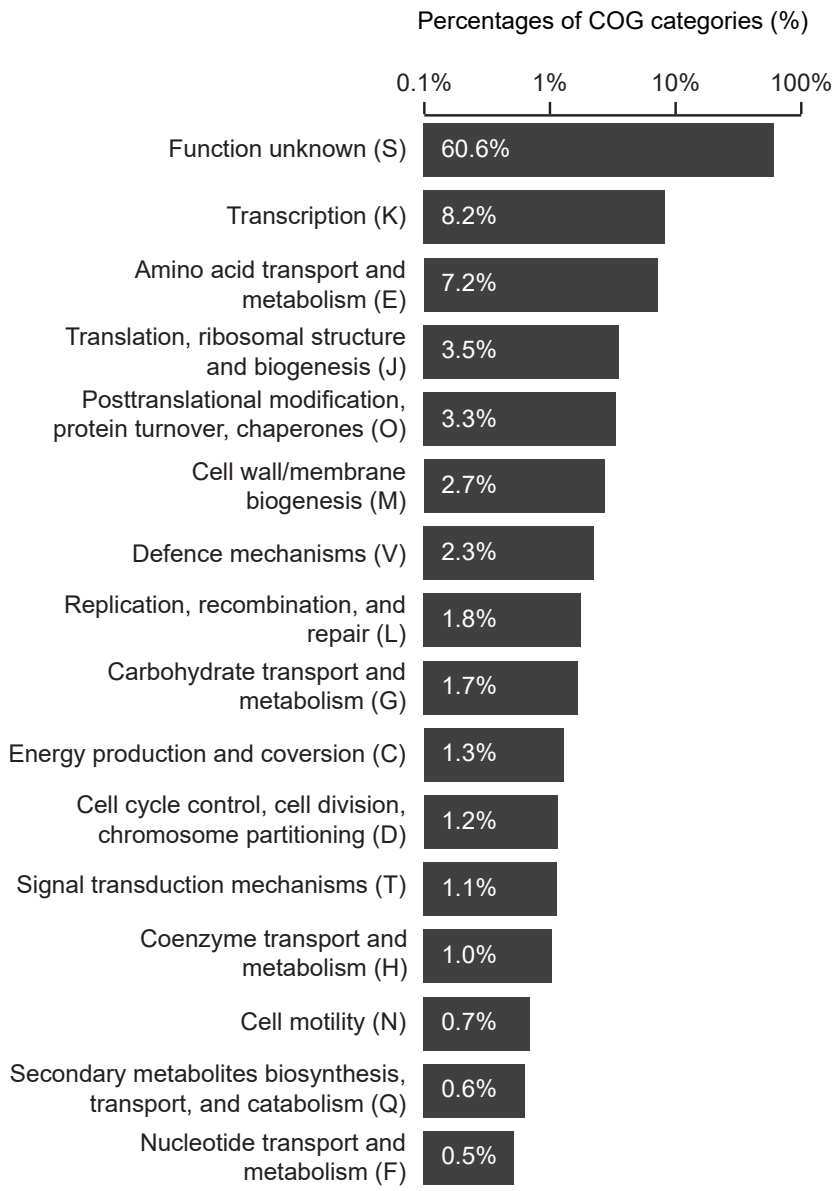
Supplementary Figure A.2 Richness (Chao1) of bacterial taxa as classified by **a**) the 16S rDNA analysis or **b**) the metagenomic analysis in macrolide antibiotic-exposed and untreated control soil. There were no statistically significant differences between the antibiotic-exposed and -unexposed groups. † $n = 4$ for the antibiotic-exposed groups, $n = 3$ for the untreated control group.



Supplementary Figure A.3 PCA ordination plots (PC1, PC2) of the CLR-transformed relative abundances of bacterial taxa as classified by **a**) the 16S rDNA analysis or **b**) the metagenomic analysis in macrolide antibiotic-exposed and untreated control soil. PERMANOVA pseudo-F and p-values with 999 permutations are displayed. Shaded areas correspond to 95% confidence ellipses of treatment groups. Percentages of variance explained by each axis are displayed in the axis titles.



