

A DECADE OF MACROLIDE ANTIBIOTIC EXPOSURE AFFECTS
THE SOIL BACTERIAL COMMUNITY, RESISTOME, AND MOBILOME

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Abstract

Biosolids are produced from treated wastewater and can be used as agricultural fertilizer. Due to the nature of their production, however, biosolids are frequently contaminated with macrolide antibiotics, to which drug resistance is rising among historically susceptible bacteria. To determine if the land-application of biosolids could increase clinically relevant antibiotic resistance in soil bacteria, we established soil plots and exposed them annually to an environmentally realistic or unrealistically high dose of macrolides for ten years. We sequenced the bacterial 16S ribosomal DNA, metagenomic DNA, and integron gene cassettes within the treated and untreated soil to compare the compositions and diversities of the bacterial communities, mobile genetic elements, and antibiotic resistance genes to that of antibiotic-free soil. We determined that the high dose but not the realistic dose of macrolides increased the diversity of clinically relevant antibiotic resistance genes and mobile genetic elements in soil and decreased the abundance of Cyanobacteria.

Keywords: biosolids, antibiotic resistance, agriculture, soil, ecotoxicology, metagenomics.

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Chapter 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 adopted quickly into human medicine (Hutchings, Truman, and Wilkinson, [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, resulting 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 only recently been banned in several countries such as the United States in 2017 (H. M. Scott et al., 2019), Canada in 2018 (Finlay et al., 2019), and China in 2020 (Hu and Cowling, 2020), but this practice still continues in many countries with few restrictions on usage (R. 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 (Marathe et al., 2019; Bielen et al., 2017), municipal sewage (Pärnänen, Narciso-da-Rocha, et al., 2019), aquaculture (Reverter et al., 2020), and animal agriculture (Kirchhelle, 2018). Antibiotic pollution in the environment selects for antibiotic resistance genes (Lau, Tien, et al., 2020; Jechalke et al., 2014; Bielen et al., 2017; Yi et al., 2019) which could be transferred to the human microbiome through the interconnected health of humans, animals, and the environment (Berendonk et al., 2015; Hernando-Amado et al., 2019; Tiedje et al., 2019; T. P. Robinson et al., 2016).

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 (Traxler and Kolter, 2015; Fajardo and Martínez, 2008). 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 (G. D. Wright, 2007; Dunivin et al., 2019). 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; B. G. Hall and 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 can be selected for by anthropogenic antibiotic pollution (Lau, Tien, et al., 2020; Jechalke et al., 2014).

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; Public Health Agency of Canada, 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 (T. P. Robinson et al., 2016).

1.3.1 The shared human-soil resistome

Under 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 (Forsberg, Reyes, et al., 2012). The human microbiome and human bacterial pathogens share antibiotic resistance genes with environmental bacteria (Forsberg, Reyes, et al., 2012; Smillie et al., 2011; Pal et al., 2016), but the frequency and context of this exchange is poorly understood (Berendonk et al., 2015; Huijbers et al., 2015) due to the challenges associated with source attribution — i.e. determining the exact pathway of a resistance gene from environment to the human microbiome (Tiedje et al., 2019; L.-G. Li, Yin, and T. Zhang, 2018). 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 (Forsberg, Reyes, et al., 2012; Lau, van Engelen, et al., 2017; Allen et al., 2009).

Antibiotic resistance genes may be transmitted from soil bacteria to the human microbiome through the consumption of produce (Maeusli et al., 2020; Blau et al., 2018). 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 (Maeusli et al., 2020; Krych et al., 2013). 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, Marti, A. Scott, Tien, Murray, Sabourin, Duenk, et al., 2016; Rahube, Marti, A. Scott, Tien, Murray, Sabourin, Y. Zhang, 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 embedded within the bacterial chromosome and are therefore inherited vertically (Dunivin et al., 2019; Forsberg, Patel, et al., 2014). A high soil bacterial diversity has been proposed to “act as a biological barrier” for increased antibiotic resistance as a loss in soil bacterial species diversity is correlated with increased antibiotic resistance gene abundance (van Goethem et al., 2018; Chen et al., 2019; 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 (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, Kwong, et al., 2018; Perry and G. D. Wright, 2013). The mobilome facilitates the horizontal gene transfer of antibiotic resistance genes between bacteria, and includes elements such as plasmids and transposons (Partridge, Kwong, et al., 2018), bacteriophages (Subirats et al., 2016); (Colomer-Lluch, Jofre, and Muniesa, 2011), and membrane vesicles (Chattopadhyay and Jaganandham, 2015). Horizontal gene transfer occurs through three main mechanisms: conjugation (physical interaction between bacteria), transformation (intake of extracellular DNA), and transduction (phage-mediated) (Partridge, Kwong, 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 and G. D. Wright, 2013), though transformation and transduction likely also play important roles (Perry and G. D. Wright, 2013; Aminov, 2011). Of all of the known non-plasmid mobile genetic elements to mobilize antibiotic resistance in soil, integrons may be the most genetically diverse (Ghaly, Geoghegan, Alroy, et al., 2019).

1.4.2 Integrons

Integrans 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 their selves, relying upon other mobile genetic elements such as plasmids and transposons for mobility (Gillings, 2014). Integrans sample their environment for gene cassettes (Ghaly, Geoghegan, Tetu, et al., 2020) — pieces of DNA that usually contain one open reading frame followed by a cassette-associated recombination site (*attC*). Gene cassettes 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 (Ma et al., 2017; Ghaly, Geoghegan, Alroy, et al., 2019; Ghaly, Geoghegan, Tetu, et al., 2020). In a recent sequencing study of the gene cassette metagenomes of 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, Geoghegan, Alroy, et al., 2019).

Vera: The leading researchers in the field refer to the totality of gene cassettes in the environment as the "cassette metagenome", so I think it'd be a good idea to keep this terminology for consistency with the literature. E.g. <http://www.ncbi.nlm.nih.gov/pubmed/31948729>

Integrans are characterized by i) an integrin-integrase gene (*intl*) encoding a site-specific tyrosine recombinase, ii) an integrin-associated recombination site (*attI*), where incoming gene cassettes are inserted with the help of *Intl*, and iii) an integrin-associated promoter (P_C) which expresses downstream gene cassettes (Figure 1) (Gillings, 2014). *Intl* 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 integrin structure. The recombination event produces two daughter molecules: a duplicate of the original integrin structure, and the other with the integrated gene cassette (Ghaly, Geoghegan, Tetu, 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-embedded antibiotic resistance gene, maintain the antibiotic resistance phenotype if it confers a selective advantage (Ghaly, Geoghegan, Tetu, et al., 2020).

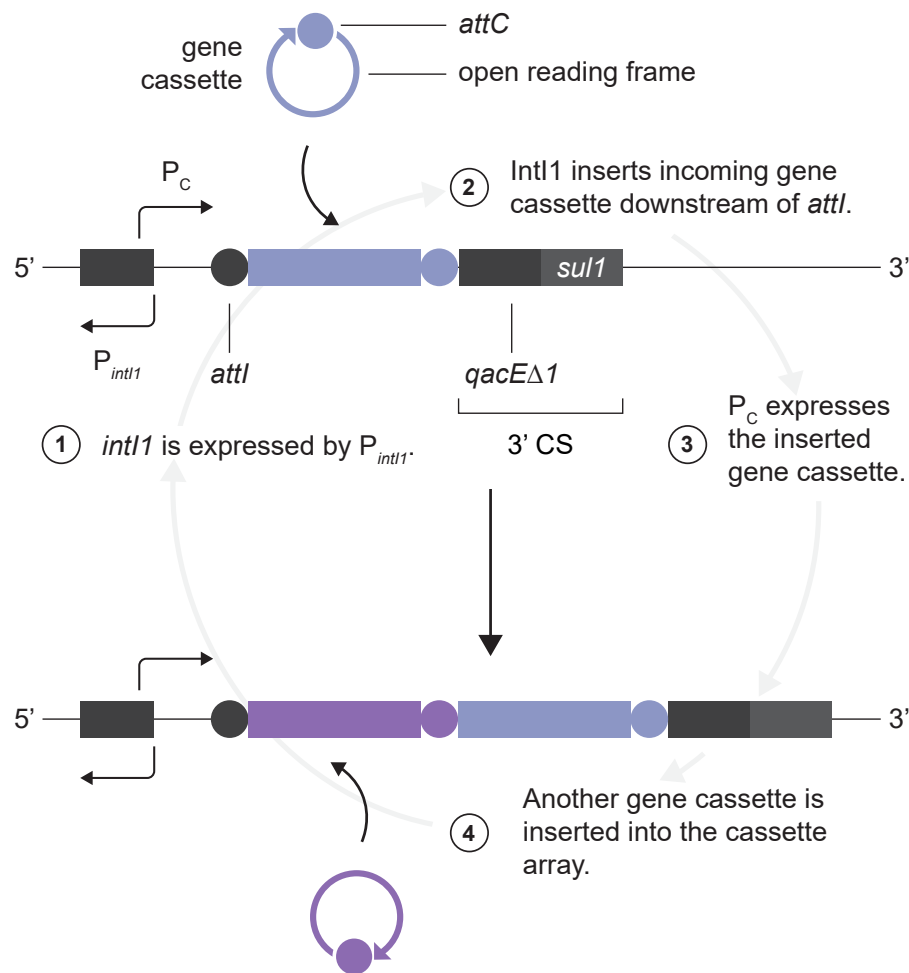


Figure 1 Class 1 integrons are clinically-relevant integrons that are ubiquitous in human-impacted environments, and are characterized by their class 1 integron-integrase gene (*intI1*) and 3' conserved sequence (3'-CS) which contains a partially deleted biocide resistance gene (*qacEΔ1*) and a sulfonamide 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. This process may continue for up to eight gene cassettes.

