MANURE-BASED SPREAD OF ANTIMICROBIAL RESISTANCE IN SOIL AND WATER ECOSYSTEMS

GONÇALO MACEDO

Manure-based Spread of Antimicrobial Resistance in Soil and Water Ecosystems

Gonçalo Nuno Barroca de Macedo

Manure-based Spread of Antimicrobial Resistance in Soil and Water Ecosystems.

Printing of this thesis was financially supported by:
Faculty of Veterinary Medicine of Utrecht University; Department of Infectious Diseases and Immunology.
Wetsus, European Centre of Excellence for Sustainable Water Technology.

Ph.D. Thesis, Utrecht University the Netherlands

ISBN: 978-90-393-7416-0
DOI: 10.33540/860 (https://doi.org/10.33540/860)
Cover design by: Simone Golob
Layout by: ProefschriftMaken (proefschriftmaken.nl)
Printed by: ProefschriftMaken

Manure-based Spread of Antimicrobial Resistance in Soil and Water Ecosystems

Verspreiding van antimicrobiële resistentie in bodem- en waterecosystemen als gevolg van bemesting

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht
op gezag van de
rector magnificus, prof.dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op

dinsdag 9 november 2021 des middags te 2.15 uur

door

Gonçalo Nuno Barroca De Macedo

geboren op 9 februari 1989
te Porto, Portugal

Promotoren:
Prof. dr. D.J.J. Heederik
Prof. dr. D.J. Mevius

Copromotoren:
Dr. H. Schmitt
Dr. P. van der Maas

This work was performed in the cooperation framework of Wetsus, European Centre of excellence for sustainable water technology (www.wetsus.nl) and Utrecht University. Wetsus is co-funded by the Dutch Ministry of Economic Affairs and Ministry of Infrastructure and Environment, the European Union Regional Development Fund, the Province of Friesland and the Northern Netherlands Provinces. Wetsus is also the coordinator of the WaterSEED project that received funding from the European Union's Horizon 2020 Research and Innovation Program under the Marie Skłodowska-Curie [grant agreement no. 665874].

To my inherited, to my adopted, and to my chosen family

Table of Contents

CHAPTER 1

"It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body."

Alexander Fleming
Nobel Prize acceptance speech, 1945

General Introduction

1.1 From antibiotics to antimicrobial resistance

1.1.1 Antibiotic characteristics and consumption

Antibiotics are compounds originally produced by bacteria and fungi that can destroy or prevent the growth of other microorganisms that coexist in the same habitat. Since the discovery of penicillin in 1928 by Alexander Fleming, they have been used widely in modern medicine. Antibiotics are complex molecules with different functional groups and depending on their chemical structure and mechanism of action, they are divided into several classes.

In general, antibiotics were used extensively in human and veterinary medicine, but recent international policies have decreased antimicrobial consumption. For example, in 1999, the use of valuable human antibiotics as growth promoters in animal feed was banned by the European Union, and in 2006, this growth promoter ban was extended to all antibiotics. This effort resulted in decreased overall antimicrobial sales by 32% (50% in some countries) between 2011 and 2017 (ECDC et al., 2017). On the other hand, a more considerable portion of the world population has been adopting a high-protein diet, particularly in low- and middle-income countries. Since 2000, meat production increased by 68%, 64%, and 40% in Asia, Africa, and South America, respectively (FAO; http://www.fao.org/faostat/en/#home), and this increase is accompanied by an increase in intensive animal production systems in which antibiotics are used routinely to maintain health and productivity (Silbergeld et al., 2008). Consequently, from 2000 to 2018, the proportion of antimicrobials to which high resistance levels were recorded increased from 0.15 to 0.41 in chickens and from 0.13 to 0.34 in pigs (Van Boeckel et al., 2019).

One of the significant industries related to high levels of antibiotic consumption is the veterinary/food sector. Livestock antibiotic consumption can range between 50–80%, while crops, pets, and aquaculture collectively account for roughly 5%, and humans the rest (Cully, 2014). These compounds can be used at farms: i) therapeutically to treat existing disease conditions, ii) prophylactically to prevent infections, or iii) sub-therapeutically to enhance growth. While the latter is prohibited in Europe, more than 90 countries worldwide currently do not limit critically essential antimicrobials (human and animal) for growth promotion in agriculture (WHO et al., 2018).

Antibiotic usage is the cause of increased percentages of resistant bacteria isolated from animals (Angulo et al., 2004). Although antibiotics as growth promoters have been banned in the EU, the total amount of active ingredients consumed for food-producing animals reached 8,927 tons, considering 28 countries (ECDC et al., 2017). In the Netherlands, antibiotic consumption at farms has been declining over the last decade to values below 200 tons of active substance sold (total active substance sales peak was achieved in 2007

with 565 tonnes) (de Greef et al., 2019). Among others, tetracyclines, penicillins, and trimethoprim/sulphonamides are the most consumed antibiotics.

If applied to the extreme, cutting antibiotic prescriptions to decrease consumption may be considered unethical because sick animals need to be treated. In addition, there is also differential use related to the income level of a country, with higher income countries tending to use more antimicrobials. Therefore, such a decision should consider economic factors as well as animal wellbeing. In 2015, the EU guidelines for prudent antimicrobial use in veterinary medicine had been implemented. The current best practice combines regulatory policies with animal health management where disease-preventive measures have replaced the non-rational use of antimicrobials, thereby safeguarding productivity (Magnusson, 2016).

1.2 Antimicrobial resistance mechanisms

Antibiotics have revolutionized modern medicine, but their use, and in many cases overuse, have promoted the rapid appearance of resistance traits and resistant bacterial strains (Davies and Davies, 2010). The rise of antimicrobial-resistant bacteria (ARB), antimicrobial resistance genes (ARG), and their potentially hazardous effects on human health led the World Health Organization to consider it one of the major challenges to global public health.

The term antimicrobial resistance (AMR) is applied when "microorganisms change in ways that render the medications used to cure the infections they cause ineffective" (WHO). Microorganisms employ multiple strategies to resist the selective pressure imposed by antibiotics, and resistance can be achieved by preventing the drug from reaching its target. Having an impermeable barrier (e.g., cell wall) or actively pumping the antibiotics to the outside of the cell (e.g., efflux pump) are effective ways of protecting the bacteria. For example, *Mycobacteria tuberculosis* and *M. leprae* cell walls' low permeability help them resist antimicrobial compounds' activity (Brennan and Nikaido, 1995). Also, the AcrAB-TolC and Mex pump systems of *Escherichia coli* are relevant to mediate intrinsic and acquired multidrug resistance (Li et al., 2015). Other mechanisms involve mutations that modify the antibiotic target protein, for example, by disabling the antibiotic-binding site and leaving the protein's cellular functionality intact. The production of antibiotic inactivation proteins (e.g., β-lactamases) or the induction of temporary conformational changes in the antibiotic target are also effective resistance mechanisms.

The nomenclature of antimicrobial resistance genes is very diverse. It may be potentially confusing because it is based on the percentage of amino acid identity compared with

previously characterized genes that confer resistance to a particular antibiotic class, not on the underlined action mechanism. For example, the tetracycline resistance genes *tet(A), tet(B)*, and *tet(C)* confer resistance by efflux, while *tet(M)* and *tet(W)* act by ribosomal protection (Chopra and Roberts, 2001). Another example of the diversity of mechanisms for the same antibiotic class regards fluoroquinolones. Mutations in DNA gyrase are known to confer a strong level of resistance to ciprofloxacin (target alteration) (Ruiz, 2003). However, genes such as *qnrS* (target protection), *qepA* (efflux), and *aac(6')-Ib-cr* (mutated enzyme) also significantly increase the minimum inhibitory concentration (MIC) of its strain (Strahilevitz et al., 2009).

1.3 Antimicrobial resistance – development and environmental dissemination

1.3.1 Development of resistance

AMR is usually measured by the minimum inhibitory concentration (MIC) of antibiotics required to limit bacterial growth. However, it is known that antibiotics at sub-MICs can select ARB (Gullberg et al., 2011). The minimal selective concentration (MSC) refers to the lowest concentration of an antibiotic that selects a resistant mutant in a population over a susceptible strain. Exposing bacteria to levels between the MSC and the MIC is known to accelerate the emergence and spread of ARB among humans and animals (Andersson and Hughes, 2014). Based on this information, predicted no-effect concentrations (PNECs) for resistance selection for all common antibiotics had been proposed by Bengtsson-Palme and Larsson (2016). Their work proposed individual emission limits for each compound based on its particular selective potential. In 2017, the AMR Industry Alliance, a private-sector coalition representing research-based pharmaceutical companies and associations, was created to provide sustainable solutions to the global AMR crisis. The members of this partnership have accepted the list of PNECs, defined by the scientific community, and started to implement policies to reduce antibiotic residue discharge emissions at manufacturing sites (AMR Industry Alliance, 2020). This unprecedented commitment represents an outstanding achievement in the fight against AMR, but this step alone is not enough.

Antibiotics are undoubtedly connected with the emergence of AMR (Goossens, 2009). Microorganisms can develop resistance through vertical inheritance or by horizontal gene acquisition. The former refers to the accumulation of genetic changes during the natural replication process, while horizontal gene transfer (HGT) is based on the exchange of genetic traits between microorganisms. During replication, mutations that allow the bacteria to tolerate higher concentrations of antibiotics can be advantageous, and if exposed to antibiotics, these mutants may become prevalent in the bacterial population.

The accumulation of mutations can lead bacteria to tolerate high antibiotic concentrations to which it was previously susceptible (Baym et al., 2016). However, vertical inheritance alone is not enough to explain AMR's rapid rise (Cantón and Coque, 2006). Some resistance traits can persist in the environment even in the absence of selective pressures (Andersson and Hughes, 2014; Martínez and Baquero, 2014).

HGT can occur through different mechanisms: i) transformation – the uptake, integration, and functional expression of extracellular DNA; ii) transduction – the introduction of foreign DNA into another cell via a bacteriophage vector; and iii) conjugation – transfer of plasmid DNA mediated by cell-to-cell junctions and a pore through which DNA can pass. Plasmids are defined as DNA molecules, linear or circular, with autonomous replication from the bacterial core genome (Carattoli, 2011; Novick, 1987) that can be transferred between distinct hosts. Plasmids can be highly diverse in size, structure, transmission capacity (Rozwandowicz et al., 2018; Smillie et al., 2010). Plasmid-mediated gene transfer (conjugation) is considered the most relevant HGT mechanism (Norman et al., 2009; Thomas and Nielsen, 2005). Plasmids may not be conjugative or even mobilizable, but when they are, they significantly contribute to the communal gene pool (Norman et al., 2009).

Furthermore, plasmids can be classified into incompatibility families. Plasmid incompatibility refers to the inability of two plasmids to coexist stably over several generations in the same bacterial cell line (Novick, 1987). Closely related plasmids tend to be incompatible due to the possession of a similar group of genes responsible for cell maintenance (replicon). This replicon defines the incompatibility family (Inc). Together, more than 60 Inc types have been described for *Enterobacteriaceae*, *Pseudomonas*, and *Staphylococcus* (Shintani et al., 2015).

The role of plasmids on AMR spread is well studied in human pathogens (Cantón and Coque, 2006; Woerther et al., 2013). Plasmids are also known to mediate gene transfer to environmental bacteria (Davison, 1999; von Wintersdorff et al., 2016). Ultimately, plasmids contribute to disseminating clinically relevant ARGs and maintaining high AMR levels in the environment.

1.3.2 Environmental dissemination of AMR

AMR is now seen as a One Health concept (Robinson et al., 2016), under which people's health is connected to the health of animals and their shared environment (Figure 1). However, AMR research focused primarily on clinically relevant pathogens for many years, namely methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE). The scientific perception towards AMR was changed only with the emergence of multidrug- or extensively drug-resistant Enterobacteriaceae (Hawkey and Jones, 2009; Walsh, 2018).

Figure 1. Schematic evidencing the complex interaction across environments and potential routes of AMR transmission. Retrieved from (Walsh, 2018).

Despite being considered an emerging threat, AMR is acknowledged as an ancient attribute (D'Costa et al., 2011) developed by bacteria to survive the selective pressure created by other antimicrobial-producing microorganisms. This fact implies that AMR is a natural phenomenon, intrinsically related to the environment, which is also the natural source of ARGs (Allen et al., 2010; Nesme and Simonet, 2015), harboring both commensal and pathogenic microorganisms. Interestingly, the first report of a (penicillin) resistance mechanism was published several years before penicillin was introduced as a therapeutic worldwide (Abraham and Chain, 1940). In his work, Fleming noted that certain bacteria's growth was not inhibited by penicillin, and subsequent experiments confirmed the activity of a penicillin modifying enzyme.

