Final Report for: Investigation of bacterial community structure and antibiotic resistance and genetic mobility gene abundance in soils fertilized with swine manure

E2016-08

Shannon Hinsa-Leasure- PI
Associate Professor of Biology
Grinnell College

Shannon Hinsa-Leasure
1116 8th Ave.
1207 Noyce Science Center
Grinnell College
Grinnell, IA 50112

641-269-4646
fax: 641-269-4984
Hinsa@grinnell.edu

Budget request- Year 1 and extended to a second year- $20,262

This is the final report for the E2016-08 project that ran from January 2016-December 2017.

Aims of this study

1. Quantify the relative amount of 7 antibiotic resistance and genetic mobility genes in manure and agricultural soil samples pre- and post- manure application. Samples will be collected prior to manure spread and at set intervals following manure application. Five genes will be the same as those used by Marti and colleagues and in addition we will test *tetM* and *cfiA,* which we have found to be present in our manure (30).

2. Characterize the bacterial community diversity in manure and agricultural soils pre- and post- manure application.

3. One medium-range outcome is to share this work with the scientific community to build a more complete picture of the fate of antibiotic resistance genes in the environment.

4. Another medium-range goal is to provide farmers and community members with our results to help them determine relative time needed between manure application and vegetable planting to decrease the spread of antibiotic resistance genes.

Due to impending legal actions at the farm we sampled at in the fall of 2015, we decided to move to a farm that was not involved in the lawsuit and hold the samples collected in 2015. We began collecting samples at the Lynneville/Sully farm in the fall of 2016. We collected soil samples prior to manure spreading on 11/08/16, manure during spreading on 11/12/16, and following manure application on 11/15/16, both manure line and soil between manure line, and tilled soil on 2/17/17 and 3/22/17. Samples were collected from the top 15 cm of soil, stored in sterile bags, and frozen upon return to the lab.

DNA was extracted individually from each sample in triplicate for qPCR analysis. This work was undertaken in the summer of 2017 by three undergraduate students and myself. After working through many technical challenges, we were able to gather reproducible qPCR results for *erm*B, *sul*I, *int*I, *erm*C, *str*B and *inc*W*rep*A. For each reaction were determined the amount of DNA present in the samples based on our standard control, which was each product in a vector tested at known concentrations. All samples were normalized for 16S rRNA. This work was completed from 02/17-12/17.