1.4.3 Co-selection

Mobile genetic elements, especially integrons, facilitate the co-selection of antibiotic resistance genes in soil (Pal et al., 2015). Co-selection occurs when antibiotic exposure results in increased resistance to an environmentally absent antibiotic drug class. Co-selection can be explained through two main processes: i) cross-resistance, when an antibiotic resistance gene is selected by an environmentally present drug class and 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 and Davies, 2015). Class 1 integrons, which are known to possess gene cassettes that are heavily biased towards conferring antibiotic resistance phenotypes (Ghaly, Geoghegan, Tetu, et al., 2020; Y. 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). Furthermore, 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).

1.4.4 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; Ruiz-Martínez et al., 2011; Gillings, 2018). Class 1 integrons typically carry less than six and no more than eight gene cassettes (Gillings, 2014; Naas et al., 2001). Class 1 integrons 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, Partridge, and R. M. Hall, 2021). The “clinical” or “*sul1*-type” variant of class 1 integrons has a 3’ conserved segment with a partially deleted but semi-functional disinfectant resistance gene *qacEΔ1*, followed by the sulfonamide antibiotic resistance gene *sul1* (Figure 1) (Partridge, Kwong, et al., 2018). 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; M. S. Wright et al., 2008; Stalder et al., 2014), iii) their gene cassette content is biased towards conferring antibiotic resistance phenotypes (Y. Yang et al., 2021), iv) some antibiotics indirectly increase the transcriptional activity of *intI1*, thereby promoting gene cassette

recombination (Baharoglu, Bikard, and Mazel, 2010), 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, Tien, 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 (*Streptococcus* and *Mycoplasma*), campylobacteriosis (*Campylobacter jejuni*, *C. coli*), and as alternatives for individuals allergic to beta-lactams (Public Health Agency of Canada, 2020; Capelo-Martínez and Igrejas, 2019). Despite their prolific use in human medicine, most macrolide antibiotics that are sold are consumed by food-producing animals for chemotherapy and prophylaxis (Capelo-Martínez and Igrejas, 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 (Resistance, 2017; Public Health Agency of Canada, 2020). Risk management strategies that focus on reducing macrolide presence in the environment will mitigate future risks to human health.

1.5.2 Structure

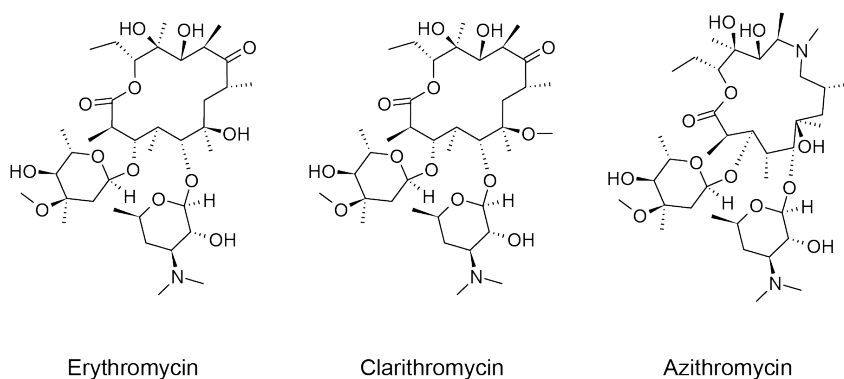


Figure 2 Chemical structures of erythromycin, clarithromycin, and azithromycin.

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 and 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) (Capelo-Martínez and Igrejas, 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 (Capelo-Martínez and Igrejas, 2019; Fyfe et al., 2016). This mechanism is usually bacteriostatic — the macrolides alone do not kill all of the bacterial cells and the host's immune system must clear the remainder of the infection (Pankey and Sabath, 2004). Macrolide antibiotic resistance mechanisms in bacteria are diverse (Fyfe et al., 2016). Resistance can be evolved through target site mutation in the 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).

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, Tien, 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 antibiotic resistance genes that were increased 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, Tien, et al., 2020). Macrolide antibiotics are also more rapidly degraded in soil with a previous exposure history to macrolides, indicating that macrolides may have an effect on soil microbial diversity and composition (Topp et al., 2016). This effect could be ecotoxic in nature and could represent a threat to agricultural productivity (Prashar, Kapoor, and Sachdeva, 2014).

1.6 Biosolids as a vector for macrolide antibiotic pollution of soil

Macrolide antibiotics 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 removes greater quantities of macrolides than other treatments, and the focus of this treatment is on disinfection rather than the removal of 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 also a hotspot of antibiotic resistance genes and mobile genetic elements (Rizzo et al., 2013; Che et al., 2019). Macrolide antibiotics from wastewater effluent can contaminate soil through the agricultural use of treated sewage sludge (McClellan and 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 (McClellan and Halden, 2010; Sabourin et al., 2012; Chenxi, Spongberg, and Witter, 2008). 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 under the One Health framework (Lau, Tien, et al., 2020; Sabourin et al., 2012).

1.6.2 Concentrations of macrolide antibiotics in biosolids and comparison to PNEC

In a survey of 74 locations producing treated biosolids in the United States, the 95th percentile concentrations of detected macrolides were 0.1 mg kg⁻¹ biosolids (dry weight) 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% dw dw⁻¹ soil, meaning that the upper-range of environmentally relevant concentrations for these macrolides in biosolids-applied soil would be 0.001 mg kg⁻¹ erythromycin, 0.002 mg kg⁻¹ clarithromycin, and 0.032 mg kg⁻¹ azithromycin (Sidhu et al., 2021). These concentrations are equal to, 8-fold, and 128-fold greater than the

Predicted No-Effect Concentrations (PNEC) for erythromycin, clarithromycin, and azithromycin in surface water, as determined by Bengtsson-Palme and Larsson, 2016. The PNEC is the concentration above which antibiotic resistance could be selected for in environmental bacteria and have been proposed as limits for the regulation of antibiotics in the environment. The 95th percentile concentrations of macrolides in municipal biosolids are equal to or exceed for **freshwater**, and biosolids could therefore realistically select for antibiotic resistance in land-applied soil.

Ed: I used Johan's paper for estimation of the PNECs, but I believe these estimates were for surface water. I found an alternative resource for PNECs which apply an equation to the surface water value to estimate the soil PNEC (NORMAN Ecotoxicology Database, <https://www.norman-network.com/nds/ecotox/>). This database was used by a paper that Thomas Berendonk was on (<https://doi.org/10.1016/j.envint.2020.105597>). Do you think I should use this database over Johan's values?

1.6.3 Critical knowledge gaps

The land-application of biosolids introduces antibiotics into agricultural soil that have carried over from the wastewater treatment process (McClellan and Halden, 2010; Sabourin et al., 2012). These antibiotics are present at concentrations that are predicted to select for resistance in the soil bacterial community (U.S. Environmental Protection Agency, 2021; Bengtsson-Palme and Larsson, 2016), 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, Tien, et al., 2020). This antibiotic exposure is also known to co-select for resistance to anthropogenically absent drug classes of antibiotics and resistance genes that are known to be associated with class 1 integron gene cassettes (Lau, Tien, et al., 2020).

The effects of macrolide antibiotic exposure on the soil bacterial community and the integron gene cassette metagenome remain 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. Because the health of humans and soil are interconnected under the One Health framework (Tiedje et al., 2019), and because biosolids are a vector for the introduction of macrolide antibiotics into the environment (Sabourin et al., 2012; McClellan and Halden, 2010), we must determine the consequences of long-term macrolide exposure on the development of antibiotic resistance in soil bacteria in order to assess if the repeated use of biosolids

in agriculture may pose a risk to human health.

1.7 Review of sequencing-based methods

1.7.1 16S rDNA sequencing

Most soil bacteria are uncultivable: 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 and Nunan, 2014; van Pham and 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 (van Elsas, 2019, 229–300). 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, Allison, et al., 2019; Isobe, Bouskill, et al., 2020). Of the different sequencing-based approaches available to investigate soil bacterial community composition, 16S rDNA sequencing and metagenomic sequencing are presently the most common.

16S rDNA sequencing involves the targeted amplicon sequencing of the 16S rRNA gene. The bacterial 16S rRNA gene is used to determine bacterial taxonomy due to i) regions of highly conserved sequence between bacterial species and ii) hypervariable regions which allow for species-specific classification (van Pham and 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 (B. Yang, Wang, and Qian, 2016), though advances in long-read technologies have made full-length 16S rDNA sequencing an attractive alternative (Shin et al., 2016; Numberger et al., 2019). First, total genomic DNA is isolated from the soil sample and the hypervariable regions (the 16S rDNA) of the bacterial 16S rRNA gene are PCR amplified using site-specific primers. Next, a DNA library is prepared from the resulting amplicons and the library is subsequently sequenced. Finally, the biological sequence data that are generated from the sequencer are analyzed using bioinformatics software. Taxonomic classification software such as QIIME 2 can cluster sequence reads based on dissimilarity thresholds into operational taxonomic units, or software such as DADA2 can attempt to infer biological sequences prior to PCR and sequencing to construct amplicon sequence variants (Bolyen et al., 2019;

Callahan, McMurdie, Rosen, et al., 2016). The use of amplicon sequence variants over operational taxonomic units is preferred, as sequence variants attempt to deal with sequencing errors and better reflect the DNA that was actually sequenced (Callahan, McMurdie, and Holmes, 2017).