Due to anthropogenic activities, the environment is also the primary recipient of human and animal gut bacteria. Wastewater treatment plants and farm activities play a significant role in the AMR spread by combining human and animal waste with environmental bacteria, which can pose a risk of transmission of ARB to humans (Huijbers et al., 2015).
At the farm level, large amounts of manure are produced from livestock. One of the most common ways to dispose of such high amounts of manure is using it as a soil fertilizer. However, manure application to soils also results in the transport of ARB, ARGs (Thanner

et al., 2016), and partially metabolized antibiotics (Jechalke et al., 2014). Many antibiotics are poorly absorbed in the animal's gut, and 30-90% of the parent compound is excreted (Sarmah et al., 2006). Their metabolites can still maintain a decreased antimicrobial activity or be transformed back to the parent compound after excretion. Other factors, such as frequency of manure application, soil texture, and bacterial diversity composition, may also contribute to increased ARG levels in water and soil ecosystems. Overall, the application of manure, or derived composts, to soils can increase soil ARGs by the direct introduction of manure-derived ARGs, enriching the intrinsic soil resistome and imposing a selective pressure on the resident microbiota with the contained antibiotic residues (Xie et al., 2018).

1.3.3 Research goals and thesis outline

Because the fate of ARGs in the environment, particularly in farmlands, is not yet clear, the main goal of this thesis is to investigate ARG proliferation in microbial communities in the agricultural chain, from the animal gut to water (manure, soil, and water), and the extent of ARG transfer potential. Understanding the main factors driving ARG dissemination in farmlands will help define concrete points of action that aim to mitigate the spread of AMR at the farm level.

Chapter 2 evaluates the soil texture's role on ARG dynamics in manured soils and surrounding surface waters. It demonstrates that rather than showing similar decay dynamics, factors such as the type of ARG and soil texture drive the ARG persistence in the environment.

Chapter 3 explores the dynamics of bacteria and ARGs introduced by manuring to soils and adjacent water ditches, both at the community and individual scale, over three weeks. It shows that the bacteriome and resistome structures are shaped by different factors, where the bacterial community composition could not explain the changes in ARG diversity or abundances. Additionally, it also highlights the potential of applying targeted metagenomic techniques, such as ResCap, to study the fate of AMR in the environment.

Chapter 4 examines the ARG removal potential of a manure treatment process using a newly designed up-flow anaerobic sludge bed reactor operating at thermophilic conditions. It indicates that this treatment successfully removes some, but not all, bacterial groups. It also shows that the abundance of the selected ARGs was not reduced.

Chapter 5 assesses how conjugation frequencies between *E. coli* strains in filter matings differ based on the temperature and nutrient availability. It shows that, despite less favorable conditions, plasmid transfer can occur in the environment. Therefore, fecal microorganisms in manure are a source of AMR transmission.

Chapter 6 investigates the fate of plasmids in manured soil microcosms introduced by fecal microorganisms over time. While being a laboratory simulation, it provides realistic quantifications of conjugation frequencies, and it identifies the new hosts of the transferred plasmids.

Chapter 7 discusses the findings reported throughout the thesis. It contextualizes the implications of research outcomes and suggests action points to reduce the farm burden in environmental AMR spread.

1.4 References

Abraham, E.P., Chain, E., 1940. An enzyme from bacteria able to destroy penicillin. Nature. https://doi.org/10.1038/146837a0

Allen, H.K., Donato, J., Wang, H.H., Cloud-Hansen, K.A., Davies, J., Handelsman, J., 2010. Call of the wild: antibiotic resistance genes in natural environments. Nat. Rev. Microbiol. 8, 251–259. https://doi.org/10.1038/nrmicro2312

AMR Industry Alliance, 2020. 2020 Progress Report [Online].

Andersson, D.I., Hughes, D., 2014. Microbiological effects of sublethal levels of antibiotics. Nat. Rev. Microbiol. 12, 465–478. https://doi.org/10.1038/nrmicro3270

Angulo, F.J., Nunnery, J.A., Bair, H.D., 2004. Antimicrobial resistance in zoonotic enteric pathogens. Rev. Sci. Tech. 23, 485–96.

Baym, M., Lieberman, T.D., Kelsic, E.D., Chait, R., Gross, R., Yelin, I., Kishony, R., 2016. Spatiotemporal microbial evolution on antibiotic landscapes. Science (80-.). 353, 1147–1151. https://doi.org/10.1126/science.aag0822

Bengtsson-Palme, J., Larsson, D.G.J., 2016. Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation. Environ. Int. 86, 140–149. https://doi.org/10.1016/j.envint.2015.10.015

Brennan, P.J., Nikaido, H., 1995. The envelope of mycobacteria. Annu. Rev. Biochem. https://doi.org/10.1146/annurev.bi.64.070195.000333

Cantón, R., Coque, T.M., 2006. The CTX-M β-lactamase pandemic. Curr. Opin. Microbiol. https://doi.org/10.1016/j.mib.2006.08.011

Carattoli, A., 2011. Plasmids in Gram negatives: molecular typing of resistance plasmids. Int. J. Med. Microbiol. 301, 654–8. https://doi.org/10.1016/j.ijmm.2011.09.003

Chopra, I., Roberts, M., 2001. Tetracycline Antibiotics : Mode of Action , Applications , Molecular Biology , and Epidemiology of Bacterial Resistance Tetracycline Antibiotics : Mode of Action , Applications , Molecular Biology , and Epidemiology of Bacterial Resistance. Microbiol. Mol. Biol. Rev. 65, 232–260. https://doi.org/10.1128/MMBR.65.2.232

Cully, M., 2014. Public health: The politics of antibiotics. Nature 509, S16–S17. https://doi.org/10.1038/509S16a

D'Costa, V.M., King, C.E., Kalan, L., Morar, M., Sung, W.W.L., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G.B., Poinar, H.N., Wright, G.D., 2011. Antibiotic resistance is ancient. Nature 477, 457–461. https://doi.org/10.1038/nature10388

Davies, J., Davies, D., 2010. Origins and evolution of antibiotic resistance. Microbiol. Mol. Biol. Rev. 74, 417–33. https://doi.org/10.1128/MMBR.00016-10

Davison, J., 1999. Genetic Exchange between Bacteria in the Environment. Plasmid 42, 73–91. https://doi.org/10.1006/plas.1999.1421

de Greef, S., Mouton, J., Schoffelen, A., Verduin, C., 2019. NethMap 2019: Consumption of antimicrobial agents and antimicrobial resistance among medically important bacteria in the Netherlands /

MARAN 2019: Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands in 2018 251.

ECDC, EFSA, EMA, 2017. ECDC/EFSA/EMA second joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals. EFSA J. 15, 4006. https://doi.org/10.2903/j.efsa.2017.4872

Goossens, H., 2009. Antibiotic consumption and link to resistance. Clin. Microbiol. Infect. https://doi.org/10.1111/j.1469-0691.2009.02725.x

Gullberg, E., Cao, S., Berg, O.G., Ilbäck, C., Sandegren, L., Hughes, D., Andersson, D.I., 2011. Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog. 7, 1–9. https://doi.org/10.1371/journal.ppat.1002158

Hawkey, P.M., Jones, A.M., 2009. The changing epidemiology of resistance. J. Antimicrob. Chemother. 64, i3–i10. https://doi.org/10.1093/jac/dkp256

Huijbers, P.M.C., Blaak, H., De Jong, M.C.M., Graat, E.A.M., Vandenbroucke-Grauls, C.M.J.E., De Roda Husman, A.M., 2015. Role of the Environment in the Transmission of Antimicrobial Resistance to Humans: A Review. Environ. Sci. Technol. 49, 11993–12004. https://doi.org/10.1021/acs.est.5b02566

Jechalke, S., Heuer, H., Siemens, J., Amelung, W., Smalla, K., 2014. Fate and effects of veterinary antibiotics in soil. Trends Microbiol. 22, 536–545. https://doi.org/10.1016/j.tim.2014.05.005

Klein, E.Y., Van Boeckel, T.P., Martinez, E.M., Pant, S., Gandra, S., Levin, S.A., Goossens, H., Laxminarayan, R., 2018. Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. Proc. Natl. Acad. Sci. U. S. A. 115, E3463–E3470. https://doi.org/10.1073/pnas.1717295115

Li, X.Z., Plésiat, P., Nikaido, H., 2015. The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. Clin. Microbiol. Rev. 28, 337–418. https://doi.org/10.1128/CMR.00117-14

Magnusson, U., 2016. Sustainable global livestock development for food security and nutrition including roles for Sweden. Stockholm.

Martínez, J.L., Baquero, F., 2014. Emergence and spread of antibiotic resistance: setting a parameter space. Ups. J. Med. Sci. 119, 68–77. https://doi.org/10.3109/03009734.2014.901444

Nesme, J., Simonet, P., 2015. The soil resistome: a critical review on antibiotic resistance origins, ecology and dissemination potential in telluric bacteria. Environ. Microbiol. 17, 913–930. https://doi.org/10.1111/1462-2920.12631

Norman, A., Hansen, L.H., Sørensen, S.J., Sorensen, S.J., 2009. Conjugative plasmids: vessels of the communal gene pool. Philos. Trans. R. Soc. B Biol. Sci. 364, 2275–2289. https://doi.org/10.1098/rstb.2009.0037

Novick, R.P., 1987. Plasmid incompatibility. Microbiol. Rev. 51, 381–95.

Robinson, T.P., Bu, D.P., Carrique-Mas, J., Fèvre, E.M., Gilbert, M., Grace, D., Hay, S.I., Jiwakanon, J., Kakkar, M., Kariuki, S., Laxminarayan, R., Lubroth, J., Magnusson, U., Thi Ngoc, P., Van Boeckel, T.P., Woolhouse, M.E.J., 2016. Antibiotic resistance is the quintessential One Health issue. Trans. R. Soc. Trop. Med. Hyg. https://doi.org/10.1093/trstmh/trw048

Rozwandowicz, M., Brouwer, M.S.M., Fischer, J., Wagenaar, J.A., Gonzalez-Zorn, B., Guerra, B., Mevius, D.J., Hordijk, J., 2018. Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae. J. Antimicrob. Chemother. 73, 1121–1137. https://doi.org/10.1093/jac/dkx488

Ruiz, J., 2003. Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection. J. Antimicrob. Chemother. 51, 1109–17. https://doi.org/10.1093/jac/dkg222

Sarmah, A.K., Meyer, M.T., Boxall, A.B.A., 2006. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. Chemosphere 65, 725–759. https://doi.org/10.1016/j.chemosphere.2006.03.026

Shintani, M., Sanchez, Z.K., Kimbara, K., 2015. Genomics of microbial plasmids: classification and identification based on replication and transfer systems and host taxonomy. Front. Microbiol. 6, 1–16. https://doi.org/10.3389/fmicb.2015.00242

Silbergeld, E.K., Graham, J., Price, L.B., 2008. Industrial Food Animal Production, Antimicrobial Resistance, and Human Health. Annu. Rev. Public Health 29, 151–169. https://doi.org/10.1146/annurev.publhealth.29.020907.090904

Smillie, C., Garcillán-Barcia, M.P., Francia, M.V., Rocha, E.P.C., de la Cruz, F., 2010. Mobility of plasmids. Microbiol. Mol. Biol. Rev. 74, 434–52. https://doi.org/10.1128/MMBR.00020-10

Strahilevitz, J., Jacoby, G.A., Hooper, D.C., Robicsek, A., 2009. Plasmid-mediated quinolone resistance: A multifaceted threat. Clin. Microbiol. Rev. 22, 664–89. https://doi.org/10.1128/CMR.00016-09

Thanner, S., Drissner, D., Walsh, F., 2016. Antimicrobial Resistance in Agriculture. MBio 7, 1–7. https://doi.org/10.1128/mBio.02227-15

Thomas, C.M., Nielsen, K.M., 2005. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nat. Rev. Microbiol. 3, 711–721. https://doi.org/10.1038/nrmicro1234

Van Boeckel, T.P., Gandra, S., Ashok, A., Caudron, Q., Grenfell, B.T., Levin, S.A., Laxminarayan, R., 2014. Global antibiotic consumption 2000 to 2010: An analysis of national pharmaceutical sales data. Lancet Infect. Dis. 14, 742–750. https://doi.org/10.1016/S1473-3099(14)70780-7

Van Boeckel, T.P., Pires, J., Silvester, R., Zhao, C., Song, J., Criscuolo, N.G., Gilbert, M., Bonhoeffer, S., Laxminarayan, R., 2019. Global trends in antimicrobial resistance in animals in low- and middle-income countries. Science (80-.). 365, eaaw1944. https://doi.org/10.1126/science.aaw1944

von Wintersdorff, C.J.H., Penders, J., van Niekerk, J.M., Mills, N.D., Majumder, S., van Alphen, L.B., Savelkoul, P.H.M., Wolffs, P.F.G., 2016. Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer. Front. Microbiol. 7, 173. https://doi.org/10.3389/fmicb.2016.00173

Walsh, T.R., 2018. A one-health approach to antimicrobial resistance. Nat. Microbiol. 3, 854–855. https://doi.org/10.1038/s41564-018-0208-5

WHO, FAO, OIE, 2018. Monitoring Global Progress on Addressing Antimicrobial Resistance.