Supplementary Figure A.4 Number of antibiotic resistance genes and mobile genetic elements detected within each compartment formed between the untreated control, low-, and high-dosed soil groups, arranged by compartment size. Shaded dots below the bar plots correspond to the compartment.



Supplementary Figure A.5 Prevalence (in percentages) of COG functional categories among integron gene cassette open reading frames that were assigned a COG function category (n = 5,206). Only COG functional categories with a prevalence over 0.5% are shown. Y-axis is logarithmically scaled and begins at 0.1% for visual clarity.

Target	Primer	Sequence (5' → 3')	Amplicon size (bp)	Annealing temperature (°C)	Reference
attC	Adapter + HS286	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCSGCTKGARCGAMTTGTTAGVC	Variable	55	Stokes et al. (2001)
	Adapter + HS287	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCSGCTKANCTCVRRCGTTAGSC			
16S rRNA	Adapter + S-D-Bact-0341-b-S-17	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG	~ 460	55	Klindworth et al. (2013)
	Adapter + S-D-Bact-0785-a-A-21	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC			

Supplementary Table A.1 PCR primer sequences, annealing temperatures, and expected amplicon sizes for integron gene cassette and 16S rDNA PCR amplification (Klindworth et al., 2013; Stokes et al., 2001). Degenerate bases follow the IUPAC standard ambiguity code. Red text indicates location of adapter overhang sequences.

		Sequencing statistics		
Analysis step (sample size)	**Antibiotic dose (mg kg^{-1})**	**Total no. reads**	**Mean percentage duplicated reads ± SD**	**Total Mbp**
Raw (*n* = 4)	0	1,707,298	88.9 ± 2.7%	513.9
	0.1	2,169,960	90.2 ± 2.0%	653.2
	10	2,337,176	90.6 ± 2.0%	703.5
		Total no. reads remaining	**Mean read length ± SD (bp)**	
Post-trimming (*n* = 4)	0	1,700,062	185 ± 23	
	0.1	2,161,602	188 ± 22	
	10	2,327,980	189 ± 21	
		Mean percentage input reads merged ± SD		
DADA2 (*n* = 4)	0	6.8 ± 6.1%		
	0.1	7.1 ± 2.2%		
	10	16.5 ± 13.7%		

Supplementary Table A.2 16S rDNA amplicon sample sequence statistics for unprocessed (raw) and trimmed reads, and the percentages of input sequence reads merged by DADA2, with samples grouped by macrolide antibiotic dose. Mean values are reported along with standard deviations (SD).

		Sequencing statistics		
Analysis step (sample size)	**Antibiotic dose (mg kg^{-1})**	**Total no. reads (million)**	**Mean percentage duplicated reads ± SD**	**Total Gbp**
Raw (n = 3)	0	495.1	6.0 ± 0.7%	74.8
	0.1	467.1	5.1 ± 0.3%	70.5
	10	530.4	5.8 ± 0.3%	80.1
		Total no. reads remaining (million)	**Mean read length ± SD (bp)**	
Post-trimming (*n* = 3)	0	402.9	146 ± 0.1	
	0.1	374.4	146 ± 0.1	
	10	419.8	145 ± 0.2	

Supplementary Table A.3 Metagenomic DNA sample sequence statistics for unprocessed (raw) and trimmed reads, with samples grouped by macrolide antibiotic dose. The number of biological replicates within each treatment group are shown beside each analysis step. Mean values are reported along with standard deviations (SD).