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. In metagenomic sequencing, total genomic DNA is isolated from the soil, a DNA library is prepared from the total genomic DNA, and the DNA library is then sequenced. Metagenomic sequencing has several advantages over 16S rDNA sequencing for determining bacterial community composition: 16S rDNA sequencing suffers from primer bias during PCR, as the primers amplify different ribosomal sequences with different efficiencies, resulting in a bias of sequence reads to taxa with rRNA genes that are more similar to the primer-binding site (Tremblay et al., 2015). In addition, metagenomic sequencing generates data covering multiple genes and possibly entire bacterial genomes, allowing for a metagenomic functional analysis in addition to taxonomic analysis (D. 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 in order to achieve a detailed picture of the bacterial community (Scholz, Lo, and Chain, 2012).

Metagenomic sequencing can also be used to identify antibiotic resistance genes and mobile genetic elements within a bacterial community (Boolchandani, D'Souza, and Dantas, 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, D'Souza, and Dantas, 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 sequencing

Integrations 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 integrations or could be made more sensitive to detect novel classes of integrations. 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 integrations by identifying *intI1* wouldn't capture the diversity of the hundreds of other known classes of integrations (Altschul et al., 1990; Buchfink, Xie, and Huson, 2015). The targeted amplicon sequencing of integron gene cassettes is a PCR-based approach that can be used 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 integrations, and similar to 16S rDNA amplicons, these amplicons can then be sequenced and analyzed using bioinformatics software for antibiotic resistance gene identification (Y. Yang et al., 2021; Ghaly, Geoghegan, Alroy, et al., 2019). Cassette-embedded genes could also be assigned more general functions using databases of orthologous groups such as eggNOG (Huerta-Cepas et al., 2019).

1.7.4 Compositional data analysis

Much statistical software has been developed to help identify biologically meaningful differences in the diversity and compositions of groups from sequence data. For example, DESeq and edgeR both accept a matrix of samples versus counts as input (also known as a feature table) and then attempt to identify differentially abundant features between groups of samples (e.g. treatments) in the table (Anders and Huber, 2010; M. D. Robinson, McCarthy, and Smyth, 2010). This feature table could describe the counts of any genomic feature of interest, including bacterial amplicon

sequence variants, antibiotic resistance genes, mobile genetic elements, or cassette-embedded genes. DESeq and edgeR both assume that sequence reads can be normalized based upon sequence depth (conversion of counts to proportions); however, sequence data is compositional by nature, as sequencing instruments have constrained capacities to sequence samples, and therefore generate counts that can themselves be described as proportions of a constrained, unknown sum (Gloor et al., 2017). More recently, bioinformatics tools such as ALDEx2 and ANCOM with Bias Correction (ANCOM-BC) have been developed which use statistical techniques that are appropriate for identifying differentially abundant features in sequencing datasets (Fernandes et al., 2014; Lin and Peddada, 2020). This software can be used to investigate differences in the compositions of sequence datasets that are relevant to the analysis of soil microbiomes.

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 remained to be determined, as did the potential for these effects to occur at an environmentally realistic dose for a biosolids land-application scenario (Lau, Tien, 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, we obtained soil DNA from field plots treated with macrolide antibiotics for ten years and from untreated plots. The 16S rDNA and class 1 integron gene cassettes were PCR amplified and sequenced, and the total soil metagenome was sequenced.

I hypothesized 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, would affect the composition and diversity of the soil bacterial community, resistome, and mobilome.

I predicted that:

1. Antibiotic resistance genes and mobile genetic elements would increase in response to antibiotic exposure,

2. Bacterial community composition and diversity would differ between antibiotic-exposed and -unexposed soil, and
3. Integron gene cassette composition and diversity would differ between antibiotic-exposed and -unexposed soil.

Chapter 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 m² 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 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 99% 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 using the DNeasy PowerSoil Kit (Qiagen) and eluted in 100 μ L 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

2.3.1 Integron gene cassette 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* recombination sites of gene cassettes (Figure XX). Total genomic DNA isolated from the microplot soils were diluted 10-fold in Tris-EDTA buffer and used as template DNA for five technical replicates of 25 μ L PCR reactions (125 μ L 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 μ L of diluted template DNA, 0.25 μ L of Q5® High-Fidelity DNA Polymerase (New England BioLabs), 0.2 μ L of 25 mM dNTPs, 5 μ L of 5X Q5® Reaction Buffer, and 1.13 μ L of each 10 μ M 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 μ L of nuclease-free water. DNA concentration of the cleaned PCR product was determined using the Qubit™ dsDNA HS Assay Kit (Invitrogen).



A small diagram of where the primers anneal to within an integron (don't use the class 1 integron structure; just use a generic integron).

The Nextera® XT DNA Library Preparation Kit was used to prepare DNA libraries of the gene cassette amplicons for sequencing by following the manufacturer's protocol. The DNA libraries were indexed using the Nextera® XT Index Kit (Illumina)

following the tagmentation step. Bead purification of the DNA libraries was performed using a 1.8X bead-supernatant ratio of HighPrep™ PCR solution (MAGBIO Genomics), quantified using Qubit™ dsDNA HS Assay Kit (Invitrogen), and sized using the Agilent High Sensitivity DNA Kit on a Bioanalyzer 2100 (Agilent). Individual libraries were diluted to 10 nM in nuclease-free water and 15 µL of each diluted library were pooled together for multiplex sequencing. The pooled DNA library was sent to The Hospital for Sick Children in Toronto, Ontario for 2 x 125 bp sequencing on a HiSeq 2500 instrument (Illumina).

2.3.2 16S rDNA amplicon sequencing

For 16S rDNA sequencing, the total genomic DNA was diluted 10-fold in Tris-EDTA buffer and used as template for PCR amplification of the V3 and V4 regions of the bacterial 16S rDNA gene (Supplementary Table [A.1](#)). The MiSeq Reagent Kit v3 (600 cycle; Illumina) was used to prepare the amplicon libraries, and the libraries were indexed using the Nextera® XT Index Kit (Illumina) by following the manufacturer's protocol. The amplicon library was sent to the Canadian Food Inspection Agency (CFIA) in Ottawa, Ontario for 2 x 300 bp sequencing on a MiSeq instrument (Illumina).

2.3.3 Metagenomic sequencing

Only three of four biological replicates were used for downstream metagenomic sequencing. For metagenomic sequencing, the DNA concentrations of each sample were determined using the Qubit™ dsDNA HS Assay Kit (Invitrogen) and then sent to The Hospital for Sick Children in Toronto, Ontario for library preparation and shotgun sequencing across two lanes on a HiSeq 2500 instrument (Illumina).

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, Lohse, and Usadel, 2014). To denoise 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 (Callahan, McMurdie, Rosen, et al., 2016; Bolyen et al., 2019). 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 (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 clade (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, Karkman, 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 and 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 (D. 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 surviving gene cassette contigs, which were then clustered at 97% identity using CD-HIT (v4.8.1) to obtain unique open reading frames (Seemann, 2014; Fu et al., 2012).

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 ARGs, 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 and Eddy, 2013).

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

- High confidence: CARD-RGI 'strict' hits; Meta-MARC hits with E-value $\leq 1\text{E-}10$.

- 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 ≤ 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 a Cluster of Orthologous Groups (COG) functional category using the web implementation of eggNOG-mapper (v2.0) (Huerta-Cepas 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 in Python (v3.9.2) unless otherwise stated (Python Software Foundation, [n.d.](#)). Data visualizations were generated using plotly (v4.14.3) and matplotlib (v3.4.1) packages and were exported for editing in Adobe Illustrator 2020 (Plotly Technologies Inc., [2015](#); Hunter, [2007](#); Adobe Inc., [2020](#)).

2.5.1 Alpha diversity

Alpha diversity (within-group diversity using the Chao1 richness estimator) was computed using the sci-kit 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 (Pauli 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 feature table of abundances (taxa, genes, open reading frames) prior to center log ratio (CLR) transformation to obtain samplewise Aitchison distances — or CLR-transformed relative abundances. A PCA was performed on the resulting table of CLR-transformed relative abundances to investigate differences in antibiotic resistance gene, mobile genetic element, integron gene cassette open reading frame, and bacterial community composition between treatment groups using the sci-kit learn package (v0.24.1). Permutational multivariate ANOVA (PERMANOVA) was used to determine if differences in the dispersion between treatment groups within the PCA was 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 was statistically significant using ANCOM-BC (v1.2.0) with Holm-Bonferroni correction as implemented in R (v4.1.0) (R Core Team, [2021](#); Lin and Peddada, [2020](#)). 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 was statistically significant as implemented by the SciPy package (Pauli Virtanen et al., [2020](#)).

Chapter 3

Results

Bacterial 16S rDNA, metagenomic DNA, and integron gene cassettes were sequenced from total genomic DNA obtained 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.

Vera: "Should start with overview of the sequence data that you generated, from your three sources (integron, 16S, metagenome). Then provide overview description of what info was extracted from these data. You have a lot of data, so I think would be very helpful for readers to know what is coming, what type of comparisons were made (e.g. comparing richness, differences in community composition of ARGs, and taxonomically, etc...) by treatment level relative to control." I implemented your suggestion to provide a brief overview of the data that were generated at the beginning of this section, but I decided to make a separate section 3.1 to introduce the purpose of each dataset before describing the sequencing statistics.