Woerther, P.L., Burdet, C., Chachaty, E., Andremont, A., 2013. Trends in human fecal carriage of extended-spectrum β-lactamases in the community: Toward the globalization of CTX-M. Clin. Microbiol. Rev. https://doi.org/10.1128/CMR.00023-13

Xie, W.Y., Shen, Q., Zhao, F.J., 2018. Antibiotics and antibiotic resistance from animal manures to soil: a review. Eur. J. Soil Sci. https://doi.org/10.1111/ejss.12494

CHAPTER 2

"Original thinkers doubt the default."
Adam Grant

The impact of manure and soil texture on antimicrobial resistance gene levels in farmlands and adjacent ditches

Gonçalo Macedo[*1,2], Lucia Hernandez-Leal[2], Peter van der Maas[3], Dick Heederik[4], Dik Mevius[1,5], Heike Schmitt[1,2,4]

[1] Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands
[2] Wetsus, European Centre of Excellence for Sustainable Water Technology, Oostergoweg 9, 8911 MA Leeuwarden, The Netherlands
[3] Van Hall Larenstein, University of Applied Sciences, Agora 1, 8901 BV Leeuwarden, The Netherlands
[4] Institute for Risk Assessment Sciences, Utrecht University, Yalelaan 2, 3584 CM Utrecht, The Netherlands
[5] Wageningen Bioveterinary Research, Houtribweg 39, 8221 RA Lelystad, The Netherlands

Published in Science of the Total Environment 737 (2020) 139563
(https://doi.org/10.1016/j.scitotenv.2020.139563)

2.1 Abstract

Manure application can spread antimicrobial resistance (AMR) from manure to soil and surface water. This study evaluated the role of the soil texture on the dynamics of antimicrobial resistance genes (ARGs) in soils and surrounding surface waters. Six dairy farms with distinct soil textures (clay, sand, and peat) were sampled at different time points after the application of manure, and three representative ARGs *sul1*, *erm(B)*, and *tet(W)* were quantified with qPCR. Manuring initially increased levels of *erm(B)* by 1.5 ± 0.5 log copies/kg of soil and *tet(W)* by 0.8 ± 0.4 log copies/kg across soil textures, after which levels gradually declined. In surface waters from clay environments, regardless of the ARG, the gene levels initially increased by 2.6 ± 1.6 log copies/L, after which levels gradually declined. The gene decay in soils was strongly dependent on the type of ARG (*erm(B)* < *tet(W)* < *sul1*; half-lives of 7, 11, and 75 days, respectively), while in water, the decay was primarily dependent on the soil texture adjacent to the sampled surface water (clay < peat < sand; half-lives of 2, 6, and 10 days, respectively). Finally, recovery of ARG levels was predicted after 29 – 42 days. The results thus showed that there was not a complete restoration of ARGs in soils between rounds of manure application. In conclusion, this study demonstrates that rather than showing similar dynamics of decay, factors such as the type of ARG and soil texture drive the ARG persistence in the environment.

2.2 Introduction

Antimicrobial resistance (AMR) is considered as one of the most significant challenges to global public health ("WHO," 2016). AMR is currently approached from a "One Health" perspective, which includes exploring the occurrence of AMR in animals, humans, the environment, and its transmission between reservoirs ("One Health," 2019). The presence of antimicrobial resistance genes (ARGs) in manure, related to the usage of antibiotics in veterinary practices (Hoelzer et al., 2017; Topp et al., 2018), results in environmental contamination of manured soils and surface water.

In the Netherlands, per year, approximately 1.6 million cows are present in the dairy industry, and 57 million chickens in layer poultry farming (de Greeff and Mouton, 2017), annually producing over 40.8 million tons of manure. While it is known that manure application introduces antibiotics and other pharmaceuticals to soils (Heuer et al., 2011; Jechalke et al., 2014b) and can result in antimicrobial-resistant bacteria and ARGs entering the environment in soil and water systems (Agga et al., 2015; Chee-Sanford et al., 2009), the contribution of the soil texture to the spread of AMR to the environment has not yet been thoroughly addressed. The soil texture (relative content of particles from different size classes) comprises a set of physicochemical parameters, which may interact in a non-independent manner. Whether by protection against predation, limited organic carbon availability, or other, the soil texture has proven effects on the survivability of fecal bacteria in soils (Franz et al., 2014; van Veen et al., 1997) and on the structure of the soil bacterial community (Blau et al., 2018; Girvan et al., 2003). Thus, one can also assume that the soil texture will also play a significant role on the fate of ARGs. Changes in soil bacterial communities and resistomes have been studied after the application of different types of manure (Han et al., 2018; Zhang et al., 2017), of different manure loads (Gou et al., 2018), or after multiple manure applications (Chessa et al., 2016; F. Wang et al., 2018). However, these studies were performed under microcosm settings. Only a few recent studies investigated the fate and transport of ARGs after manure application in soils and water runoffs simultaneously in field experiments (Fahrenfeld et al., 2014; He et al., 2016; Joy et al., 2013; Luby et al., 2016; Muurinen et al., 2017; Soni et al., 2015), and even fewer studies aimed to model the spread of resistance (Baker et al., 2016; Volkova et al., 2013), while no study so far addressed the role of soil texture in a field setting.

The soil resistome has been correlated with the microbial phylogenetic and taxonomic structure across soil textures, indicating that the soil native bacterial composition is the primary determinant of the ARG content in agricultural and grassland soils (Forsberg et al., 2014). Additionally, recent field studies focus on changes in resistome diversity after manure application measured with high-throughput qPCR (Chen et al., 2019; Cheng et al., 2019; Pu et al., 2018; Wan-Ying Xie et al., 2018), but this approach does not provide the quantitative data needed to determine the fate of the different ARGs in the environment in terms of decay rates.

This study analyzed the role of the soil texture on the dynamics of AMR in soils and adjacent surface waters (runoff). To achieve this goal, we (i) evaluated the impact of manure application on selected ARG levels, over time, in manured soil and watercourses adjacent to the soil; and (ii) tested the role of soil texture on the dynamics of ARG decay in soils and surrounding surface water over time. Manure, soil, and water samples were examined by qPCR to quantify β-lactam (bla_{TEM}), sulfonamide (*sul1*), macrolide (*erm(B)*), and tetracycline (*tet(W)*) resistance genes levels. These ARGs were selected because they represent resistance to the most consumed antimicrobial families in animal health (de Greeff and Mouton, 2018; ECDC et al., 2017), and are measured in manured soils worldwide (Blau et al., 2018; McKinney et al., 2018; Tien et al., 2017). In this work, we focus on qPCR on a large number of samples, as qPCR – in contrast to metagenomics or resistance gene arrays which can generate data on a wide scale of resistance genes – can provide information on the absolute gene concentrations per g of soil which are needed to determine resistance gene kinetics. We hypothesized that the soil texture would have a significant impact on the decay rates of ARGs introduced by manure application in soils and water streams.

2.3 Materials and Methods

2.3.1 Sampling locations and soil characteristics

In the Netherlands, manure application to soils is only permitted between February and August (spring and summer; RVO 2017). During the rest of the year, cattle manure is collected and stored below the stables until the compartment's holding capacity is full, after which it is transferred to a storage silo until the manuring season starts. The manure is then applied by injection, and no-tillage is performed. For this study, six dairy farms were selected, each of which had distinct soil textures (clay, sand, or peat; Table 1). For the last five years before the sampling campaign, the sampled fields had similar usage (grassland) and fertilization rates (farmers' personal communication). In these farms, the animals pasture during the day, but not in the parcels to which manure was applied. Farmers regularly perform soil analysis on their fields every four to five years; however, the results were not available to us. Therefore, the physicochemical properties of the soils were determined, following standardized procedures (Eurofins Agro; Netherlands), and detailed information about the soil characteristics can be found in Table S1.

Table 1. Characteristics of the farms included in the study.

Farm Code	Soil Texture	Clay (%)	Silt (%)	Sand (%)	Org. Matter (%)	Nr. animals	Manure applied (tons/ha)			Area (ha)*
							Round 1	Round 2	Round 3	
F01	Clay	18	32	45	4,8	700	32	25	--	5,0
F02	Clay	35	41	9	14,6	200	30	--	--	8,7
F03	Sand	5	15	70	10,3	340	30	15	--	1,7
F04	Sand	2	10	79	8,6	100	25	15	15	1,0
F05	Peat	36	28	17	19,3	120	40	10	--	7,0
F06	Peat	31	34	9	25,1	400	30	20	15	10,0

Note: Even though the chemical analysis of the farm F05 classified it as clay, the previous analysis performed by the farmers indicated that its soil texture was peat. Similarly, the analysis classified the farm F01 as loam, but the previous analysis indicated that its texture is clay. *Estimated via https://boerenbunder.nl.

2.3.2 Sample collection

Sampling occurred from February to August 2017, during the manuring season. The manure samples were collected shortly before being applied to the field (after mixing). Soil and water samples were collected within one week before manuring (time point T0) and at defined time intervals after manuring (1, 4, 7, 14, and 21 days; time points T1, T2, T3, T4, and T5, respectively). In addition, never manured soil samples from each soil texture (NM) were used as controls and were collected from gardens nearby the sampled farms due to the inability to find buffer or forest areas of the same soil types. Each sampling cycle was repeated after each round of manure application, and occurred 34 – 80 days after the previous round, except for the first round. Composite soil samples were collected and prepared according to ISO guidelines (ISO 10381-6:2009). Briefly, after walking the fields in a "W" pattern, in which >25 grab samples of each field (0-10 cm soil depth; 4 – 8 samples taken on the manure bands) were collected every 40 steps with a soil probe. To avoid an excess of plant biomass and rhizosphere, the grass turfs were pushed aside by foot before collection with the probe. The manure bands were visible until two weeks after manure application. After collection, the soil was homogenized with a regular 3-prong gardening tool and enclosed in plastic zip-lock bags. Water samples were collected with sterile 2-L bottles, from the adjacent discharge ditch (closed and independent systems). Because the drainage pipes ended below the ditch water surface, the water samples were collected as close as possible to the drainage pipe exit, except the first round of farm F01, which was collected directly from the drainage pipe. All samples were kept on ice during transport and were processed for *E. coli* enumeration within 24 hours and stored at -20°C before DNA extraction and further chemical analysis.

2.3.3 *E. coli* enumeration

Manure samples (10 g) were homogenized in a blender with 10 mL of sterile saline solution (0.85% NaCl, m/v) and were diluted before being plated on Tryptone Bile X-glucuronide (TBX)

agar media (Oxoid, UK). Soil samples (100 g) were homogenized for 1 minute with 100 mL sterile saline solution and then diluted 10-fold. The homogenate and dilution were plated on TBX, in duplicate, and incubated. Volumes of 1, 3, 10, 30- and 100-mL of ditch water were filtered through 0.45 µm pore cellulose nitrate membranes (Merck-Millipore, USA), and filters were placed on TBX. All plates were prepared in duplicate and were incubated between 16-24 hours at 37 °C before counting. Bacterial enumeration was calculated according to ISO guidelines (ISO 8199:2018). Briefly, the final bacterial concentration (CFUs/kg or L) resulted from the sum of the total CFUs obtained in a sample divided by the total amount of the same sample tested. The limit of quantification (LOQ) was calculated by assuming a count of 10 CFUs in the highest volume of the original sample.

2.3.4 DNA extraction and qPCR

Total DNA extracts were obtained from 200 mg of manure with the QIAamp DNA Stool Mini Kit (QIAGEN, Germany), 250 mg of soil with the FastDNA™ Spin Kit for Soil (MP Biomedicals, USA), and 100 mL of water samples with the DNeasy® PowerWater® Kit (QIAGEN), in triplicate. The water samples were filtered through 0.22 µm pore PVDF filters (Merck-Millipore, USA) prior to DNA extraction. At each sampling cycle, an internal standard was spiked in at least one sample type from each farm to assess the DNA extraction efficiency (Figure S1). The spike consisted of 50 µL containing 6.34 x 10^6 gene copies of a synthetic blue fluorescence protein as a 720 bp DNA fragment (BFP; gBlocks; IDT technologies, Belgium) added at the first step of the extraction procedure, before cell lysis. As the after-lysis recoveries were comparable within soil and water samples (Figure S1), the concentrations were not corrected for after-lysis recovery, and no lysis efficiency was measured. DNA extraction proceeded according to the manufacturer's instructions with one adaptation, which consisted of adding or adjusting the bead-beating step with Precellys Evolution (Bertin Instruments, France) for enhanced cell lysis. DNA quantification was performed using Quantus Fluorometer (Promega, USA) according to the manufacturer's protocol, and checked for purity with NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA).