		Sequencing statistics			
Analysis step (sample size)	**Antibiotic dose (mg kg^{-1})**	**Total no. reads**	**Mean percentage duplicated reads ± SD**	**Total Gbp**	
Raw (n = 4)	0	27,043,396	43.5 ± 7.7%	3.4	
	0.1	25,015,412	41.6 ± 7.4%	3.4	
	10	25,936,620	40.0 ± 7.1%	3.3	
		Total no. contigs	**Total Mbp**	**Mean contig N50 ± SD (bp)**	**Largest contig (bp)**
Post-assembly (n = 4)	0	86,281	42.6	4,010 ± 412	5,350
	0.1	89,436	44.7	4,115 ± 333	5,648
	10	94,651	48.2	4,294 ± 946	5,774
		Total no. remaining contigs	**Percentage contigs remaining**	**Contig N50**	**Largest contig (bp)**
Post-cassette filtering (n = 4)	0 + 0.1 + 10	75,850	28.1%	27,956	4,744

Supplementary Table A.4 Integron gene cassette amplicon sample sequence statistics for unprocessed (raw) reads, and assembly statistics for unfiltered (post-assembly) and filtered (post-cassette filtering) contigs, with samples grouped by macrolide antibiotic dose. The number of biological replicates within each treatment group are shown beside each analysis step. Mean values are reported along with standard deviations (SD).

	Confidence level		
Drug class	Low	Moderate	High
Acridine dye	59	9	0
Aminocoumarin	55	6	1
Aminoglycoside	958	298	29
Antibacterial free fatty acids	1	0	0
Benzalkonium chloride	4	0	0
Beta-lactam	14	12	0
Carbapenem	328	16	3
Cephalosporin	732	34	5
Cephamycin	191	9	0
Diaminopyrimidine	154	14	1
Elfamycin	1	0	0
Fluoroquinolone	235	29	1
Fosfomycin	89	6	0
Fusidic acid	24	0	0
Glycopeptide	190	10	0
Glycylcycline	40	7	0
Lincosamide	179	12	3
Macrolide	344	27	4
Monobactam	100	7	2
Mupirocin	5	4	2
Nitrofuran	4	2	0
Nitroimidazole	2	1	0
Nucleoside	37	6	0
Oxazolidinone	22	4	1
Penam	757	34	4
Penem	143	8	2
Peptide	93	5	0
Phenicol	204	24	8
Pleuromutilin	19	4	1
Polyamine	6	1	0
Pyrazinamide	1	0	0
Rhodamine	3	0	0
Rifamycin	85	19	12
Streptogramin	150	8	2
Sulfonamide	43	1	0
Sulfone	1	0	0
Tetracycline	243	34	1
Triclosan	42	4	0

Supplementary Table A.5 Counts of target drug classes for predicted antibiotic resistance genes within integron gene cassettes at each confidence level. Values in the table are shaded based upon the minimum and maximum values in each column.

Curriculum Vitae

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Post-Secondary Education and Degrees:	M.Sc. in Biology The University of Western Ontario London, ON 2019 – 2021
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	Western Scholarship of Excellence 2014
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Publications:

1. Yost, R. T., Robinson, J., Baxter, C. M., Scott, A. M., **Brown, L. P.**, Aletta, M., Hakimjavadi, R., Lone, A., Cumming, R. C., Dukas, R., Mozer, B., & Simon, A. F. (2020). Abnormal social interactions in a *Drosophila* mutant of an autism candidate gene: *Neuroligin 3*. *International Journal of Molecular Sciences*, *21*(13), 4601. https://doi.org/10.3390/ijms21134601

Conferences:

1. **Poster**: Brown, L. P., Murray, R., Scott, A., Tien, Y.-C., Lau, C. H.-F., Tai, V., & Topp, E. (2021). A decade of macrolide antibiotic exposure impacts the soil bacterial community, resistome, and mobilome. 70th Annual Conference of the Canadian Society of Microbiologists (CSM 2021). Online.

2. **Presentation (Keynote)**: Brown, L. P., Tai, V., Topp, E. (2021). The impact of long-term antibiotic exposure on soil bacteria: A human health perspective. Western Research Forum 2021. Online.

3. **Poster**: Brown, L. P., Murray, R., Scott, A., & Topp, E. (2019). Do benzalkonium chloride biocides promote antimicrobial resistance in environmental bacteria? 10th Annual Biology Graduate Research Forum. Western University, London, ON.

4. **Presentation**: Brown, L. P., Yost, R. T., & Simon, A. F. (2019). Chasing the *neuroligins*: Quantification of low copy transcripts in *Drosophila melanogaster*. Ontario Biology Day 2019. Western University, London, ON.