3.1 Sequencing statistics

Bacterial 16S rDNA was 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 $50.7 \pm 12.8 \text{ K}$ unique reads per sample over 12 samples ($n = 4$ for each treatment group). 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 (2019-SEQ-0924) 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, $F = 2.3$, $p = 0.16$) (see [Supplementary File 1](#) for more information on sequencing statistics). The resulting 16S rDNA sequence reads were used to establish amplicon sequence variants which were taxonomically classified, and the abundance of soil bacterial taxa were obtained.

Next, metagenomic DNA was sequenced to investigate differences in the soil bacterial community, antibiotic resistance gene, and mobile genetic element composition and diversity in response to antibiotic exposure. Metagenomic HiSeq sequencing generated 1.49 B reads with an average of 153 ± 18.7 M unique reads per sample over nine samples ($n = 3$ for each treatment group). The resulting metagenomic sequence reads were i) taxonomically classified to obtain a second set 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 ± 0.18 M unique reads per sample. 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 (50%) 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

At the phylum level, the relative abundance of Cyanobacteria was decreased in the high-dosed soil (Holm-Bonferroni-adjusted p -value < 0.001 , $W = -5.7$) but not in the low-dosed soil, relative to the control. This result was observed only in the metagenomic analysis (Figure 3) and not in the 16S rDNA analysis (Supplementary

Figure A.1). This difference was driven by the cyanobacteria *Microcoleus vaginatus* ($p < 0.001$, $W = -5.2$) and *Oscillatoria nigro-viridis* ($p < 0.001$, $W = -5.3$) in the high-dosed soil. 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 and metagenome taxonomic datasets. 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; (Figure XX)).

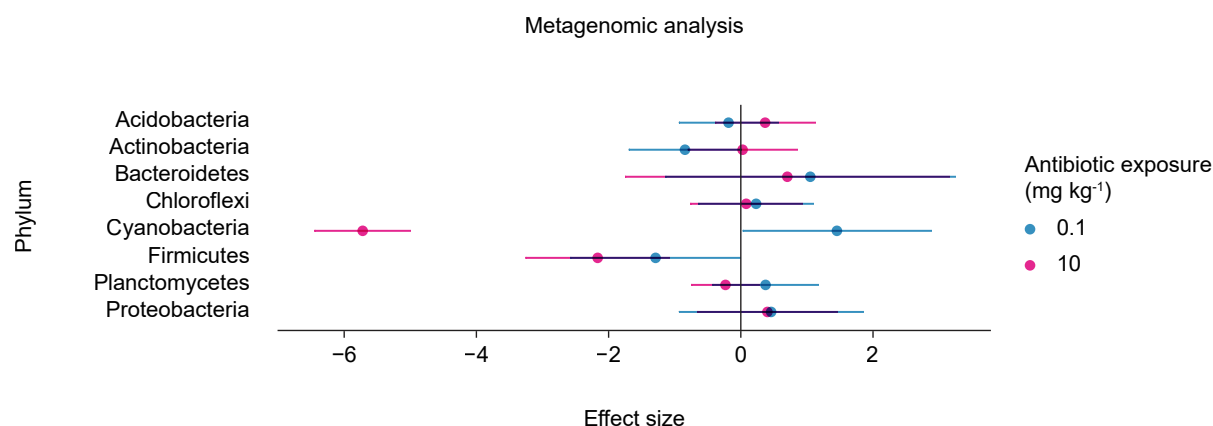


Figure 3 Effect sizes (fold-changes) of differences in the relative abundances of bacterial phyla classified in the metagenomic analysis relative to the untreated control soil ($n = 3$). Horizontal lines intersecting with circles are error bars, indicating the extent of Bonferroni-adjusted 95% confidence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

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 4a; Supplementary Figure A.3). Similarly, high exposure but not low exposure changed the composition

Table 1 Effect sizes 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, as identified by ANCOM-BC in the 16S rDNA analysis (16S) or metagenomic analysis (M). No taxa were identified as differentially abundant by both analyses.

Vera: I changed all mention of "ASVs" and "species" in this table to "taxa" to avoid confusion, as MetaPhlAn3 does classify taxa to the species-level whereas QIIME 2 does not.

Differentially abundant bacterial taxon	Effect size	Adjusted <i>p</i> - value	Analysis (M, 16S)
0.1 mg kg⁻¹			
<i>Mycolicibacterium tusciae</i>	-7.08	1.59E-10	M
<i>Sphingomonas</i> sp. Leaf20	4.51	7.17E-04	M
Unknown <i>Gaiella</i> sp.	4.4	2.82E-02	M
Unknown Acidobacteria Subgroup 6 sp.	45.79	0	16S
10 mg kg⁻¹			
<i>Arthrobacter globiformi</i>	-5.23	1.87E-05	M
<i>Arthrobacter</i> sp. Leaf69	-5.56	2.99E-06	M
<i>Microcoleus vaginatus</i>	-5.22	2.01E-05	M
<i>Oscillatoria nigro-viridis</i>	-5.29	1.34E-05	M
<i>Ramlibacter</i> sp. Leaf400	-7.82	5.89E-13	M
Unknown Chloroflexi Gitt-GS-136 sp.	23.02	6.95E-114	16S
Unknown Chloroflexi Gitt-GS-136 sp.	58.58	0	16S

of antibiotic resistance genes in soil bacteria (PERMANOVA pseudo- $F = 1.49$, $p < 0.05$, 999 permutations) (Figure 5a). These differences in composition were largely driven by 21 increased ARGs in the high dosed soil ($p < 0.05$) (Figure 6). Only five ARGs were differentially abundant (two decreased, three increased) in the low-dosed soil and no ARG was differentially abundant in both treatment groups.

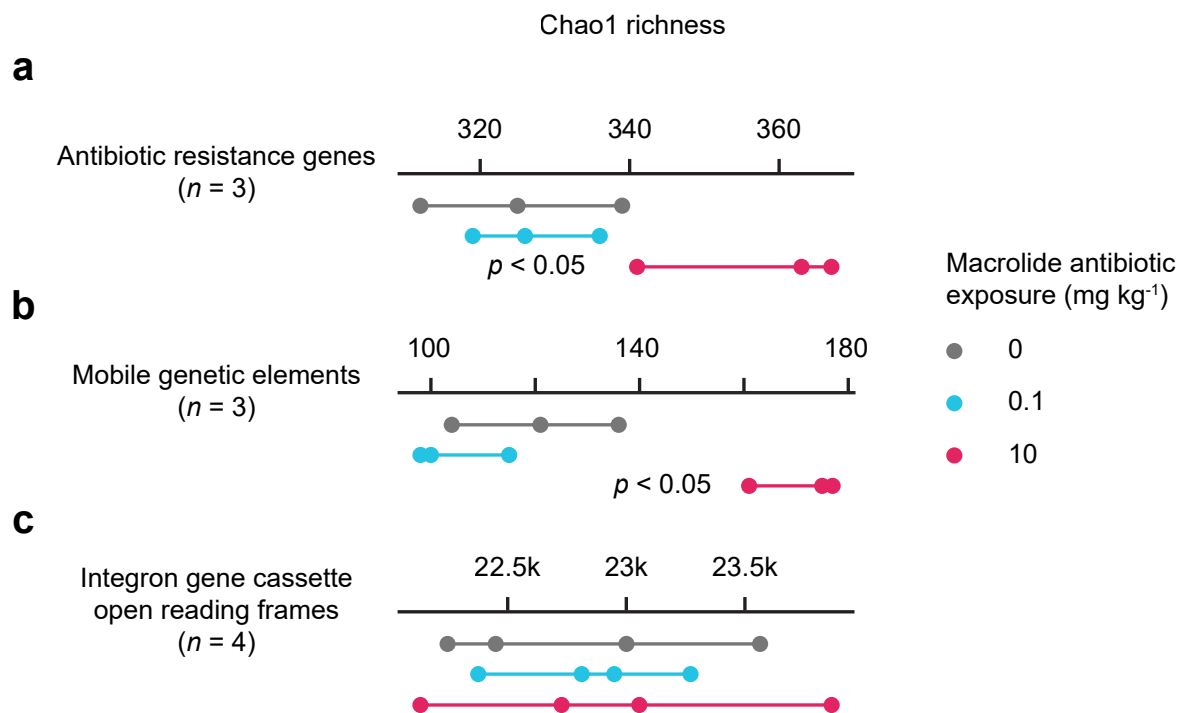


Figure 4 Richness (Chao1) of **a**) antibiotic resistance genes, **b**) mobile genetic elements, and **c**) integron gene cassette open reading frames from bacteria in macrolide antibiotic-exposed and -unexposed soil. Statistically significant comparisons between the antibiotic-exposed and untreated control soil are displayed (Kruskal-Wallis test, $p < 0.05$).

Vera: I decided to avoid the use of asterisks as significance indicators here and kept the legend the same for consistency with the other plots.