The 16S rRNA gene and the selected ARGs were quantified by qPCR in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA). Each qPCR reaction contained 1x iQ™ SYBR Green Supermix (Bio-Rad), 300 mM of both forward and reverse primers (except for *ermB* and bla_{TEM} where 400 mM was used), 2 µg of bovine serum albumin (BSA; Thermo Scientific, USA), and 2 µL of 10-fold diluted DNA (1-10 ng), in a final volume of 20 µL (Table M2). The following thermal cycling conditions were applied: 95 °C for 10 min (1 cycle); 95 °C for 15 s, 60 °C for 1 min (except for *sul1*, which required 65 °C), in 40 cycles. Samples were tested with a standard curve ranging between 10^{-3} – 10^{-7}, or 10^{-5} – 10^{-9} target copies of standardized gene fragments (gBlocks) for the 16S rRNA gene, or ARGs, respectively, and non-template controls for each run. Cut-off values were calculated based on the lowest amount of genes included in the calibration curve of all plates. All calibration curves had

a signal intensity of >2 ct difference to the non-template controls (only for 16S rRNA gene, other genes did not show amplification in non-template controls). The average of ct obtained from this point across all plates was taken, and the standard deviation added to it. Melting curves were performed to confirm the specificity of each reaction, starting at 65 °C with successive increments of 0.5 °C, up to 95 °C. Quantifications for each extract was performed in duplicates, following the Standard Curve method described elsewhere (Brankatschk et al., 2012). Possible qPCR inhibition was verified by quantifying the 16S rRNA gene using 10-, 100-, and 1000-fold diluted extracts. The primer sets and concentrations used in this study can be found in the supplemental information (Table S2). Predicted values were estimated based on the average ARG concentration in manure and soil previous to amendment, on the amount of manure applied and soil sampled (top 10 cm) per hectare, and on assumed densities of 1.0 and 1.5 kg/dm3 for manure and soil, respectively.

2.3.5 Data Analysis

For the comparison of gene levels (log copies/kg soil) before and after manuring, outcome variables were tested for normality, and when verified, an analysis of variance (ANOVA) was applied, using Tukey post-hoc analysis. When normality was not achieved, group comparison was performed using the equivalent non-parametric test (Kruskal-Wallis). For analysis of decay rates of gene levels (log copies/kg soil), linear mixed models were used treating the farms and the rounds of manuring as random effects. Because higher gene levels were observed at T2 than at T1, the first three days (i.e., T0 and T1) after manure application were excluded from the models for soil and water models. Relevant factors were identified through model reduction. The full model included the following variables as fixed effects: days after manure application (4-21 days), gene type, the soil texture, the amount of rain, the interaction between days after manuring and soil texture, and interaction between days after manuring and gene type. Resulting models were inspected for normality of residues, and significance scores of $p < 0.05$ were considered for all performed tests. The half-lives were calculated based on the slope of the models. The statistical analyses were performed with R version 3.5.1 (R Core Team, 2020) and RStudio (Version 1.1.456; https://www.rstudio.com/) using the software packages *dplyr* (Wickham et al., 2015), *tidyr* (Wickham and Henry, 2019) and *reshape2* (Wickham, 2007) to trim, clean, and transform data; *lme4* (Bates et al., 2015), *lmerTest* (Kuznetsova et al., 2017), and *MuMIn* (Barton, 2018) to create the models; *Rcmdr* (Fox, 2005), and *sjstats* (Lüdecke, 2019) to perform the group comparisons; and *ggplot2* (Wickham, 2016) to produce the graphs.

2.4 Results and Discussion

2.4.1 Gene identity determines ARG persistence in soil fertilized with dairy manure

In this study, ARG levels were measured to understand the role of manure and soil type on the persistence of AMR in grasslands and adjacent watercourses after manure application. Across soil textures, a significant increase of *erm(B)* and *tet(W)* was observed directly after manuring (Figure 1), followed by a gradual decrease, while *sul1* levels remained roughly constant throughout time (Figure 2). Overall, the decay rates differed between genes, but the soil textures did not affect resistance gene decay rates. bla_{TEM} was only incidentally detected in the manure samples used in the first round of manuring from farms F01 and F05 (8.75 and 8.83 log copies/kg, respectively). Thus, it was excluded from further analysis.

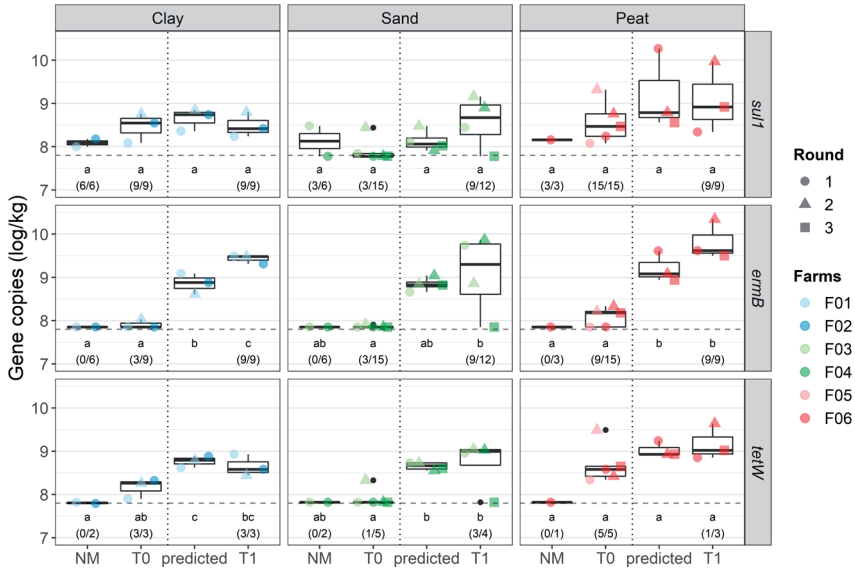


Figure 1. ARG concentrations in soil samples that were never manured (NM), and before (T0) and after (T1) manure application on farms using different manure types and manure application systems. Predicted values were estimated based on the average ARG concentration in manure and soil previous to amendment, on the amount of manure applied and soil sampled, and on assumed densities of 1.0 and 1.5 kg/dm^3 for manure and soil, respectively. The horizontal dashed bar represents the limits of quantification (LOQ), which were determined based on the experimentally defined cut-off values for qPCR (7.78, 7.85, and 7.82 log copies/kg of soil, for *sul1*, *erm(B)*, and *tet(W)*, respectively). Values between brackets represent the number of DNA replicates above the LOQ used to calculate the represented averages. $^{a, b, c}$ Indicate significantly different groups ($p < 0.05$) of gene concentrations between each time point.

The *erm(B)* levels increased significantly after manuring ($p < 0.01$). After being transported from manure (manure concentrations 10.25 – 11.30 log copies/kg; Table S3) and reaching its highest concentrations in soils, *erm(B)* decreased by roughly -0.05 log copies/kg per day across soil textures ($t_{1/2}$ = 7 days; Table 2; $p < 0.01$), as predicted from linear mixed models that were used to relate the gene decay to soil texture and gene identity (Table 2). These findings are corroborated by Tien et al. (2017), who found an identical reduction of this ARG in soil 30 days after manure application. In the present study, *erm(B)* levels in soils immediately after manuring differed between the rounds of manure application, but ARG decay was identical.

Moreover, *erm(B)* was not detected in never-manured soils (NM), nor before the first spring manure amendment (T0; Figure 1). Recently, it has been found that *erm(B)* was only detected in soils amended with either pig or cattle manure, and not in soils that either received chemical fertilizer or were not fertilized (Peng et al., 2017). Others were able to detect *erm(B)* in composite and band samples of manure-injected soils, but not between the manure bands of the same soils (Luby et al., 2016). This suggests that *erm(B)* is mainly introduced to soils via manure application, and our findings support this hypothesis. According to the Comprehensive Antibiotic Resistance Database (CARD) (Jia et al., 2017), *erm(B)* is often found in Gram-positive bacteria, namely in *Enterococcus* spp. and the obligate anaerobic *Clostridium* spp., which are common manure microbiota (Hodgson et al., 2016; Leclercq et al., 2016). The relatively fast decay of *erm(B)* could be related to the decline of *Clostridia*, as they are less fit to thrive in aerobic soil habitats (Pourcher et al., 2007).

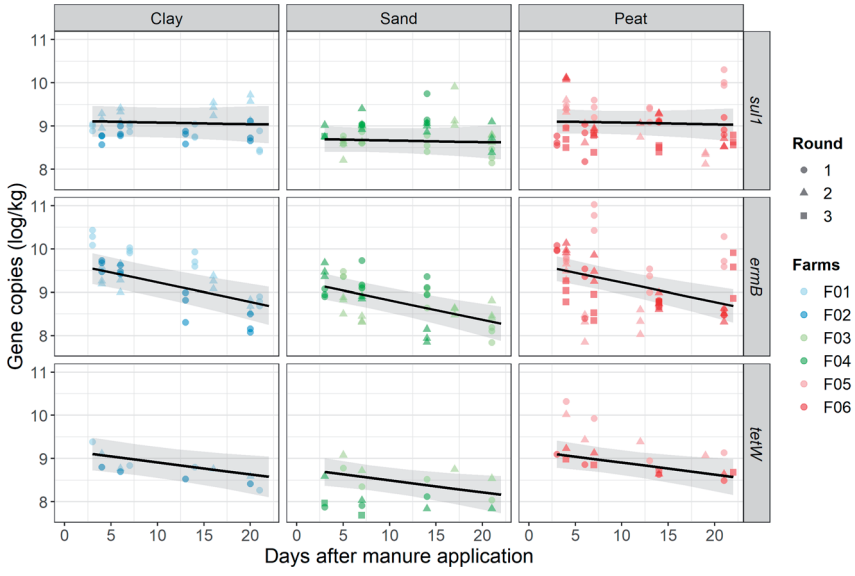


Figure 2. ARG level in soil samples after manure application in farms from different soil textures.

Despite being found in manure (10.20 – 10.83 log copies/kg), and in contrast to *erm(B)* findings, *tet(W)* was present in NM samples and at T0 (Figure 1) and showed a slower decay rate than *erm(B)* ($t_{1/2}$ = 11 days; Table 2). This finding is in accordance with recent publications (Fahrenfeld et al., 2014; McKinney et al., 2018), where *tet(W)* could be quantified in soils before manure application, but at higher levels than the ones described here. However, in two studies focusing on the prevalence of ARG after long-term manure application (Li et al., 2017; Peng et al., 2017), *tet(W)* was not found in the non-manured soils used as controls. A similar decay of *tet(W)* was found 20 days after the application of poultry litter in untilled soils (Cook et al., 2014), and after the application of cattle manure in soils with different pastures (Kyselková et al., 2015), although in these studies fewer time points were measured. In one of those long-term studies, where soils were continuously applied with different manures for 30 years (Peng et al., 2017), *Firmicutes*, *Gammaproteobacteria,* and *Bacteroidetes* were found positively correlated with most of the ARGs, including *tet(W)*. These phyla represent bacteria commonly found in the gut, whose adaptability to agricultural environments (e.g., manure, soils) differs – *Clostridia* and *Bacteroidetes* represent obligate anaerobes that will quickly die-off in mostly aerobic soils, in contrast to *Bacilli*. Given this condition, the relatively moderate decay rate of *tet(W)* could be explained by the survival potential of its host bacteria. Recently, different bacterial hosts have been associated with single types of ARGs (Stalder et al., 2018), reinforcing the argument that the bacterial hosts can vary greatly, and that fate of genes will depend to a large extent on the fate of host bacteria. An alternative hypothesis would be that the application of manure, and its organic compounds and other components, could stimulate the soil bacterial communities that carry the targeted ARGs (Udikovic-Kolic et al., 2014; W.-Y. Xie et al., 2018), thus contributing to the maintenance (slow decay) of the ARGs. This might be particularly relevant for the case of *tet(W)* since it was detected before manure application (T0) and in NM samples.

The *sul1* gene was present in manure (9.33 – 11.96 log copies/kg), but did not increase after manure application, and consequently, the decay rate was the lowest of the targeted ARGs in this study (-0,01 log units per day; $t_{1/2}$ = 75 days; Table 2; $p < 0.01$). The constant levels of *sul1* (Figure 2; $p = 0.15$) can be explained by the high prevalence of this ARG in soils previous to manure application (NM and T0; Figure 1), which confirms the ubiquity of *sul1* in the environment (Gillings et al., 2008). Recently, Wang *et al.* (2017) also observed that *sul1* had the lowest decay rates in manure-amended soil microcosms, over 96 days. Nevertheless, that decay rate was calculated based on the ARG relative abundance (ARG copies/16S rRNA copies), which limits the comparison with the results of this study. However, in contrast to our findings, during a microcosm study where manure, with and without antibiotics, was applied to different soil textures (Heuer and Smalla, 2007), an increase of *sul1* in both soils was observed after manure application, and after 32 days, the *sul1* levels had decreased close to 1 log, which corresponded to a much higher decay rate than in the current study (roughly -0.03 log units per day). In other field studies, the *sul1*

levels after application of dairy manure were similar to the ones in the current study, even though it was increased after manuring (Munir and Xagoraraki, 2011; Nõlvak et al., 2016). Increases of *sul1* after manuring were also observed by (McKinney et al., 2018).

Furthermore, the results showed that in most cases, there was not a complete recovery of soil resistome from one round to the next. According to the model-predicted decay rates, it would take on average 42 days (47 and 33 days in round 1 and 2, respectively) for the levels of *erm(B)* to decrease to the LOQ levels before manure application in clayey soils. In sandy soils, the model predicted that it would take an average of 29 days for *erm(B)* levels to decrease back to original levels, while in peaty soils, *erm(B)* levels were predicted to decrease to LOQ in roughly 40 days. Similar trends were also found for *tet(W)*, as it would take on average 38, 36, and 37 days for the *tet(W)* levels to decrease to the levels found before the first application of manure in clayey, sandy, and peaty soils, respectively. As the pasture soils are often repeatedly manure-fertilized after 34 – 80 days, soil resistance levels do not recover entirely between manuring rounds. Decay at later timepoints might be related to the input of fresh manure from grazing cows.