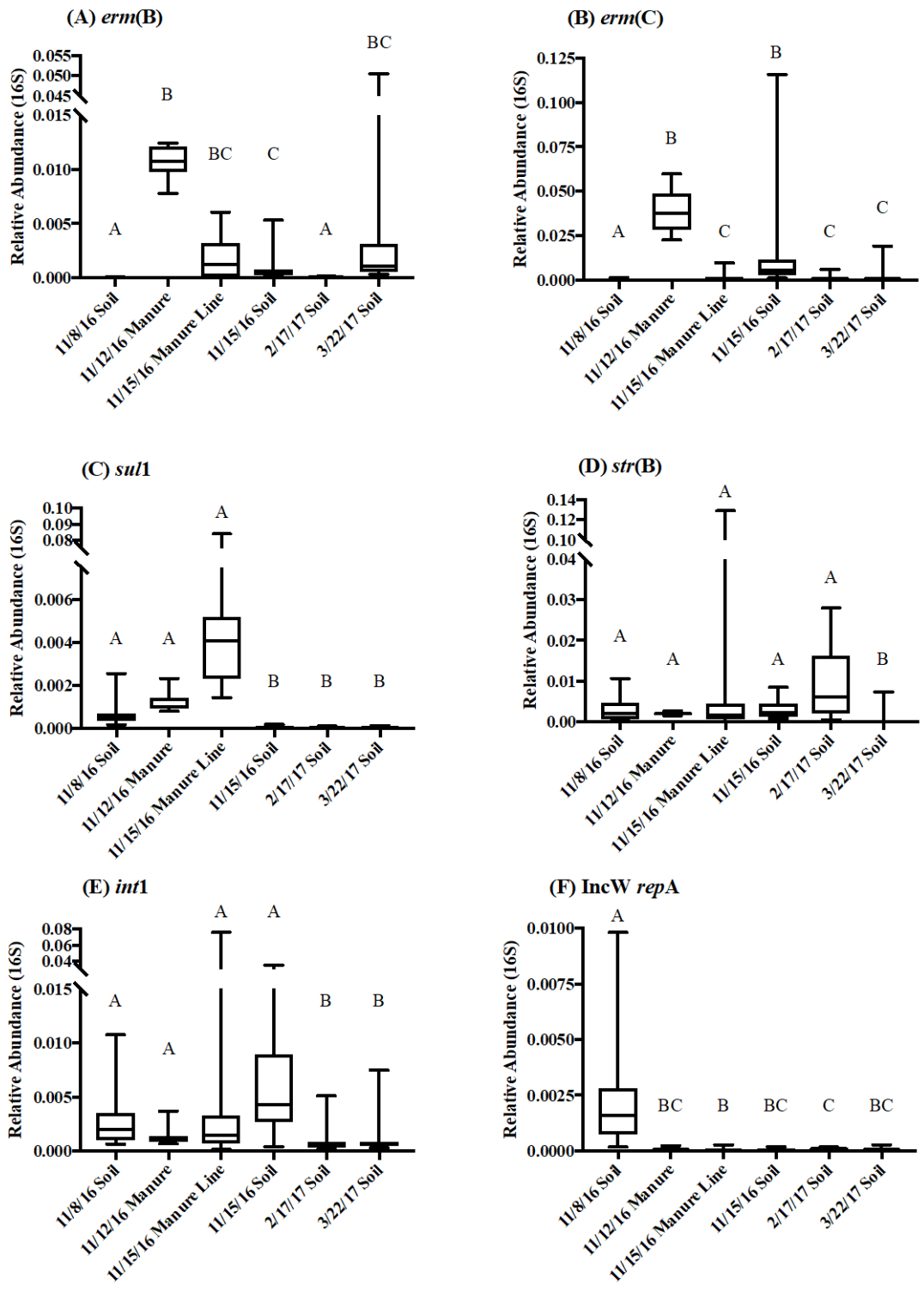

Figure 1. Relative abundance to 16S of *(A) erm*(B), *(B) erm*(C), *(C) sul*1, *(D) str*(B), *(E) int*1, *(F)* IncW *rep*A in soil, manure, and manure line samples. N=20 for soil and manure line. N=10 for manure. Different letters designate statistical significance within each target gene (P<0.05).

Gene abundances were evaluated for the 2016 fall and 2017 spring relative to the *rrnS* gene copy number. Every gene was able to be detected in at least one sample (Fig. 1). However, gene abundance ranged varied by about 100 fold and each gene abundance pattern was unique. The gene target *erm(C)* had the highest abundance of all the genes.

Gene targets *erm(B)* and *erm(C)* had similar abundance dynamics (Fig.1). For both genes, manure had the highest gene abundance. Soil prior to manure application had almost no quantifiable gene copies of *erm(B)* or *erm(C)*, and both gene abundances significantly increased in the manure line and soil samples after manure application. The target *ermB* and *erm(C)* abundances returned to pre-manure application levels in the spring. *Sul1* abundance was overall very low. Soil before manure application, manure, and manure line samples all had similar abundances of the gene target *sul*1 (Fig.1). *sul1* abundance decreased in the soil after manure application. In the 11/15/16 soil sample, was below the limit of quantification (BLQ) in all but one replicate, and was BLD in about half of the spring soil sample dates. Abundance of *str(B)* was similar in all the samples, with a significant decrease in the 3/22/17 soil where *str(B)* abundance was mostly BLQ. Similarly, *int1* abundance was unchanged throughout samples until a dcrease in abundance in the spring soil samples. *int1* abundance was also very low. IncW *rep*A had little significant difference between abundances throughout all the samples as all samples except for the 11/8/16 soil were mostly BLQ.

Abundance of select antibiotic resistance and mobile element target genes were measured to determine the effect of manure application on their dynamics. Gene target abundances in almost all the soil samples were low and many were unquantifiable. Even with the difficulty measuring and analyzing the abundances, there were two sets of genes that had similar abundance dynamics. The targets *erm(*B), *erm(C),* and *sul1* abundances significantly increased after manure application, but returned to pre-manure abundance in the spring, while manure application had no significant effect on the abundance of *str(B)*, *int1* and IncW *rep*A genes. A decrease in gene abundance may be attributed to either natural decay of the genes or weather conditions, including precipitation and temperature. An increase in gene abundance at any sampling time point may be attributed to as an increase in bacteria that carry that gene either due to direct addition from the manure microbiome or proliferation of resistant bacteria previously in the soil. Interestingly, the two sets of patterns may be explained by the hogs previous exposure to antibiotics. The manure producing hogs were given sulfamethazine, which *sul1* resists, and the macrolide tylosin phosphate, which both *erm(B), erm(C)* may provide resistance (46, 47). Abundance of *sul1* has been previously shown to be positively correlated with the amount of sulfonamide in soil (48). The hogs were not given any streptomycin or other aminoglycides of which *str(B)* provides resistance (49). While the abundance patterns may be explained by antibiotic exposure, which is thought to be the mechanism that promotes resistance, it is important to note *str(B)* was still detected all samples (17). This may mean the *str(B)* abundances may be residues from a previous course of antibiotics as resistance is known to persist for years after antibiotics have been administered, or be due to naturally occurring resistance (50, 51).