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 7). Sixteen of these ARGs 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 largest effect size of the antibiotic

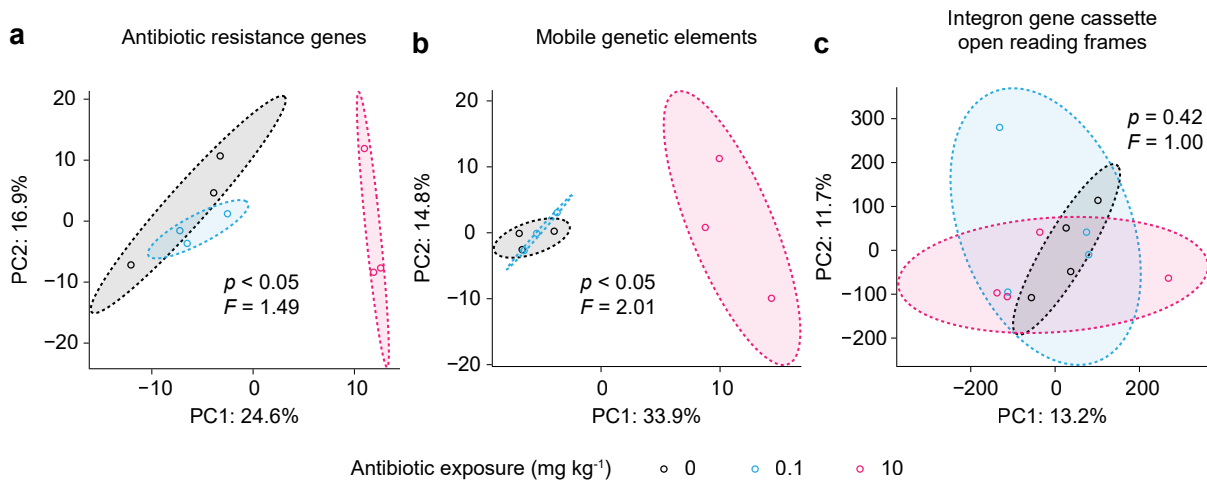


Figure 5 PCA ordination plots (PC1, PC2) of the CLR-transformed relative abundances of **a)** metagenomic antibiotic resistance genes, **b)** metagenomic mobile genetic elements, and **c)** integron gene cassette open reading frames in antibiotic-exposed and -unexposed soil bacteria. PERMANOVA pseudo- 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.

resistance genes that had increased relative abundances in the high dose was $W = 22.9 \pm 0.5$ for *aph(3'')-Ib / strA* ($p < 0.05$).

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 8). 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 ARGs had increased relative abundances in both doses.

Seven ARGs had significantly decreased relative 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 ARGs were predicted to encode beta-lactamases (Figure 6). *bla*_{SHV-71} ($W = -24.1 \pm 0.3$), *bla*_{SHV-165} ($W = -11.0 \pm 0.3$), *bla*_{CTX-M-117} ($W = -7.6 \pm 0.5$), *E. coli ampC* ($W = -5.7 \pm 0.4$), and *bla*_{PEDO-1} ($W = -4.4 \pm 0.2$) were decreased in the high dose, and *bla*_{TEM-1} ($W = -4.9 \pm 0.3$) and *bla*_{TEM-22} ($W = -4.1 \pm 0.8$) were decreased in the

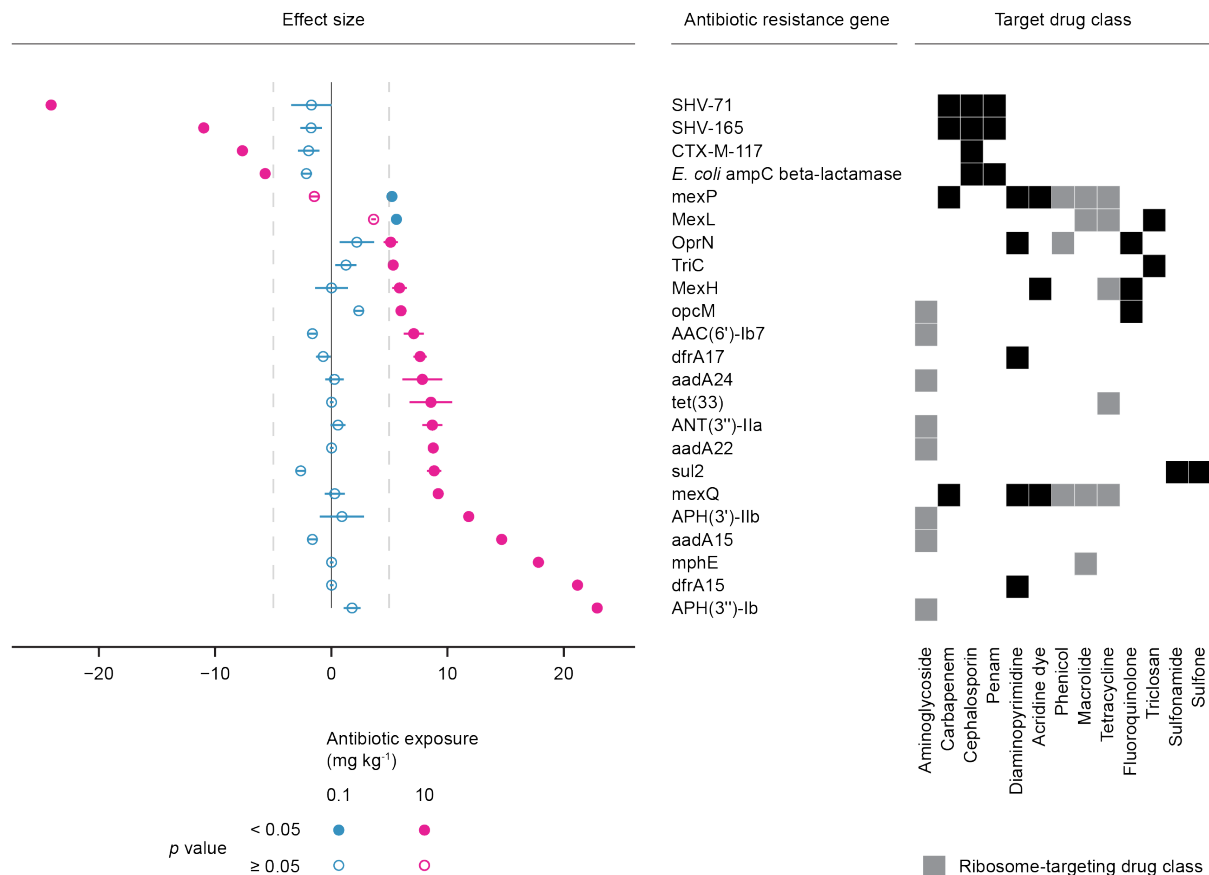


Figure 6 **a)** Effect sizes (fold-changes) of differences in the relative abundances of antibiotic resistance genes in antibiotic-exposed soil metagenomes and **b)** their target drug classes relative to the untreated control soil ($n = 3$). **a.** Only the genes that were differentially abundant ($p < 0.05$) 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.** Black squares indicate target drug classes for which resistance is predicted for each antibiotic resistance gene, and grey squares indicate that resistance to a ribosome-targeting drug class is predicted.

Ed: Hopefully my modifications to the text (with a lot of help from Vera) clarified that metagenomic ARGs were not subject to the confidence-level approach that we used for the integron gene cassette ORFs — All of the ARG hits in this plot are of quite high-confidence. Re: *mexP* and other ARGs with multi-drug resistance in the heatmap plot, this information is all determined by CARD (e.g. <https://card.mcmaster.ca/ontology/40350>). Re: The ARGs associated with quinolones being identified as ribosome-targeting, I think you may be looking at the tetracycline column to the left of the quinolones. I could add a grid to this heatmap to help with visually discerning the columns.

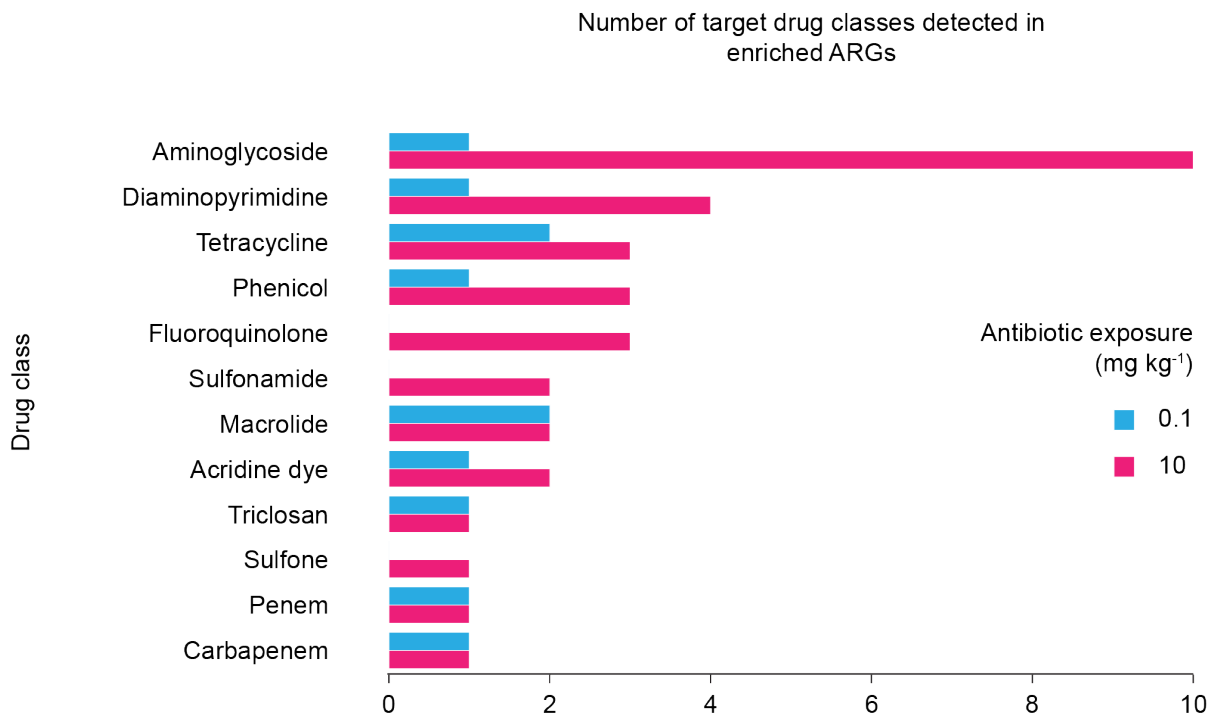


Figure 7 Counts of target drug classes for which resistance was predicted to be encoded by antibiotic resistance genes within the soil metagenome. Only counts for antibiotic resistance genes that were enriched in response to macrolide antibiotic exposure are displayed ($p < 0.05$, $n = 3$).