Table 2. Best models for soil and water samples in farms with different soil textures (excl. 0-3 days after manuring).

Sample Type	Explanatory Factor	Estimate (log/kg or log/L)	Std. Error	p-score
Soil	(Intercept)	9.6	0.19	<0.01
	Gene decay per day	-0.05	0.01	<0.01
	tet(W)	-0.5	0.1	<0.01
	sul1	-0.5	0.1	<0.01
	Soil Texture: Sand	-0.4	0.2	0.09
	Soil Texture: Peat	-0.0	0.2	0.99
	Rainfall	0.04	0.01	<0.01
	Interaction: Days and *tet(W)*	0.02	0.01	0.08
	Interaction: Days and *sul1*	0.04	0.01	<0.01
Water	(Intercept)	7.8	0.5	<0.01
	Gene decay	-0.15	0.02	<0.01
	tet(W)	-0.26	0.25	0.29
	sul1	-0.4	0.2	0.01
	Soil Texture: Sand	-1.7	0.7	0.04
	Soil Texture: Peat	-1.7	0.7	0.03
	Rainfall	0.04	0.02	0.01
	Interaction: Days and *tet(W)*	0.04	0.02	0.07
	Interaction: Days and *sul1*	0.05	0.01	<0.01
	Interaction: Days and Sand	0.12	0.03	<0.01
	Interaction: Days and Peat	0.10	0.03	<0.01

In contrast to gene identity, the soil texture did not affect gene decay rates in soil samples. This was shown in the linear mixed models by non-significant interactions between soil texture and time after manuring, and from the fact that this interaction was not included in the final, best models after model reduction (Table 2). It was initially hypothesized that the soil texture would play a significant role in the fate of ARGs in manure-amended soils. The fate of added microorganisms can vary with soil texture, as shown for *E. coli*, for which survival was lower in organically managed sandy soils (Franz et al., 2005). Recently, it has been found that physicochemical properties, such as heavy metals, moisture content, and organic matter, can affect the decay kinetics of some ARGs in soils (Sui et al., 2019). However, according to the results observed in this study, the type of ARG rather than the texture of soil was the determining variable affecting the decay of the measured ARGs (Table 2). This discrepancy might indicate that the fate of the resistance gene host and the background of resistance genes has stronger effects than the soil textures. Additionally, the rhizosphere might also contribute to the maintenance of the gene in soils as it is a known hotspot for horizontal gene transfer because they promote the occurrence of high densities of active cells (Jechalke et al., 2013; Kopmann et al., 2013; Van Elsas et al., 2003).

E. coli is commonly used as an indicator organism of fecal contamination, and it was tested to evaluate whether fecal bacteria would be able to remain viable in manured soils throughout the sampling time frame, complementing resistance gene measurements, which also detect genes from dead cells posing smaller public health risks. *E. coli* was only detected after manure application (except in round 3 of farms with peaty soil), and although no clear survival trends were observed, *E. coli* was more abundant at round 2, and it was still quantifiable three weeks after manuring in all soil types, except in farms with sandy soils (Figure S2).

Rainfall had an overall increasing effect on the levels of ARG found in soils according to the linear mixed models (Table 2). Rainfall has been linked to the transport of *erm* and *tet* genes in agricultural runoffs (Joy et al., 2013; Soni et al., 2015), supposedly through mobilization from the upper soil fraction through infiltration and surface transportation. Also, the shorter survival of *E. coli* has been found in soils with higher moisture content (Oliver et al., 2006; Rothrock et al., 2012). Given this, one would assume that the ARG levels in topsoil would decrease after rainfall; however, that was not the case. On the other hand, the water content of a soil microcosm set-up had a negligible effect on the decay of ARGs (Sandberg and LaPara, 2016). Also, the findings of this study are consistent with the findings of Joy et al. (2013), where the levels of *erm* and *tet* genes increased in the top manure-broadcasted soils even after three rainfall events. This increase is likely to be due to the dissemination of the manure bands in the field caused by the rainfall, making it more homogeneous, but it can also be due to ARG-carrying bacterial growth.

2.4.2 Soil texture determines ARG persistence in water

In the ditch water samples, the rates of the ARG decrease (i.e., slope) were similar within the same soil texture, regardless of the ARG (Figure 3). The ARG levels decreased quicker in ditches than in soil: by roughly -0.15 ($t_{1/2}$ = 2 days; $p < 0.01$), -0.03 ($t_{1/2}$ = 10 days; $p < 0.01$), and -0.05 ($t_{1/2}$ = 6 days; $p < 0.01$) log copies / L per day in clay, sand, and peat, respectively (Table 2).

ARG transport from the fields to the ditches can depend on rainfall as well as on the strength of bacterial and gene sorption to soil particles, which in turn can depend on the soil type. Here, water samples from two clayey soils showed to have the highest ARG decline. This might be related to rainfall-induced transport of ARG to the ditches shortly after manure fertilization: on two of the three sampling occasions in clayey soils, more than 8 mm precipitation per day occurred shortly after manuring. As the permeability of clay particles is low (Schramm et al., 1986), surface run-off from clay might, therefore, have resulted in peaks of resistance genes in the receiving water early on. On the other hand, high levels of ARGs have also been found in farm F01 in round 1 in water, although this represented the only water sample taken at the outlet of a drainage pipe and, therefore, representing soil infiltrate. Thus, in clay soils, infiltration is another mechanism of transfer to adjacent ditches next to surface run-off, in contrast to high sorption of bacteria (Cho et al., 2016; Pachepsky et al., 2006) and nucleic acids (Ogram et al., 1988) to clayey soils. Arguably, even though the water ditches were surrounded solely by fields owned by one farmer, adjacent soil parcels owned by the same farmer can follow a different manure application cycle, possibly leading to additional leaching into the sampled ditches. It should be noted that the ARG persistence within ditches depends on hydrological parameters that determine the dilution of soil run-off, such as flow and volume of ditches, which were not recorded in this study.

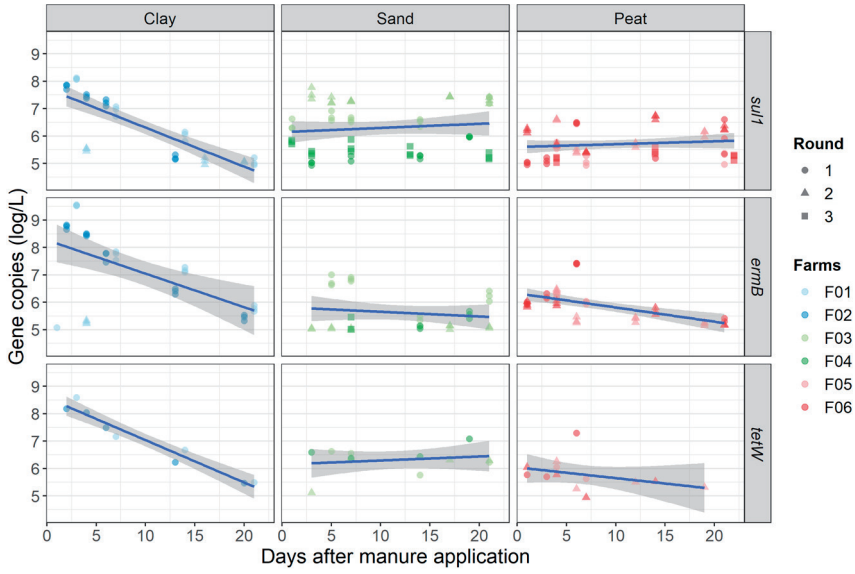


Figure 3. ARG level in water samples after manure application in farms from different soil textures.

2.5 Conclusions

In this field study, the role of the soil texture on the dynamics of AMR in soils and adjacent surface waters was addressed. Overall, this study demonstrated that the persistence of the measured ARGs in soils differed, and largely depended on the type of gene. The descending decay rates (*sul1* > *tet(W)* > *erm(B)*) were related to the level of the ARG prior to manure application and possibly influenced by the different survival capabilities of the bacteria hosting these ARGs in soils. Nonetheless, in water samples, the texture of soil to which manure was applied determined the persistence of the targeted ARGs (clay < peat < sand), thus affecting the fate of AMR in the environment. Finally, ARG levels were predicted to recover to levels before manure fertilization after 29 – 42 days of manure application, i.e., do not recover between rounds of manure application. To conclude, this study demonstrates that rather than showing similar dynamics of decay, factors such as the type of ARG and soil texture drive the ARG persistence in the environment.

2.6 Acknowledgments

This work was performed in the cooperation framework of Wetsus, European Centre of excellence for sustainable water technology (www.wetsus.nl). Wetsus is co-funded by the Dutch Ministry of Economic Affairs and Ministry of Infrastructure and Environment, the European Union Regional Development Fund, the Province of Fryslân and the Northern Netherlands Provinces. This work has also received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie [grant agreement No. 665874]. The authors like to thank the participants of the research theme Source Separated Sanitation for the fruitful discussions and their financial support. Additionally, the authors gratefully acknowledge the support of the farmers who voluntarily accepted to participate and allowed sample collection; the veterinarians Bas Landerman and Maaike van den Berg for helping to recruit and persuade the participants; the former students Harm de Haan, Esther Meinderts, and Lydia Speijker for their technical assistance in collecting samples and qPCR measurements; and Rebeca Pallares-Vega for the support during qPCR method development and data processing.

2.7 Supplementary Information

Supplementary data can be found online at
https://doi.org/10.1016/j.scitotenv.2020.139563.

2.8 References

Agga, G.E., Arthur, T.M., Durso, L.M., Harhay, D.M., Schmidt, J.W., 2015. Antimicrobial-resistant bacterial populations and antimicrobial resistance genes obtained from environments impacted by livestock and municipal waste. PLoS One 10, 1–19. https://doi.org/10.1371/journal.pone.0132586

Baker, M., Hobman, J.L., Dodd, C.E.R., Ramsden, S.J., Stekel, D.J., 2016. Mathematical modelling of antimicrobial resistance in agricultural waste highlights importance of gene transfer rate. FEMS Microbiol. Ecol. 92, fiw040. https://doi.org/10.1093/femsec/fiw040

Barton, K., 2018. MuMIn: Multi-Model Inference. R Packag. version 1.42.1.

Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting Linear Mixed-Effects Models Using lme4. J. Stat. Softw. 67, 1--48. https://doi.org/10.18637/jss.v067.i01

Blau, K., Casadevall, L., Wolters, B., Van den Meersche, T., Kreuzig, R., Smalla, K., Jechalke, S., 2018. Soil texture-depending effects of doxycycline and streptomycin applied with manure on the bacterial community composition and resistome. FEMS Microbiol. Ecol. 94, 1–11. https://doi.org/10.1093/femsec/fix145

Brankatschk, R., Bodenhausen, N., Zeyer, J., Burgmann, H., 2012. Simple absolute quantification method correcting for quantitative PCR efficiency variations for microbial community samples. Appl. Environ. Microbiol. 78, 4481–4489. https://doi.org/10.1128/AEM.07878-11

Chee-Sanford, J.C., Mackie, R.I., Koike, S., Krapac, I.G., Lin, Y.-F., Yannarell, A.C., Maxwell, S., Aminov, R.I., 2009. Fate and Transport of Antibiotic Residues and Antibiotic Resistance Genes following Land Application of Manure Waste. J. Environ. Qual. 38, 1086. https://doi.org/10.2134/jeq2008.0128

Chen, Z., Zhang, W., Yang, L., Stedtfeld, R.D., Peng, A., Gu, C., Boyd, S.A., Li, H., 2019. Antibiotic resistance genes and bacterial communities in cornfield and pasture soils receiving swine and dairy manures. Environ. Pollut. 248, 947–957. https://doi.org/10.1016/j.envpol.2019.02.093

Cheng, J.-H., Tang, X.-Y., Cui, J.-F., 2019. Effect of long-term manure slurry application on the occurrence of antibiotic resistance genes in arable purple soil (entisol). Sci. Total Environ. 647, 853–861. https://doi.org/10.1016/j.scitotenv.2018.08.028

Chessa, L., Jechalke, S., Ding, G.-C., Pusino, A., Mangia, N.P., Smalla, K., 2016. The presence of tetracycline in cow manure changes the impact of repeated manure application on soil bacterial communities. Biol. Fertil. Soils 52, 1121–1134. https://doi.org/10.1007/s00374-016-1150-4

Cho, K.H., Pachepsky, Y.A., Oliver, D.M., Muirhead, R.W., Park, Y., Quilliam, R.S., Shelton, D.R., 2016. Modeling fate and transport of fecally-derived microorganisms at the watershed scale: State of the science and future opportunities. Water Res. 100, 38–56. https://doi.org/10.1016/j.watres.2016.04.064

Cook, K.L., Netthisinghe, A.M.P., Gilfillen, R.A., 2014. Detection of Pathogens, Indicators, and Antibiotic Resistance Genes after Land Application of Poultry Litter. J. Environ. Qual. 43, 1546. https://doi.org/10.2134/jeq2013.10.0432

de Greeff, S.C., Mouton, J.W., 2018. Nethmap 2018: Consumption of antimicrobial agents and antimicrobial resistance among medically important bacteria in the Netherlands / Maran 2018:

Monitoring of antimicrobial resistance and antibiotic usage in animals in the Netherlands in 2017. https://doi.org/10.21945/RIVM-2018-0046

de Greeff, S.C., Mouton, J.W., 2017. Nethmap 2017: Consumption of antimicrobial agents and antimicrobial resistance among medically important bacteria in the Netherlands / Maran 2017: Monitoring of antimicrobial resistance and antibiotic usage in animals in the Netherlands in 2016. https://doi.org/10.21945/RIVM-2017-0056

ECDC, EFSA, EMA, 2017. ECDC/EFSA/EMA second joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals. EFSA J. 15, 4006. https://doi.org/10.2903/j.efsa.2017.4872

Fahrenfeld, N., Knowlton, K., Krometis, L.A., Hession, W.C., Xia, K., Lipscomb, E., Libuit, K., Green, B.L., Pruden, A., 2014. Effect of Manure Application on Abundance of Antibiotic Resistance Genes and Their Attenuation Rates in Soil: Field-Scale Mass Balance Approach. Environ. Sci. Technol. 48, 2643–2650. https://doi.org/10.1021/es404988k

Forsberg, K.J., Patel, S., Gibson, M.K., Lauber, C.L., Knight, R., Fierer, N., Dantas, G., 2014. Bacterial phylogeny structures soil resistomes across habitats. Nature 509, 612–616. https://doi.org/10.1038/nature13377

Fox, J., 2005. The R Commander: A Basic Statistics Graphical User Interface to R. J. Stat. Softw. 14, 1--42.

