Master of Science Thesis

The University of Western Ontario

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Chapter 1

Introduction

1.0.1 Describe a situation

Antimicrobial resistance (**AMR**) is the natural phenomenon by which microorganisms acquire defences against harmful chemicals within their environment. All microorganisms have the potential to acquire AMR under various environments, including terrestrial and aquatic ecosystems, agri-food, hospitals, and the human body. In the context of an infection, resistant microorganisms limit available treatment options. Acquired antimicrobial resistance is estimated to have caused 5,400 Canadian fatalities in 2015 — a number which is expected to climb to 13,700 deaths per year by 2050, while disproportionally affecting populations that are at a greater risk of acquiring infections (Council of Canadian Academies, 2019).

AMR is ancient and is prolific in the environment. AMR likely arose from competition between bacteria for natural resources. As Buongermino Pereira et al. (2020) has said...

Antimicrobial discovery and synthesis revolutionized the treatment of bacterial and fungal infections, but the overruse and misuse of antimicrobials in healthcare and agriculture has led to widespread AMR.

1.0.2 Describe a problem or question that arises from that situation

One pertinent problem is the introduction of antimicrobials into the environment, and the selective pressure that these antimicrobials impose on environmental microorganisms. The introduction of antibiotics, heavy metals, and biocides into the environment promotes AMR. These resistant microorganisms find their way into people and into the clinic.

Agriculture represents a pathway for antibiotics to enter the environment. Antibiotics are used to treat infections in farm animals, sometimes used as prophylactics, and were historically used as growth promoting substances (still used in some countries today).

Biosolids are used as agricultural fertilizer and are known to contain trace amounts of antibiotics. Biosolids are produced from the separation of wastewater into water and solids, followed by the treatment of the solids portion to reduce pathogens and odour. These biosolids may be treated using a combination of chemical, biological, or physical processes. The purpose of applying biosolids to agricultural soil is to improve the soil quality and fertility: Soil that is more fertile requires less inorganic fertilizer, which reduces the risk of runoff into adjacent water sources, and soil that has more organic matter has increased moisture retention and *better structure*. In Canada, biosolids are suitable for the growth of crops which require... such as cere-

als, hay, field corn, and soybeans (http://www.omafra.gov.on.ca/english/nm/nasm/info/brochure.htm#7). Currently, most wastewater treatment plants do not have technologies to remove micropollutants (such as antibiotics) from influent.

We previously determined that biosolids contain sub-mg/kg concentrations of macrolide antibiotics. Macrolide antbiotics are used in both healthcare and agri-food to treat infections, and have been deemed critically important by the WHO for their use as first-line and sole treatments of serious human bacterial infections. Macrolide antibiotic resistance is rising. There is a need to safeguard this antibiotic supply.

1.0.3 Describe how others have approached that problem or question

To investigate whether or not biosolids pose a risk for promoting AMR in the environment via the introduction of macrolide antibiotics into the environment, we performed an experiment investigating the consequences of long-term exposure of antibiotics on antimicrobial resistance gene (**ARG**) and mobile genetic element (**MGE**) abundance. The results of this experiment indicated that some ARGs and MGEs were increased in response to macrolide antibiotic exposure, but only at the unrealistically high concentration (10 mg/kg). Due to the technique used, it was unclear how the ARGs were increasing in the 10 mg/kg treated soil microplots. It was also unclear whether or not ARGs and MGEs that weren't pre-selected for quantitative PCR were actually elevated in the 0.1 mg/kg treatment (realistic exposure scenario) but remained undetected using this targeted technique.

1.0.4 Explain a need to approach it in a different way or expand upon what's been done

In order to investigate AMR in the environment without restricting one's self to using techniques that can only detect X ARGs and MGEs, we can use total metagenoic sequencing and targeted amplicon sequencing of informative DNA elements.

There is also a growing need to analyze this data using compositionally appropriate techniques (CoDA). Sequencing data is constrained by an unknown sum, and thus resides within a simplex. Non-Euclidean data cannot be analyzed using traditional statistical tests unless a transformation has been performed. Typically this transformation aims to... As the field rapidly evolves, a transformation to put the data back into Euclidean space is becoming the gold-standard for analyzing this data.

1.0.5 Say what you aim to do...

In order to determine if AMR was increased in the soil microplots that were exposed to 0.1 mg/kg antibiotics, and to investigate if other ARGs and MGEs were increased in 10 mg/kg-exposed microplots, we aimed to perform a comprehensive metagenomic investigation of the soil microplots, focusing on bacterial community composition and the abundance and diversity of ARGs and MGEs within the soil. This approach involved targeted amplicon sequencing of the 16S bacterial rDNA to investigate bacterial community composition, targeted amplicon sequencing of class 1 integrons to investigate MGE abundance and diversity, and total metagenomic sequencing to investigate both ARG and MGE abundance and diversity. We also aimed to analyze the data using recently 'remembered' CoDA techniques.