Ed: I changed the title of this plot to clarify the meaning of the counts here. Re: "So there were 10 aminoglycoside genes that were more abundant in the high rate relative to the control, for example?" Yes, there were 10 aminoglycoside resistance genes that were more abundant in the high rate relative to the control, here. Re: "Here and elsewhere, does the conservative vs liberal CARD approach need to be spelled out?" Please see my comment under Figure 6.

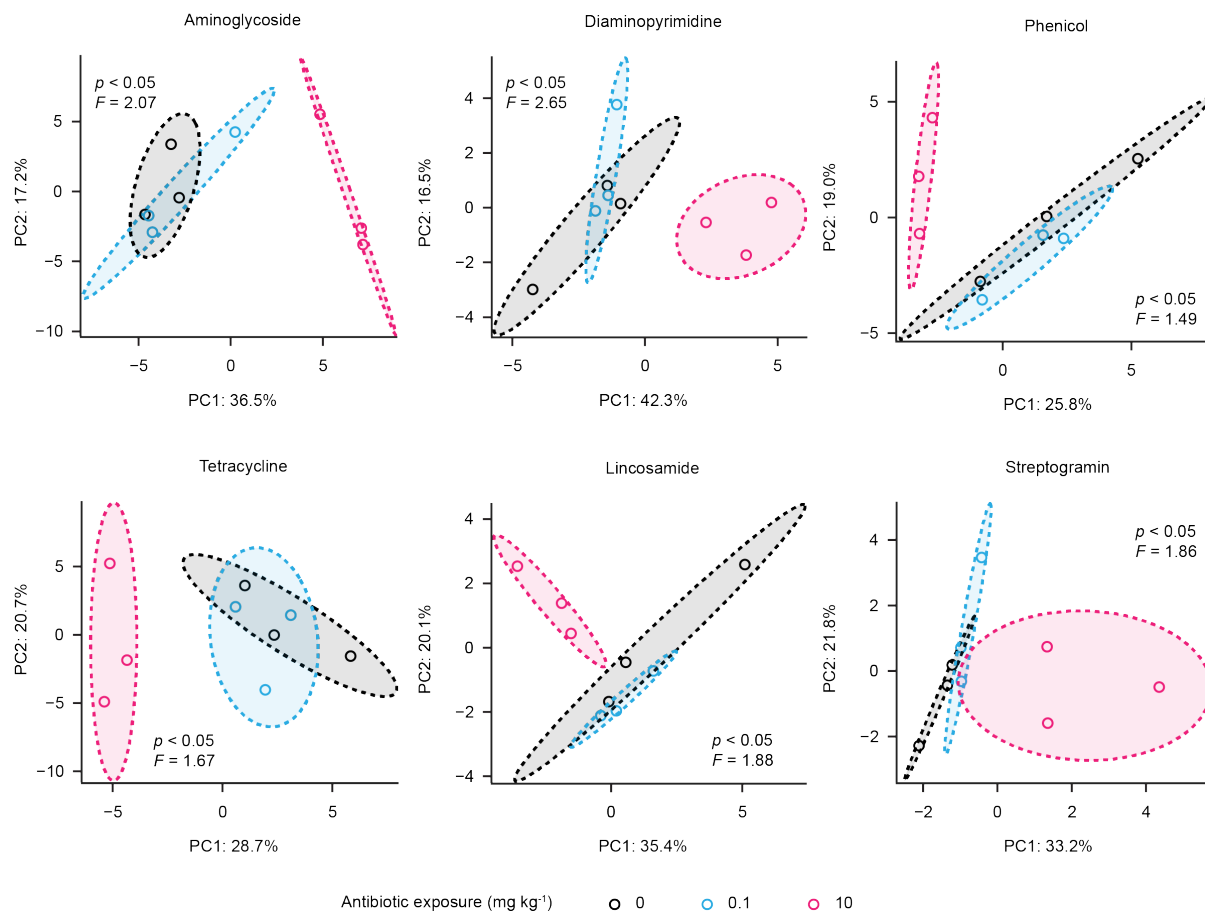


Figure 8 PCA ordination plots (PC1, PC2) of CLR-transformed relative abundances of antibiotic resistance genes in the untreated control soil and in the low- and high-dosed soil, grouped by their target drug class. PERMANOVA pseudo- F and p -values with 999 permutations are displayed. Shaded areas correspond to 95% confidence ellipses of treatment groups. Percentages of variance explained by each axis are displayed in the axis titles.

Increase font size for this plot.

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 4b; Supplementary Figure A.3), 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 5b).

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 9). Of these 23 increased mobile genetic elements, 15 were identified as *tnpA*, three as *intI1*, three as *qacEΔ1*, 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 9). Of the three mobile genetic element variants that were 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 Integron gene cassette composition and diversity

Integron gene cassette amplicon sequencing generated 9.83 Gb of reads with an average of 0.94 ± 0.18 M unique reads per sample. 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 35,809 (49%) were considered unique. Integron gene cassette amplicon short reads were mapped back onto the unique gene cassette open reading frames to obtain relative abundances.

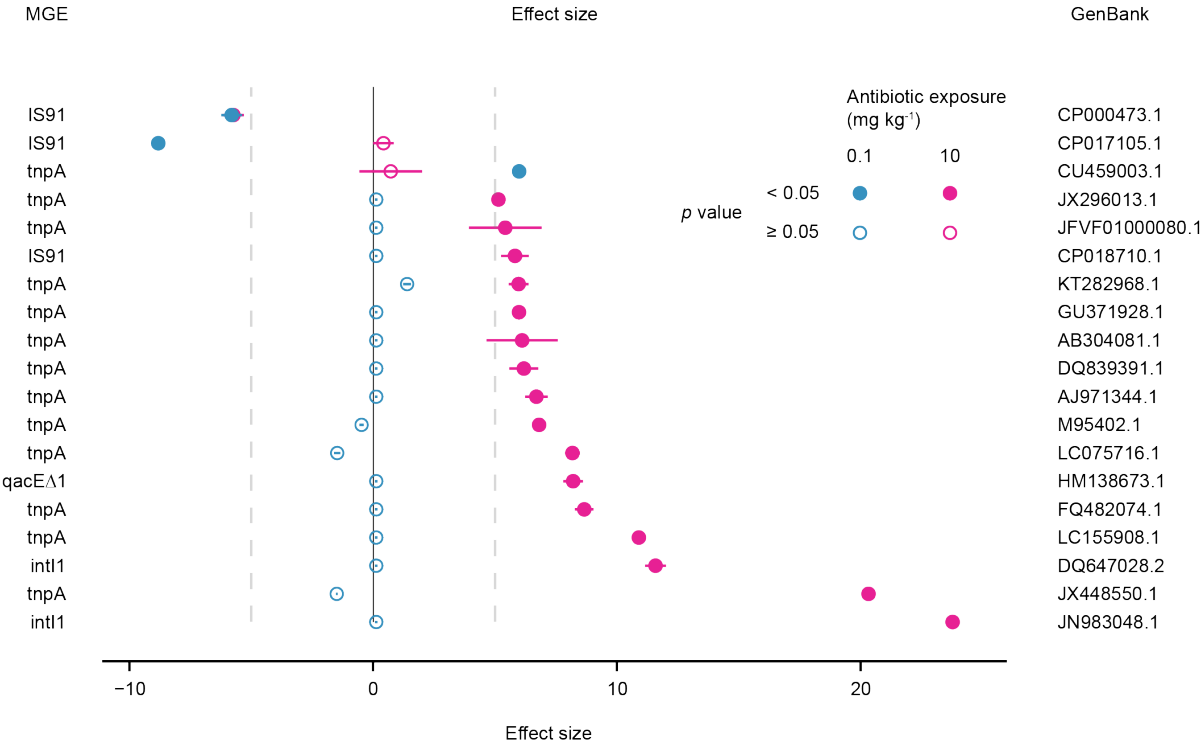


Figure 9 Effect sizes (fold-changes) of differences in the relative abundances of mobile genetic elements in antibiotic-exposed soil metagenomes relative to the untreated control soil ($n = 3$). Only the mobile genetic elements that were differentially abundant ($p < 0.05$) with an absolute effect size of at least 5 (vertical dashed bars), for either treatment group, are shown. The name of the mobile 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.

Vera: I changed the wording of this figure caption to hopefully clarify what the GenBank number is representing. Does this make sense / was this phrased correctly?

Increase font size of this plot.

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 4c; Figure 5c). Overall, 370 open reading frames (1%) were identified as differentially abundant relative to the untreated control soil ($p < 0.05$); similar percentages of differentially abundant gene cassette open reading frames were identified in the low-dosed ($n = 144$, 54%) and high-dosed ($n = 246$, 55%) soil bacteria.

The FDR of ANCOM-BC is probably close to 1% ... Would it be interesting/useful to test this?

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 2.4.3). For the antibiotic resistance genes predicated 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 XX). Additionally, no putative antibiotic resistance genes were increased or decreased in both treatment groups at any confidence level.

Design a table to summarize the results for each confidence level for the integron gene cassette ORF ARG analysis.