Franz, E., Schijven, J., de Roda Husman, A.M., Blaak, H., 2014. Meta-Regression Analysis of Commensal and Pathogenic Escherichia coli Survival in Soil and Water. Environ. Sci. Technol. 48, 6763–6771. https://doi.org/10.1021/es501677c

Franz, E., van Diepeningen, A.D., de Vos, O.J., van Bruggen, A.H.C., 2005. Effects of Cattle Feeding Regimen and Soil Management Type on the Fate of Escherichia coli O157:H7 and Salmonella enterica Serovar Typhimurium in Manure, Manure-Amended Soil, and Lettuce. Appl. Environ. Microbiol. 71, 6165–6174. https://doi.org/10.1128/AEM.71.10.6165-6174.2005

Gillings, M.R., Boucher, Y., Labbate, M., Holmes, A., Krishnan, S., Holley, M., Stokes, H.W., 2008. The Evolution of Class 1 Integrons and the Rise of Antibiotic Resistance. J. Bacteriol. 190, 5095–5100. https://doi.org/10.1128/JB.00152-08

Girvan, M.S., Bullimore, J., Pretty, J.N., Osborn, A.M., Ball, A.S., 2003. Soil type is the primary determinant of the composition of the total and active bacterial communities in arable soils. Appl. Environ. Microbiol. 69, 1800–1809. https://doi.org/10.1128/AEM.69.3.1800–1809.2003

Gou, M., Hu, H.-W., Zhang, Y.-J., Wang, J.-T., Hayden, H., Tang, Y.-Q., He, J.-Z., 2018. Aerobic composting reduces antibiotic resistance genes in cattle manure and the resistome dissemination in agricultural soils. Sci. Total Environ. 612, 1300–1310. https://doi.org/10.1016/j.scitotenv.2017.09.028

Han, X.-M., Hu, H.-W., Chen, Q.-L., Yang, L.-Y., Li, H.-L., Zhu, Y.-G., Li, X.-Z., Ma, Y.-B., 2018. Antibiotic resistance genes and associated bacterial communities in agricultural soils amended with different sources of animal manures. Soil Biol. Biochem. 126, 91–102. https://doi.org/10.1016/j.soilbio.2018.08.018

He, L.Y., Ying, G.G., Liu, Y.S., Su, H.C., Chen, J., Liu, S.S., Zhao, J.L., 2016. Discharge of swine wastes risks water quality and food safety: Antibiotics and antibiotic resistance genes from swine sources to the receiving environments. Environ. Int. 92–93, 210–219. https://doi.org/10.1016/j.envint.2016.03.023

Heuer, H., Schmitt, H., Smalla, K., 2011. Antibiotic resistance gene spread due to manure application on agricultural fields. Curr. Opin. Microbiol. 14, 236–243. https://doi.org/10.1016/j.mib.2011.04.009

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. https://doi.org/10.1111/j.1462-2920.2006.01185.x

Hodgson, C.J., Oliver, D.M., Fish, R.D., Bulmer, N.M., Heathwaite, A.L., Winter, M., Chadwick, D.R., 2016. Seasonal persistence of faecal indicator organisms in soil following dairy slurry application to land by surface broadcasting and shallow injection. J. Environ. Manage. 183, 325–332. https://doi.org/10.1016/j.jenvman.2016.08.047

Hoelzer, K., Wong, N., Thomas, J., Talkington, K., Jungman, E., Coukell, A., 2017. Antimicrobial drug use in food-producing animals and associated human health risks: what, and how strong, is the evidence? BMC Vet. Res. 13, 211. https://doi.org/10.1186/s12917-017-1131-3

Jechalke, S., Heuer, H., Siemens, J., Amelung, W., Smalla, K., 2014. Fate and effects of veterinary antibiotics in soil. Trends Microbiol. 22, 536–545. https://doi.org/10.1016/j.tim.2014.05.005

Jechalke, S., Kopmann, C., Rosendahl, I., Groeneweg, J., Weichelt, V., Krögerrecklenfort, E., Brandes, N., Nordwig, M., Ding, G.-C., Siemens, J., Heuer, H., Smalla, K., 2013. Increased Abundance and Transferability of Resistance Genes after Field Application of Manure from Sulfadiazine-Treated Pigs. Appl. Environ. Microbiol. 79, 1704–1711. https://doi.org/10.1128/aem.03172-12

Jia, B., Raphenya, A.R., Alcock, B., Waglechner, N., Guo, P., Tsang, K.K., Lago, B.A., Dave, B.M., Pereira, S., Sharma, A.N., Doshi, S., Courtot, M., Lo, R., Williams, L.E., Frye, J.G., Elsayegh, T., Sardar, D., Westman, E.L., Pawlowski, A.C., Johnson, T.A., Brinkman, F.S.L., Wright, G.D., McArthur, A.G., 2017. CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. Nucleic Acids Res. 45, D566–D573. https://doi.org/10.1093/nar/gkw1004

Joy, S.R., Bartelt-Hunt, S.L., Snow, D.D., Gilley, J.E., Woodbury, B.L., Parker, D.B., Marx, D.B., Li, X., 2013. Fate and Transport of Antimicrobials and Antimicrobial Resistance Genes in Soil and Runoff Following Land Application of Swine Manure Slurry. Environ. Sci. Technol. 47, 12081–12088. https://doi.org/10.1021/es4026358

Kopmann, C., Jechalke, S., Rosendahl, I., Groeneweg, J., Krögerrecklenfort, E., Zimmerling, U., Weichelt, V., Siemens, J., Amelung, W., Heuer, H., Smalla, K., 2013. Abundance and transferability of antibiotic resistance as related to the fate of sulfadiazine in maize rhizosphere and bulk soil. FEMS Microbiol. Ecol. 83, 125–134. https://doi.org/10.1111/j.1574-6941.2012.01458.x

Kuznetsova, A., Brockhoff, P.B., Christensen, R.H.B., 2017. lmerTest Package: Tests in Linear Mixed Effects Models. J. Stat. Softw. 82, 1--26. https://doi.org/10.18637/jss.v082.i13

Kyselková, M., Kotrbová, L., Bhumibhamon, G., Chroňáková, A., Jirout, J., Vrchotová, N., Schmitt, H., Elhottová, D., 2015. Tetracycline resistance genes persist in soil amended with cattle feces

independently from chlortetracycline selection pressure. Soil Biol. Biochem. 81, 259–265. https://doi.org/10.1016/j.soilbio.2014.11.018

Leclercq, S.O., Wang, C., Sui, Z., Wu, H., Zhu, B., Deng, Y., Feng, J., 2016. A multiplayer game: species of Clostridium , Acinetobacter , and Pseudomonas are responsible for the persistence of antibiotic resistance genes in manure-treated soils. Environ. Microbiol. 18, 3494–3508. https://doi.org/10.1111/1462-2920.13337

Li, J., Xin, Z., Zhang, Y., Chen, J., Yan, J., Li, H., Hu, H., 2017. Long-term manure application increased the levels of antibiotics and antibiotic resistance genes in a greenhouse soil. Appl. Soil Ecol. 121, 193–200. https://doi.org/10.1016/j.apsoil.2017.10.007

Luby, E.M., Moorman, T.B., Soupir, M.L., 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. https://doi.org/10.1016/j.scitotenv.2016.01.132

Lüdecke, D., 2019. sjstats: Statistical Functions for Regression Models (Version 0.17.4). https://doi.org/10.5281/zenodo.1284472

McKinney, C.W., Dungan, R.S., Moore, A., Leytem, A.B., 2018. Occurrence and abundance of antibiotic resistance genes in agricultural soil receiving dairy manure. FEMS Microbiol. Ecol. 94, 1–10. https://doi.org/10.1093/femsec/fiy010

Munir, M., Xagoraraki, I., 2011. Levels of Antibiotic Resistance Genes in Manure, Biosolids, and Fertilized Soil. J. Environ. Qual. 40, 248. https://doi.org/10.2134/jeq2010.0209

Muurinen, J., Stedtfeld, R., Karkman, A., Pärnänen, K., Tiedje, J., Virta, M., 2017. Influence of Manure Application on the Environmental Resistome under Finnish Agricultural Practice with Restricted Antibiotic Use. Environ. Sci. Technol. 51, 5989–5999. https://doi.org/10.1021/acs.est.7b00551

Nõlvak, H., Truu, M., Kanger, K., Tampere, M., Espenberg, M., Loit, E., Raave, H., Truu, J., 2016. Inorganic and organic fertilizers impact the abundance and proportion of antibiotic resistance and integron-integrase genes in agricultural grassland soil. Sci. Total Environ. 562, 678–689. https://doi.org/10.1016/j.scitotenv.2016.04.035

Ogram, A., Sayler, G.S., Gustin, D., Lewis, R.J., 1988. DNA adsorption to soils and sediments. Environ. Sci. Technol. 22, 982–984. https://doi.org/10.1021/es00173a020

Oliver, D.M., Haygarth, P.M., Clegg, C.D., Heathwaite, A.L., 2006. Differential E. coli Die-Off Patterns Associated with Agricultural Matrices. Environ. Sci. Technol. 40, 5710–5716. https://doi.org/10.1021/es0603249

One Health [WWW Document], 2019. URL http://www.euro.who.int/en/health-topics/disease-prevention/antimicrobial-resistance/about-amr/one-health (accessed 11.13.19).

Pachepsky, Y.A., Sadeghi, A.M., Bradford, S.A., Shelton, D.R., Guber, A.K., Dao, T., 2006. Transport and fate of manure-borne pathogens: Modeling perspective. Agric. Water Manag. 86, 81–92. https://doi.org/10.1016/j.agwat.2006.06.010

Peng, S., Feng, Y., Wang, Y., Guo, X., Chu, H., Lin, X., 2017. Prevalence of antibiotic resistance genes in soils after continually applied with different manure for 30 years. J. Hazard. Mater. 340, 16–25. https://doi.org/10.1016/j.jhazmat.2017.06.059

Pourcher, A.-M., Françoise, P.-B., Virginie, F., Agnieszka, G., Vasilica, S., Gérard, M., 2007. Survival of faecal indicators and enteroviruses in soil after land-spreading of municipal sewage sludge. Appl. Soil Ecol. 35, 473–479. https://doi.org/10.1016/j.apsoil.2006.10.005

Pu, C., Liu, H., Ding, G., Sun, Y., Yu, X., Chen, J., Ren, J., Gong, X., 2018. Impact of direct application of biogas slurry and residue in fields: In situ analysis of antibiotic resistance genes from pig manure to fields. J. Hazard. Mater. 344, 441–449. https://doi.org/10.1016/j.jhazmat.2017.10.031

R Core Team, 2020. R: A Language and Environment for Statistical Computing.