To our knowledge, this study is the first of its kind to examine manure application from a CAFO, but many studies have shown similar trends of increase in ARG abundance after manure application affirming the present study (13, 14, 52, 53). However, the time period that the increased abundance persists is still unknown. The present study found that gene abundances return to pre-manure application levels if applied in the fall before spring planting. Marti et al. suggests a offset time of at least one growing period is necessary to allow abundance to safely return to pre-application conditions (13). Hamscher et al. found that the abundance of ARGs can continue to accumulate for years (54). Another study from Heuer and Smalla found that ARGs persist for at least 2 months (55). These differing results suggest that gene abundances are sensitive to factors like soil type and application rates, and further studies are needed to better characterize the fate and dynamics of ARGs following manure application.

Portions of each sample were aliquoted in 0.25 g amounts for DNA extraction. The DNA was delivered to the USDA lab in Ames for DNA extraction by a robot. This DNA is for 16S rRNA community analysis. After we received low yield on 16S rRNA high throughput sequencing, it was determined that the USDA robot system and kit used did not work equally well on manure and soil samples. DNA has been extracted again from all samples using a different kit and robot at Iowa State University. The samples are currently waiting in a cue for MiSeq of the 16S rRNA. I attended the EDAMAME workshop in 08/17 to learn the tools necessary for 16S rRNA analysis and I currently have one student at the 2018 workshop to practice analysis on our first set of sequences before we get the second round of sequencing back.

We are currently writing a paper for submission to Applied and Environmental Microbiology. The last piece of data for this paper will be the 16S rRNA community analysis, which will be completed this summer. The paper will be submitted in the fall of 2018. This work has been presented at the Midstates Consortium meeting in the fall of 2017 in St. Louis. Upon completion of the last part of the work, we will meet with Doug Hoksbergen to share our findings and to explore ways to share our results with the broader farming community.

References Cited

13. Marti R, Tien Y-C, Murray R, Scott A, Sabourin L, Topp E. 2014. Safely Coupling Livestock and Crop Production Systems: How Rapidly Do Antibiotic Resistance Genes Dissipate in Soil following a Commercial Application of Swine or Dairy Manure? Appl Environ Microbiol 80:3258–3265.
14. Luby EM, Moorman TB, Soupir ML. 2016. Fate and transport of tylosin-resistant bacteria and macrolide resistance genes in artificially drained agricultural fields receiving swine manure. Sci Total Environ 550:1126–1133.

17. Heuer H, Schmitt H, Smalla K. 2011. Antibiotic resistance gene spread due to manure application on agricultural fields. Curr Opin Microbiol.

46. Antunes P, Machado J, Sousa JC, Peixe L. 2005. Dissemination of sulfonamide resistance genes (sul1, sul2, and sul3) in Portuguese Salmonella enterica strains and relation with integrons. Antimicrob Agents Chemother 49:836–839.

47. Leclercq R, Courvalin P. 1991. Bacterial resistance to macrolide, lincosamide, and streptogramin antibiotics by target modification. Antimicrob Agents Chemother.
48. Zhao X, Wang J, Zhu L, Ge W, Wang J. 2017. Environmental analysis of typical antibiotic-resistant bacteria and ARGs in farmland soil chronically fertilized with chicken manure. Sci Total Environ 593–594:10–17.
49. Srinivasan V, Gillespie BE, Nguyen LT, Headrick SI, Murinda SE, Oliver SP. 2007. Characterization of antimicrobial resistance patterns and class 1 integrons in Escherichia coli O26 isolated from humans and animals. Int J Antimicrob Agents 29:254–262.
50. Johnsen PJ, Townsend JP, Bøhn T, Simonsen GS, Sundsfjord A, Nielsen KM. 2009. Factors affecting the reversal of antimicrobial-drug resistance. Lancet Infect Dis.
51. Wellington EMH, Boxall ABA, Cross P, Feil EJ, Gaze WH, Hawkey PM, Johnson-Rollings AS, Jones DL, Lee NM, Otten W, Thomas CM, Williams AP. 2013. The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. Lancet Infect Dis 13:155–165.
52. Wu N, Qiao M, Zhang B, Cheng W Da, Zhu YG. 2010. Abundance and diversity of tetracycline resistance genes in soils adjacent to representative swine feedlots in China. Environ Sci Technol 44:6933–6939.
53. Munir M, Xagoraraki I. 2011. Levels of Antibiotic Resistance Genes in Manure, Biosolids, and Fertilized Soil. J Environ Qual 40:248.
54. Hamscher G, Pawelzick HT, Höper H, Nau H. 2005. Different behavior of tetracyclines and sulfonamides in sandy soils after repeated fertilization with liquid manure. Environ Toxicol Chem 24:861–868.
55. Heuer H, Smalla K. 2007. Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. Environ Microbiol 9:657–666.