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 (14.5%) unique open reading frames could be assigned a functional category and 2,053 (5.7%) were assigned a non- 'function unknown' (S) category — i.e. 5.7% of identified open reading frames shared homology with a gene whose function had been characterized (Supplementary Figure XX). 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) were slightly decreased ($W = -3.6 \pm 0.8$, $p < 0.05$) 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, $n = 2$) (Supplementary Figure XX).

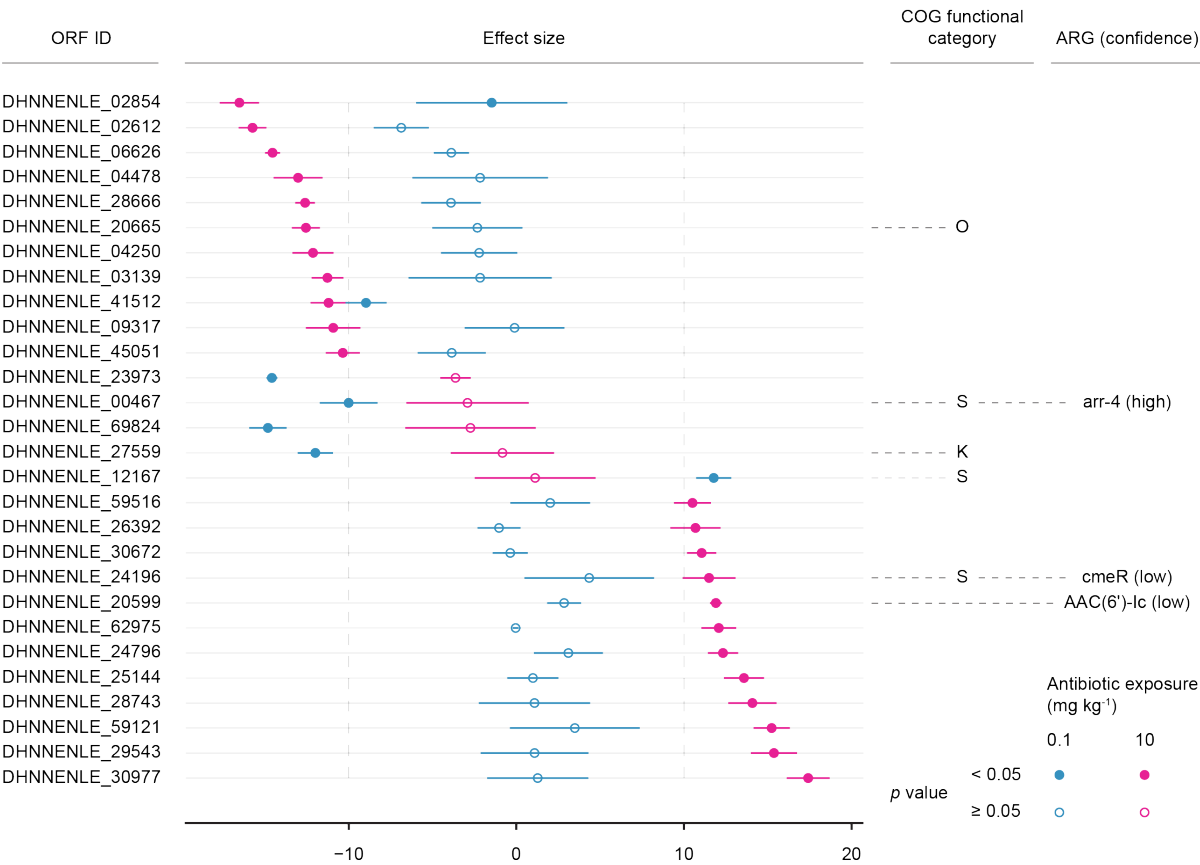


Figure 10 Effect sizes (fold-changes) of differences in the relative abundances of integron gene cassette open reading frames in antibiotic-exposed soil bacteria relative to the untreated control soil ($n = 3$). Only the open reading frames that were differentially abundant ($p < 0.05$) with an absolute effect size of at least 10 (vertical dashed bars), for either treatment group, are shown. The ID of the open reading frame is shown on the left and the assigned COG functional category is shown on the inner-right. If the open reading frame was classified as an antibiotic resistance gene under any confidence level, the name of the antibiotic resistance gene and the highest confidence level (low, moderate, or high) at which the gene was predicted is 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.



Supplementary Figure: Bar plot of counts of COG functional categories assigned to integron gene cassette open reading frames.

Chapter 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 under the One Health framework, and antibiotic resistance in soil bacteria may affect antibiotic resistance in the human microbiome or in pathogens.

I decided to present the no-effect results for the realistic dose first since I believe they're the most important for informing policy.

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

Ed: I re-introduced the 'realistic' and 'unrealistic' terminology back into some of the summarizing statements in this Discussion. Is this ok?

Overall, we detected 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. This dose is similar to what would be expected in soil following the land-application ($1\% \text{ dw dw}^{-1}$) 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

4.1. REALISTIC ANTIBIOTIC EXPOSURE DOES NOT AFFECT THE DIVERSITY OR COMPOSITION OF THE

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.

Ed: Do you think the above sentence is a fair statement? Could we be challenged by assuming to know what levels of antibiotic resistance are/aren't relevant to human health in the environment? I just want to make sure I'm not overstepping in my conclusions here.

Only 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 6). *mexL* and *mexP* are members of the *mex* gene family which are components of chromosomally encoded efflux pumps in *Pseudomonas* spp. (Mima, Sekiya, et al., 2005; Rungtip Chuanchuen 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 (Rungtip 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, Sekiya, 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 (Shaw et al., 1989; Partridge, Tsafnat, et al., 2009). Only one mobile genetic element variant, *tnpA*, was increased in the low-dosed soil (Figure 9). The *tnpA* gene encodes a transposase which is involved in the mobilization of bacterial mobile genetic elements such as transposons and insertion sequences across a broad host range (Partridge, Kwong, et al., 2018).

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 we did not detect any taxa that were both significantly increased and known to carry this assortment of genes. 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 — we detected significant effects on the diversity and composition of the soil bacterial resistome and mobilome. While this dose is considered unrealistically high for soil receiving biosolids with 95th percentile concentrations of macrolides, the maximum azithromycin concentration detected in the biosolids themselves (5 mg kg^{-1}) was within an order-of-magnitude of the high dose (U.S. Environmental Protection Agency, 2009). This high dose of macrolide antibiotics has also been detected in sediments surrounding macrolide antibiotic manufacturing facilities (González-Plaza et al., 2019). Therefore, while we note that this dose is 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 4a). 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.3a). The most likely explanation for the increased number of unique antibiotic resistance genes in the high-dosed soil is selection for antibiotic resistance genes that were below the detection limit in the control and low groups, but were raised above the limit of detection by high antibiotic exposure.

In addition, a high dose of macrolide antibiotics changed the composition of antibiotic resistance genes within the soil metagenome (Figure 5a), and this effect extended to when antibiotic resistance genes were grouped by several drug classes (Figure 8). 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

4.2. UNREALISTICALLY HIGH ANTIBIOTIC EXPOSURE ALTERS THE DIVERSITY AND COMPOSITION OF

abundances of 21 antibiotic resistance genes in the high dose — only two of which are known to confer resistance to macrolide antibiotics. The increased relative abundances of non-macrolide antibiotic resistance genes strongly suggests co-selection via co-resistance, 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 *qacEΔ1* (a component of the 3' conserved sequence) in the high dose (Partridge, Tsafnat, et al., 2009; Yan et al., 2006; Herrero et al., 2008). 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 gene cassette-associated antibiotic resistance genes (except *sul1*, which is a member of the 3' conserved sequence), integron gene cassette richness (Figure 4c) and composition (Figure 5c) were unaffected by antibiotic exposure. This may be because the transcription of *intI1*, which is responsible for acquiring, re-arranging, and excising gene cassettes, is regulated by the bacterial SOS response, and macrolide antibiotics (along with other protein synthesis-inhibiting drug classes) do not induce this response, thus the richness and composition would be unchanged (Baharoglu, Bikard, and Mazel, 2010).

Vera: Does this make sense to you? Could integron gene cassette-embedded antibiotic resistance genes be amplified in response to antibiotic exposure, detected in the metagenomic analysis, but not detected in the gene cassette analysis?

Of the remaining antibiotic resistance genes, 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* / *strA* 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*) (Hächler, Santanam, and Kayser, 1996; Mesaros et al., 2007; Mima, Sekiya, et al., 2005; Mima, Joshi, et al., 2007; Burns et al., 1996). 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.

The high dose of antibiotics similarly increased the number of unique mobile genetic element variants (Figure 4b) and altered the composition of the mobilome (Figure 5). 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.3b). Of the 23 mobile genetic element variants with increased relative abundances in the high-dosed soil (Figure 9), 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 associated with several types of mobile genetic elements were increased, and several mobile genetic elements were increased, it's plausible that multiple co-selection processes were active in the high-dosed soil simultaneously.

4.3 Unrealistically high antibiotic exposure decreased the abundance of Cyanobacteria

4.4 Low and high antibiotic exposure enriched for fastidious taxa

In this study, we detected increased relative abundances of three bacterial taxa in the low-dosed soil and two taxa in high-dosed soil (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 Subgroup 6 Acidobacterium taxon was over 45-fold more abundant in the low-dosed soil than the control. This taxon was present in both antibiotic treated groups but not in the control soil.