Chapter 2

Methods

2.0.1 Class 1 integron gene cassette sequencing

2.0.1.1 DNA isolation from soil microplots

Total genomic DNA was isolated from 2019 Day 30 post-application soil microplots using the DNeasy PowerSoil Kit (Qiagen) with an input of 250 mg of dry soil. The DNA was eluted in 100 uL of 10 mM Tris-HCl and Nanodrop readings were taken to assess quality. The isolated DNA was stored at -20°C until it was used as template for gene cassette PCR.

2.0.1.2 Gene cassette PCR

Class 1 integron gene cassettes were amplified using primers and thermocycling conditions described by Stokes et al. (2001), except with 33 and 34 bp oligonucleotides ligated onto the 5' ends (Figure **XX**). The purpose of these oligonucleotides was to provide more distance between the 5' end of the PCR product (lost during library preparation) and the desired gene cassette sequence. DNA from each soil microplot was used as template for five technical replicates of 25 uL reactions (125 uL total). PCR product was analyzed by gel electrophoresis and visualized under UV light. Technical replicates were pooled together and PCR product was purified using the GenepHlow PCR Cleanup Kit (Geneaid). Cleaned PCR product was quantified using the Qubit dsDNA HS Assay Kit (Fisher Scientific). PCR and cleanup were repeated as described above using the same template DNA for a total of two technical replicates for each soil microplot.

2.0.1.3 Library preparation

The Nextera XT DNA Library Preparation Kit (Illumina) was used to prepare the library for sequencing by following the manufacturer's protocol. The indexed PCR product was quantified using the Qubit dsDNA HS Assay Kit and sized using the High Sensitivity DNA Kit on a Bioanalyzer 2100 (Agilent). Individual libraries were diluted to 10 nM, and 15 uL of each diluted library was pooled into a single library and sent for 2 x 125 bp sequencing at Sick Kid's Hospital (Toronto, ON) on a HiSeq 2500 (Illumina).

2.0.1.4 Data processing

Adapters were trimmed using CUTADAPT (v2.10) and the sequence quality was assessed using FastQC (v0.11.9) and MultiQC (v1.8) (Martin, 2011; Andrews, 2010; Ewels et al., 2016). The adapter-trimmed reads were assembled into contigs using MEGAHIT (v1.2.9) with default options (D. Li et al., 2015). Contigs that didn't contain the terminal 9 bp of each primer sequence were removed using BBDuk with options

`copyundefined=t rcomp=f mm=f k=9`. After filtering out contigs that didn't contain the terminal primer sequences, the metagenomic assemblies of each sample were merged into a single assembly, and the open reading frames (**ORFs**) of the remaining contigs were called using Prokka (Bushnell, 2020; Seemann, 2014). The ORFs were concatenated into a non-redundant protein database at 97% identity using CD-HIT (vX.X) and then reverse translated back to nucleotides (W. Li and Godzik, 2006; Fu et al., 2012). To obtain fold-coverages, the adapter-trimmed reads of each sample were mapped back onto the concatenated nucleotide database of ORFs using BBMap (vX.X) (Bushnell, 2020). ORFs were then annotated using CARD-RGI with options `--low_quality --include_loose --clean -t protein` (vX.X) for ARGs and eggNOG-mapper (v2.0) for functional categories (Alcock et al., 2019; Huerta-Cepas, Forslund, et al., 2017; Huerta-Cepas, Szklarczyk, et al., 2019). CARD annotations with a percentage identity less than the mean percentage identity of all ARG hits, along with hits of less than 20% coverage to the subject ARG sequence were discarded.

2.0.1.5 Compositional data analysis and statistics

The fold-coverages of technical replicates were combined. A pseudocount of 0.1 was added to the resulting feature table prior to centred log-ratio (**CLR**) transformation, although we acknowledge that a non-zero count could lead to x, y, and z errors. The CLR transformation was performed using the base 2 logarithm, thereby producing relative abundances where the difference between two values is the fold-change between them. The Kruskal-Wallis test was used to determine if there were differences between the median relative abundances of class 1 integron gene cassettes ORFs between treatment groups ($P < 0.05$), followed by Dunn's post-hoc test for multiple comparisons ($P < 0.05$) with Bonferroni correction.

2.0.2 16S rDNA sequencing

Total genomic DNA was isolated exactly as described in 2.0.1.1 by Yuan-Ching Tien. The DNA was stored at -20°C until it was used as template for library preparation by Andrew Scott. The 16S rDNA library was prepared using the Illumina XX kit and was sent for 2 x 300 bp sequencing on an Illumina MiSeq at Canadian Food Inspection Agency in Ottawa, ON. The demultiplexed reads were quality controlled as described in 2.0.1.4, except trimming was performed using Trimmomatic v0.36 with options `LEADING:2 TRAILING:2 MINLEN:25 HEADCROP:25 CROP:250 SLIDINGWINDOW:4:15` (**ref**). The trimmed reads were imported into QIIME2 for denoising with DADA2 and classification with a classifier trained on the SILVA Ref NR 99 database (release 132)

(**ref**). The sample-ASV table was CLR-transformed and analyzed as described in 2.0.1.5, except a pseudocount of 0.5 was added to the table prior to CLR-transformation.