Rothrock, M.J., Frantz, J.M., Burnett, S., 2012. Effect of Volumetric Water Content and Clover (Trifolium incarnatum) on the Survival of Escherichia coli O157:H7 in a Soil Matrix. Curr. Microbiol. 65, 272–283. https://doi.org/10.1007/s00284-012-0142-3

Sandberg, K.D., LaPara, T.M., 2016. The fate of antibiotic resistance genes and class 1 integrons following the application of swine and dairy manure to soils. FEMS Microbiol. Ecol. 92, fiw001. https://doi.org/10.1093/femsec/fiw001

Schramm, M., Warrick, A.W., Fuller, W.H., 1986. Permeability of Soils to Four Organic Liquids and Water. Hazard. Waste Hazard. Mater. 3, 21–27. https://doi.org/10.1089/hwm.1986.3.21

Soni, B., Bartelt-Hunt, S.L., Snow, D.D., Gilley, J.E., Woodbury, B.L., Marx, D.B., Li, X., 2015. Narrow Grass Hedges Reduce Tylosin and Associated Antimicrobial Resistance Genes in Agricultural Runoff. J. Environ. Qual. 44, 895. https://doi.org/10.2134/jeq2014.09.0389

Stalder, T., Press, M.O., Sullivan, S., Liachko, I., Top, E.M., 2018. Linking the Resistome and Plasmidome to the Microbiome. bioRxiv 484725. https://doi.org/10.1101/484725

Sui, Q., Zhang, J., Chen, M., Wang, R., Wang, Y., Wei, Y., 2019. Fate of microbial pollutants and evolution of antibiotic resistance in three types of soil amended with swine slurry. Environ. Pollut. 245, 353–362. https://doi.org/10.1016/j.envpol.2018.11.003

Tien, Y.-C., Li, B., Zhang, T., Scott, A., Murray, R., Sabourin, L., Marti, R., Topp, E., 2017. Impact of dairy manure pre-application treatment on manure composition, soil dynamics of antibiotic resistance genes, and abundance of antibiotic-resistance genes on vegetables at harvest. Sci. Total Environ. 581–582, 32–39. https://doi.org/10.1016/j.scitotenv.2016.12.138

Topp, E., Larsson, D.G.J., Miller, D.N., Van den Eede, C., Virta, M.P.J., 2018. Antimicrobial resistance and the environment: assessment of advances, gaps and recommendations for agriculture, aquaculture and pharmaceutical manufacturing. FEMS Microbiol. Ecol. 94, 1–5. https://doi.org/10.1093/femsec/fix185

Udikovic-Kolic, N., Wichmann, F., Broderick, N.A., Handelsman, J., 2014. Bloom of resident antibiotic-resistant bacteria in soil following manure fertilization. Proc. Natl. Acad. Sci. U. S. A. 111, 15202–7. https://doi.org/10.1073/pnas.1409836111

Van Elsas, J.D., Turner, S., Bailey, M.J., 2003. Horizontal gene transfer in the phytosphere. New Phytol. 157, 525–537. https://doi.org/10.1046/j.1469-8137.2003.00697.x

van Veen, J.A., van Overbeek, L.S., van Elsas, J.D., 1997. Fate and activity of microorganisms introduced into soil. Microbiol. Mol. Biol. Rev. 61, 121–135. https://doi.org/10.1128/.61.2.121-135.1997

Volkova, V. V., Lu, Z., Lanzas, C., Scott, H.M., Gröhn, Y.T., 2013. Modelling dynamics of plasmid-gene mediated antimicrobial resistance in enteric bacteria using stochastic differential equations. Sci. Rep. 3, 2463. https://doi.org/10.1038/srep02463

Wang, F., Xu, M., Stedtfeld, R.D., Sheng, H., Fan, J., Liu, M., Chai, B., Soares de Carvalho, T., Li, H., Li, Z., Hashsham, S.A., Tiedje, J.M., 2018. Long-Term Effect of Different Fertilization and Cropping Systems on the Soil Antibiotic Resistome. Environ. Sci. Technol. 52, 13037–13046. https://doi.org/10.1021/acs.est.8b04330

Wang, M., Sun, Y., Liu, P., Sun, J., Zhou, Q., Xiong, W., Zeng, Z., 2017. Fate of antimicrobial resistance genes in response to application of poultry and swine manure in simulated manure-soil microcosms and manure-pond microcosms. Environ. Sci. Pollut. Res. 24, 20949–20958. https://doi.org/10.1007/s11356-017-9623-z

WHO [WWW Document], 2016. . WHO. URL https://www.who.int/antimicrobial-resistance/global-action-plan/en/ (accessed 12.10.18).

Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.

Wickham, H., 2007. Reshaping Data with the reshape Package. J. Stat. Softw. 21, 1--20.

Wickham, H., Francois, R., Henry, L., Müller, K., 2015. dplyr: A grammar of data manipulation [Computer software manual]. R Packag. version 0.7 1.

Wickham, H., Henry, L., 2019. tidyr: Easily Tidy Data with "spread()" and "gather()" Functions. R Packag. version 0.8.3.

Xie, W.-Y., Shen, Q., Zhao, F.J., 2018. Antibiotics and antibiotic resistance from animal manures to soil: a review. Eur. J. Soil Sci. 69, 181–195. https://doi.org/10.1111/ejss.12494

Xie, W.Y., Yuan, S.T., Xu, M.G., Yang, X.P., Shen, Q.R., Zhang, W.W., Su, J.Q., Zhao, F.J., 2018. Long-term effects of manure and chemical fertilizers on soil antibiotic resistome. Soil Biol. Biochem. 122, 111–119. https://doi.org/10.1016/j.soilbio.2018.04.009

Zhang, Y.-J., Hu, H.-W., Gou, M., Wang, J.-T., Chen, D., He, J.-Z., 2017. Temporal succession of soil antibiotic resistance genes following application of swine, cattle and poultry manures spiked with or without antibiotics. Environ. Pollut. 231, 1621–1632. https://doi.org/10.1016/j.envpol.2017.09.074

CHAPTER 3

"We also know there are known unknowns; that is to say we know there are some things we do not know. But there are also unknown unknowns — the ones we don't know we don't know."

Donald Rumsfeld
U.S. Department of Defense news briefing, 2002

Targeted metagenomics reveals inferior resilience of farm soil resistome compared to soil microbiome after manure application

Gonçalo Macedo[1,2], H. Pieter J. van Veelen[2], Lucia Hernandez-Leal[2], Peter van der Maas[3], Dick Heederik[4], Dik Mevius[1,5], Alex Bossers[4,5], Heike Schmitt[6]

[1] Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands
[2] Wetsus, European Centre of Excellence for Sustainable Water Technology, Oostergoweg 9, 8911 MA Leeuwarden, The Netherlands
[3] Van Hall Larenstein, University of Applied Sciences, Agora 1, 8901 BV Leeuwarden, The Netherlands
[4] Institute for Risk Assessment Sciences, Utrecht University, Yalelaan 2, 3584 CM Utrecht, The Netherlands
[5] Wageningen Bioveterinary Research, Houtribweg 39, 8221 RA Lelystad, The Netherlands
[6] National Institute for Public Health and the Environment, Antonie van Leeuwenhoeklaan 9, 3721 MA Bilthoven, The Netherlands

Published in Science of the Total Environment 770 (2021) 145399
(https://doi.org/10.1016/j.scitotenv.2021.145399)

3.1 Abstract

Application of animal manure to soils results in the introduction of manure-derived bacteria and their antimicrobial resistance genes (ARGs) into soils. ResCap is a novel targeted-metagenomic approach that allows the detection of minority components of the resistome gene pool without the cost-prohibitive coverage depths and can provide a valuable tool to study the spread of antimicrobial resistance (AMR) in the environment. We used high-throughput sequencing and qPCR for 16S rRNA gene fragments as well as ResCap to explore the dynamics of bacteria, and ARGs introduced to soils and adjacent water ditches, both at community and individual scale, over a period of three weeks. The soil bacteriome and resistome showed strong resilience to the input of manure, as manuring did not impact the overall structure of the bacteriome, and its effects on the resistome were transient. Initially, manure application resulted in a substantial increase of ARGs in soils and adjacent waters, while not affecting the overall bacterial community composition. Still, specific families increased after manure application, either through the input of manure (e.g., *Dysgonomonadaceae*) or through enrichment after manuring (e.g., *Pseudomonadaceae*). Depending on the type of ARG, manure application resulted mostly in an increase (e.g., *aph(6)-Id*), but occasionally also in a decrease (e.g., *dfrB3*) of the absolute abundance of ARG clusters (FPKM/kg or L). This study shows that the structures of the bacteriome and resistome are shaped by different factors, where the bacterial community composition could not explain the changes in ARG diversity or abundances. Also, it highlights the potential of applying targeted metagenomic techniques, such as ResCap, to study the fate of AMR in the environment.

3.2 Introduction

Antimicrobial resistance (AMR) has been considered as one of the major challenges to global public health. Agriculture contributes to the emergence and spread of antibiotic resistance genes (ARGs) and antibiotic residues in the environment (Thanner et al., 2016). Manure from (intensive) livestock production has been widely used as fertilizer because of its nutrient-rich and stable organic carbon composition. However, the application of animal manure also results in the introduction of manure-derived bacteria and their ARGs into the soil, enriching the already occurring set of ARGs (Jechalke et al., 2014b; Udikovic-Kolic et al., 2014).

The gene groups encoding antimicrobial resistance – defined as resistome – in manure and soils are clearly different (Noyes et al., 2016). Depending on the animal source, genes conferring tetracycline and aminoglycoside resistance have been shown to be highly prevalent in animal fecal matter, or manure (Fang et al., 2018; Han et al., 2018; Munk et al., 2018; Wan-Ying Xie et al., 2018), and *Firmicutes*, *Bacteroidetes,* and *Proteobacteria* are the predominant *phyla* in dairy manure (Lopatto et al., 2019; Wichmann et al., 2014). Recently, a strong correlation between the resistome and bacterial taxonomy has been reported in pig and poultry feces in nine European countries (Munk et al., 2018). Soil microbial communities are highly diverse in bacterial species (Fierer, 2017). Among other factors, soil texture strongly influences soil microbial communities (Girvan et al., 2003) and has proven effects on the survivability of fecal bacteria in soils (Franz et al., 2014; van Veen et al., 1997). In grasslands, the bacteriome can be dominated by *Acidobacteria*, *Actinobacteria*, and *Proteobacteria* (Kaiser et al., 2016; Lopatto et al., 2019), but through manure fertilization, the community structure may change due to the input of nutrients (Pan et al., 2014), antimicrobial compound residues (Jechalke et al., 2014a), or manure-derived taxa.

Most studies that explored the soil resistome, particularly in manured soils, report the use of ARG-targeted high-throughput qPCR (HT-qPCR) coupled with 16S rRNA gene sequencing. HT-qPCR has the advantage of analyzing significantly more resistance determinants (up to 384 primer sets) than conventional qPCR, providing resistome profile changes with relative abundance to the 16S rRNA gene population structure. However, it still does not target the majority of known ARGs (ResFinder database contains over 2700 gene variants). Metagenomic shotgun sequencing (MGSS) has the potential to sequence all genetic material of a given sample. However, MGSS analyses are constrained by low sensitivity in detecting the "rare biosphere" unless cost-prohibitive deep sequencing strategies are applied. Often the ecologically important taxa are present in concentrations below the "default" MGSS detection limits (Lynch and Neufeld, 2015). In general, the resistome constitutes the minority in the whole gene pool of environmental samples, and

is, therefore, challenging to target. Recently, ARG target-enrichment strategies, such as ResCap, have been introduced to overcome this challenge specifically for the resistome (Lanza et al., 2018).

Up to now, a limited number of metagenomic studies targeting both the bacterial community and resistome of manure and soils have been performed, sampling either dairy (Guron et al., 2019; Noyes et al., 2016) or swine farms (Fang et al., 2018; He et al., 2019; Leclercq et al., 2016). However, in these studies, samples were collected only at a single time point, and consequently, do not capture temporal variations in the soil bacteriome and resistome structure induced by the application of manure. Only two studies focused on application of dairy manure on grassland, where typically, no tillage is performed, but did not measure the microbial community (Muurinen et al., 2017; Nõlvak et al., 2016). More knowledge is available for swine or poultry manure incorporated into agricultural fields, but overall, the number of field studies characterizing both the bacteriome and/or the resistome over time is quite limited (Hong et al., 2013; Lopatto et al., 2019; Riber et al., 2014; Udikovic-Kolic et al., 2014). Therefore, changes in the bacterial community and resistome composition in a field approach remain to be thoroughly studied, particularly in a context of application of dairy manure on grassland.

We hypothesized that the application of manure would significantly alter the bacterial community, and that recovery of the community would occur after a period of time. We similarly expected that ARG diversity would also initially increase and then decrease to values similar to the ones found before manure application. The goal of this study was to evaluate the impact of manure application on the bacterial community and resistome of manured soils and nearby watercourses, with emphasis on the resilience of the bacterial community and the resistome diversity changes over time. We used high-throughput sequencing and qPCR for 16S rRNA gene fragments as well as targeted shotgun metagenomics (ResCap) (i) to evaluate the effect of the introduction of manure-derived bacterial and ARG taxa on the bacterial community and its resistome of manured soils and adjacent watercourses, over a period of three weeks; (ii) to explore the fate of individual bacterial and ARG taxa once introduced to soils or water; (iii) to correlate the bacterial community structure with the resistome structure; and finally, (iv) to compare the outcome of ResCap to qPCR results obtained previously. To the best of our knowledge, this work reports the first usage of ARG-targeted metagenomics to analyze the effect of manure application to soils.