Acidobacteria are a largely uncultivated, highly abundant bacterial phylum in Canadian agricultural soil and play an important role in shaping the soil bacterial

community through their decomposition of organic carbon (Solden, Lloyd, and Wrighton, 2016; Banerjee, Baah-Acheamfour, et al., 2016; Banerjee, Kirkby, 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 (Yi et al., 2019; Milaković et al., 2020). Conservatively, the unknown Subgroup 6 Acidobacterium taxon may represent a macrolide-resistant decomposer, but more speculatively, could represent a species 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 Acidobacteria taxon.

For the high-dosed soil, the effect sizes for both increased taxa were high ($W > 0.20$) and both were identified as unknown Gitt-GS-136 Chloroflexi 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 role of Chloroflexi in antibiotic resistance remains understudied (Razavi et al., 2017).

Although we did not detect a significant effect of macrolide antibiotic exposure at either dose on the soil bacterial community composition or diversity, some taxa do 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 may have played a role in the accelerated biodegradation of these macrolide antibiotics.

4.4.1 Strengths and limitations

4.4.2 Policy implications

I'll go through all of these references and make sure formatting/names are correct after I'm finished adding all of my references to the thesis.

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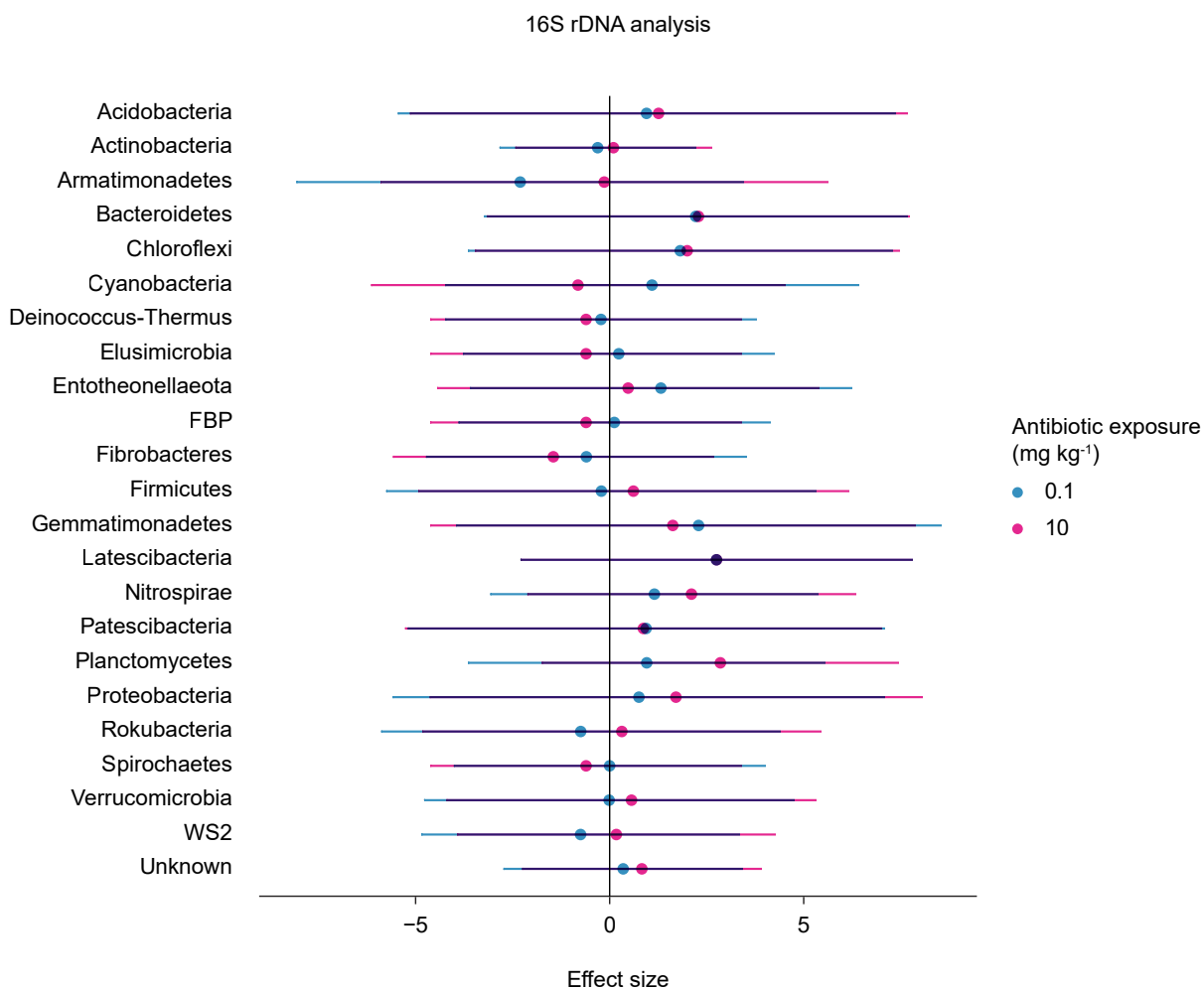
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Appendix A

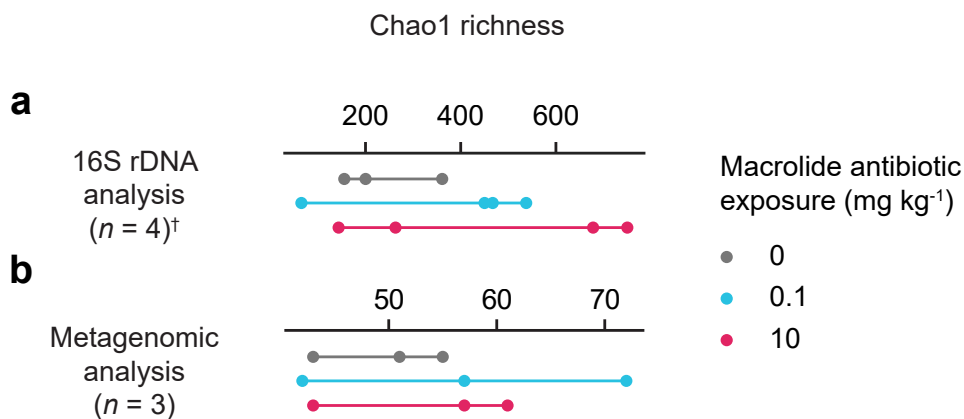
Supplementary Information

Target	Primer	Sequence (5' -> 3')	Amplicon size (bp)	Annealing temperature (°C)
<i>attC</i>	Adapter + HS286	TCGTCGGCAGCGTCAGATGTGTATAAG AGACAGTCGCTKGARCGAMTTGTTAG VC	Variable	55
	Adapter + HS287	GTCTCGTGGGCTCGGAGATGTGTATAA GAGACAGGCGCTKANCTCVRRCGTTA GSC		
16S rRNA	Adapter + S-D-Bact-0341-b-S-17	TCGTCGGCAGCGTCAGATGTGTATAAG AGACAGCCTACGGGNGGCWGCAG GTCTCGTGGGCTCGGAGATGTGTATAA	~ 460	55
	Adapter + S-D-Bact-0785-a-A-21	GAGACAGGACTACHVGGTATCTAATC C		

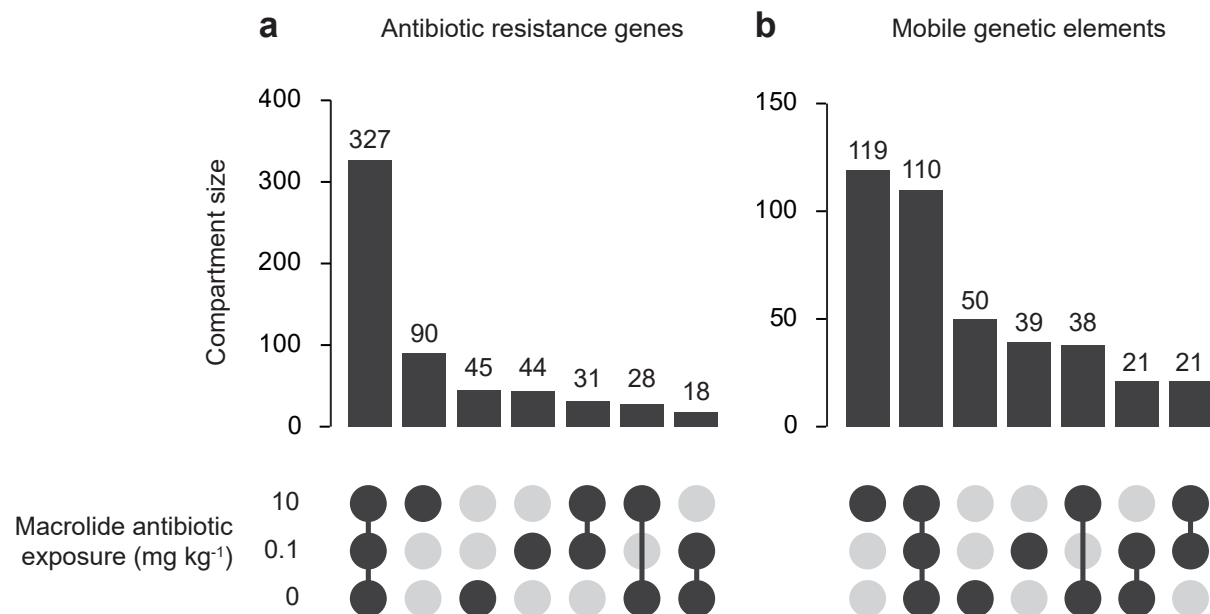
Supplementary Table A.1 PCR primer sequences, annealing temperatures, and expected amplicon sizes for integron gene cassette and 16S rDNA PCR amplification. Red text indicates location of adapter overhang sequences.



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 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, and lines are drawn between shaded dots for visual clarity.

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