2.0.3 Total metagenomic DNA sequencing

Total genomic DNA was isolated using the DNeasy PowerMax Soil Kit (Qiagen) with an input of 10 g. The DNA was eluted in X mL of 10 mM Tris-HCl and Nanodrop readings were taken to assess quality. The DNA was quantified using the Qubit dsDNA HS Assay Kit (Fisher Scientific) and was sent for 2 x 150 bp total metagenomic sequencing on an Illumina HiSeq 2500 at SickKids Hospital in Toronto, ON.

Chapter 3

Results

3.0.1 Bacterial community composition

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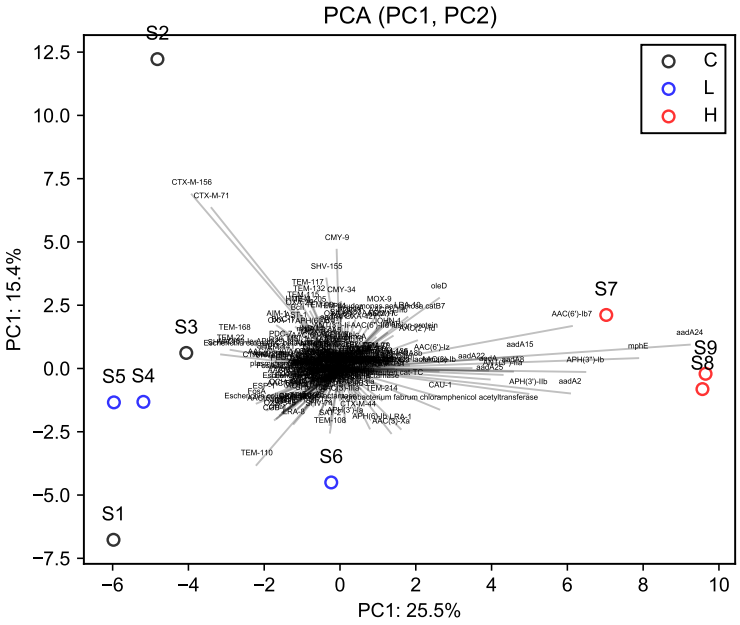

Figure 3.1 PCA ordination biplot of antibiotic target replacement mechanisms in soil unexposed (0 mg/kg) and exposed (0.1, 10 mg/kg) to macrolide antibiotics over ten years. A pseudocount of 0.1 was added to the fold-coverage table and fold-coverages were centered log-ratio (**CLR**) transformed to obtain relative abundances centered on the geometric mean of each sample.

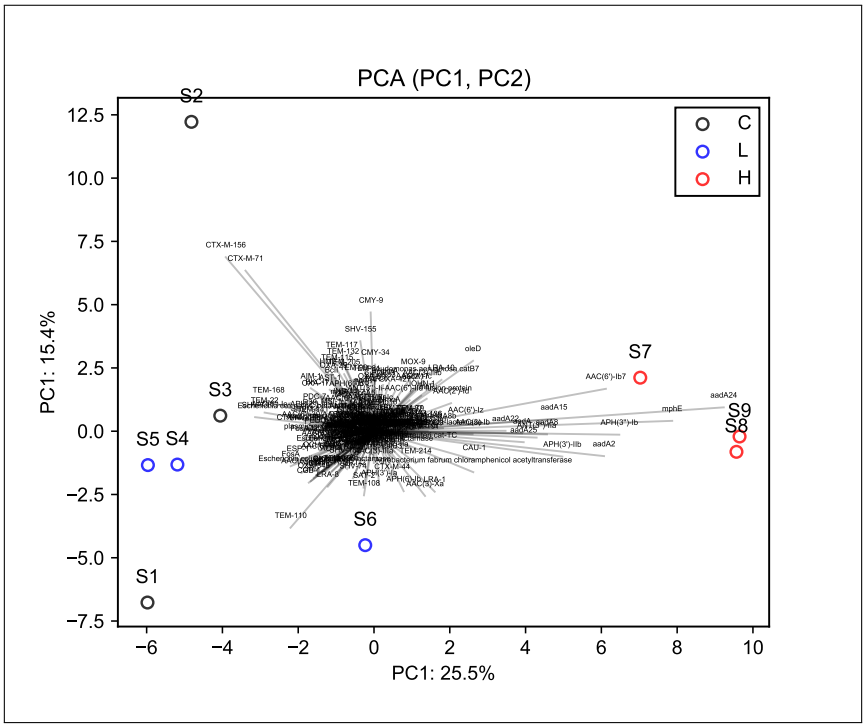

Figure 3.2 Same as 3.1 but with a boxed border.

Chapter 4

Discussion

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Chapter 5

Conclusion

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