3.3 Methodology

3.3.1 Sampling locations and sample collection

The soil and water samples used in this study were collected from six dairy farms, with different soil textures (clay, sand, or peat), during the manuring season of 2017 (between February and August). Each farm had similar soil usage (grassland) and fertilization rates (farmers' personal communication) over the five years prior to the sampling campaign.
Details on the manure properties, frequency of manure application, soil physicochemical properties, and sampling procedure have been described previously (Macedo et al., 2020). Briefly, the manure samples were collected shortly before being applied to the field and after the mixing. Manure (liquid slurry) was used as received from manure transportation trucks, without any extra processing, except for mixing, prior to soil application. Throughout the year, manure is collected and stored below the stables until the compartment's holding capacity is full, after which it is transferred to a storage silo until the start of manuring season. The manure is then applied to grassland by injection at approximately 10 cm depth, and no-tillage is performed. The soil and water samples were collected before the application of manure (time point T0) and at defined time intervals after manuring (1, 4, 7, 14, and 21 days; time points T1, T2, T3, T4, and T5, respectively). Samples from nearby gardens, with no history of manure application, but of the same texture were also collected and used as controls (NM). The NM samples were collected from gardens due to the absence of forest areas of the same soil texture close to the farms. Composite soil samples were collected and prepared according to ISO guidelines (ISO 10381-6:2009). Briefly, over 25 grab samples of topsoil (0-10 cm) were collected every 40 steps with a soil probe, while walking the fields in a "W" pattern (of those, 4 – 8 samples were collected within the visual manure application bands). After collection, the soil was homogenized and enclosed in plastic zip-lock bags. Water samples were collected with sterile 2-L bottles from the adjacent discharge ditch. Water samples were collected as close as possible to the exit of a soil drainage pipe discharging to the ditch. All samples were kept on ice during transport and stored at -20°C before DNA extraction.

3.3.2 DNA extraction and quantification

The total DNA extracts were obtained from 200 mg, 250 mg, and 100 mL of manure, soil, and water samples, respectively. The DNA from manure and soil samples was extracted using the QIAamp DNA Stool Mini Kit (QIAGEN; Hilden, Germany) and the FastDNA® Spin Kit for Soil (MP Biomedicals; Irvine, CA, USA), respectively. The DNA from the water samples was isolated with DNeasy® PowerWater® Kit (QIAGEN) after filtration through 0.22 µm pore PVDF filters (Merck-Millipore; Burlington, MA, USA). The DNA extraction of soil and water samples proceeded according to the manufacturer's instructions, but for manure samples, a bead-beating step with Precellys Evolution (Bertin Instruments; Montigny-le-Bretonneux, France) was added for enhanced cell lysis (Knudsen et al., 2016).

Each DNA extraction was performed in triplicate, after which the DNA from each sample was pooled, containing similar volumes of each original extract. For each extraction kit used, blank extracts were randomly performed as controls to monitor kit and processing contamination. The DNA quantification was performed using Quantus Fluorometer (Promega; Madison, WI, USA) according to the manufacturer's protocol, and checked for purity with NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA).

Ultimately, 140 DNA extracts were sent for 16S rRNA gene amplicon sequencing, which comprised ten from manure, 62 from soil, and 58 from water samples, as well as ten negative extraction controls. These samples covered time points NM, T0, T1, T2, T3, T4, and T5. A subset of the samples (n = 74) was sent for ResCap-enriched metagenomic shotgun sequencing. The ResCap samples included ten DNA pools from manure samples, 34 from soils, and 30 from water samples. These samples corresponded to the time points NM, T0, T2, and T5, among which the highest resistome changes were expected.

3.3.3 qPCR amplification of 16S rRNA gene and selected ARGs

Amplification of the 16S rRNA genes (*rrs*) and selected ARGs (*sul1, erm(B)*, and *tet(W)*) has been described previously (Macedo 2020). Briefly, the absolute abundance of these genes was quantified by qPCR in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA), and each qPCR run was tested with a standard curve ranging between 10^3 – 10^7, or 10^{-5} – 10^{-9} target copies of standardized gene fragments (gBlocks; IDT technologies, Belgium) for *rrs,* or ARGs, respectively, and non-template controls. Cut-off values were calculated based on the lowest quantity of genes included in the calibration curve of all plates, and all calibration curves had a signal intensity of >2 threshold cycle (C_t) difference to the non-template controls. The average of the highest C_t obtained from this point across all plates was taken, and the standard deviation was added to it.

All assays were performed on the diluted DNA extracts, in duplicate, and the results were inspected to ensure that each duplicate fell within 1 C_t. The amplification efficiencies of all qPCR assays ranged between 88% and 102%, and the melting curves were performed to confirm the amplicon specificity, starting at 65 °C with successive increments of 0.5 °C, up to 95 °C.

The quantifications occurred for each original extract, in duplicates, following the Standard Curve method described elsewhere (Brankatschk et al., 2012), and the final values for each pooled sample were obtained by averaging the results obtained for each original extract. Later, the qPCR results were used to normalize ResCap values (*rrs*) and to compare them with the quantification obtained from ResCap (ARGs).

3.3.4 Microbiome profiling using *rrs* gene amplicon sequencing

Sequencing of *rrs* amplicons was performed at MrDNA Molecular Research LP (Shallowater; TX, USA). Sequence libraries of the V4-V5 region were constructed using PCR with primers 515F/926R (Caporaso et al., 2012; Quince et al., 2011) during a single-step 30-cycle PCR using the HotStarTaq Plus Master Mix Kit (QIAGEN) under the following conditions: 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. The samples were run at 30 cycles to achieve a good signal without hitting the amplification plateau. After amplification, PCR products were checked on a 2% agarose gel to determine the success of amplification and the relative intensity of bands. Guided by expected microbial diversity, 300 bp paired-end reads were generated using the MiSeq Reagent Kit V3 (Illumina; San Diego, CA, USA) at a depth of 40,000 reads for manure and soil samples (hereafter "high-diversity samples"), and at 20,000 reads for water samples (hereafter "low-diversity samples") and negative controls. These sample sets were purified using Agencourt XP AMPure beads (Beckman Coulter; Brea, CA, USA) prior to Illumina library preparation. Sequencing was performed on an Illumina MiSeq (Illumina).

The QIIME2 workflow (version 2018.11) was used to perform quality control and filtering of sequence data (Bolyen et al., 2019). Raw sequence data were imported into QIIME2, samples were demultiplexed, and primer sequences were trimmed from the sequence reads using *q2-cutadapt* (Martin, 2011). Then, feature tables and representative sequences for unique amplicon sequence variants (ASV) were constructed for each data set after quality filtering of paired-end reads (i.e., denoising, error-correction, and chimera removal) using the *DADA2* q2-plugin (Callahan et al., 2016). Parameters for quality filtering were set as follows: to obtain a median Phred score > 30 at each base position, the first ten and the first three bases were trimmed from reads of high- and low-diversity samples, respectively, and forward and reverse reads were trimmed at position 250 and 220 and at position 265 and 205 from high- and low-diversity samples, respectively. The five feature tables and representative sequence sets were both then merged into two merged data sets. Taxonomy assignment until the genus taxonomic level was performed on representative sequences using the scikit-learn naive Bayesian classifier (Pedregosa et al., 2011) trained on full *rrs* sequences from the SILVA database version 132 (Quast et al., 2013).

Data analysis was performed using *phyloseq* version 1.22.3 (McMurdie and Holmes, 2013) in R statistical software version 3.5.0 (R Core Team, 2020). Prior to calculating bacterial diversity, ASVs were removed that were not assigned to Bacteria (n = 2,869), and when assigned to Chloroplast (n = 993) or Mitochondria (n = 1,417). Furthermore, using the *decontam* package (Davis et al., 2018), 19 predicted contaminant ASVs which were linked to blank controls were removed, retaining a total of 16,311 ASVs across all samples. Rarefaction curves and library sizes can be found in the Supplementary material

(Supplementary File 1, Figure 1 – 3). The raw reads are accessible under the NCBI Bioproject number PRJNA665747.

3.3.5 ResCap workflow and data processing

The ResCap workflow is composed of three main steps, KAPA HyperPlus Library construction (for MGSS) and enrichment, Illumina short-read sequencing, and data annotation. The ResCap method was initially described by (Lanza et al., 2018), while the followed procedure was according to the manufacturer conditions (Roche SeqCap EZ workflow version 2.3). Briefly, 100 ng of DNA per sample was enzymatically fragmented 20 minutes to a size between 200 –500 bp, after end-repair and adapter/barcode ligation using seven cycles amplification. Eight purified libraries were pooled at 125 ng library each and enriched according to the SeqCap EZ Library SR procedure (Roche). After the final ligation-mediated PCR for 14 cycles, the enriched library pools were paired-end 150 bp sequenced on Illumina NovaSeq6000 at a target sequencing depth of 2 million read-pairs (4M reads) per enriched sample.

After barcode demultiplexing, the raw reads (over 90% >= Q30) were adapter-clipped, erroneous-tile filtered, and quality-trimmed at Q20 (PHRED score) using BBduk (Bushnell, 2013). Read-pairs were subsequently mapped against the ResFinder database ((Zankari et al., 2012) version of February 2020) using the default global-alignment algorithm of BBMap (Bushnell, 2013). To normalize for library prep, enrichment, sequencing depth, and gene length, FPKM (Fragments Per Kilobase Million) values for each ResFinder gene were calculated and subsequently converted to FPKM/*rrs* gene copies. Each step of relative volume/concentration adjustment or amplification was taken into account for the relative normalization (this includes the relative volumes during library preparation, the number of samples in each pool during the library preparation, the number of PCR cycles during enrichment, and the equimolar loading of the final pools on the sequencer). Finally, the outcomes were corrected using the qPCR-obtained *rrs* copies per sample and multiplied by 10^9. This final FPKM value represents the normalized ARG count per bacterial community.

To circumvent potential problems with ambiguous mapping of read-pairs to single highly identical ARGs in the ResFinder database, we aggregated the FPKM data per identified ARG at 90% identity clustered ARGs as the lowest level of detail (Munk et al., 2018). Briefly, all ARG variants from ResFinder (accessed 19 February 2020) were clustered using at least 80% coverage and a 90% sequence identity threshold using CD-HIT-EST (v4.8.1) (Huang et al., 2010). Each cluster was manually inspected and optionally renamed to reflect their representant/reference sequence and gene members (Table S7) similar to (Munk et al., 2018). The raw reads are accessible under the NCBI Bioproject number PRJNA665747.

3.3.6 Alpha and beta diversities

For both bacteriome and resistome data, alpha diversity (Chao1 richness) was estimated after rarefaction. Other richness indexes (observed, Shannon, and Pielou's evenness) can be found in the supplementary information (Supplementary File 1, Figure 5 and 8).
In the bacteriome dataset, prior to rarefying, samples with low library sizes were excluded (< 5% of max read sum; 24 water samples removed), and then, the data was split into two datasets. The first bacteriome dataset was composed by manure and soil and the other by manure and water samples. The manure-soil dataset consisted of 10,943 ASVs, distributed in 72 samples (1,796 and 9,620 ASVs for manure and soil samples, respectively), while the manure-water dataset consisted of 4,128 ASVs, distributed in 44 samples (1,492 and 2,675 ASVs for manure and water samples, respectively). Rarefying at 14,480 reads resulted in 5,368 ASVs removed from the first dataset while rarefying at 4,022 reads resulted in 12,183 ASVs removed from the latter.

The resistome diversity was based on the NCBI accession numbers of the ARG reference sequence from ResFinder and consisted of 424 different genes, distributed in 74 samples (265, 313, and 347 different ARGs for manure, soil, and water samples, respectively). The ARG gene cluster count matrix was rarefied to the number of the lowest sample library size (183,629 hits).

The beta diversity analysis using Bray-Curtis dissimilarities were calculated using the R package *vegan* (Oksanen et al., 2019) for each dataset. The effects of time point, farm, and soil type were determined using permutational multivariate analysis of variance (PERMANOVA) and are depicted in non-metric multidimensional scaling ordination plots based on the Bray-Curtis distances with 999 permutations. For each variable tested, the homogeneity of group dispersion was confirmed by testing for multivariate homogeneity of groups dispersions (PERMDISP2).

3.3.7 Procrustes analysis

The Procrustes analysis was used to assess the correlation between the bacteriome and the resistome composition. The ordinations were created from the gene cluster FPKM/*rrs* matrix and the ASV count matrix, and NMDS was performed based on Bray–Curtis dissimilarities. The symmetric Procrustes correlation coefficients between the first two axes of the bacteriome and resistome ordinations. Significance was tested using PROTEST, with default 999 permutations, and was calculated separately for manure, soil, and water samples (alpha significance threshold: 0.01).

3.3.8 Differential abundance analysis

To identify bacteria and ARG that differed in abundance before and after manure application, the raw ASV and ARG cluster counts were analyzed separately, using the DESeq2 package (Love et al., 2014). For each analysis, samples from before manuring (T0) were used as normalization reference. For each ASV and ARG, a Wald test was used to determine whether the fold change between time points was statistically significant (alpha significance threshold: 0.01).

Additionally, manure-associated (M) bacterial ASVs absent before (NM or T0) but present in soil and in water samples after (T1 – T5) manure application were identified. In addition to this exploratory approach, the samples were screened for selected families comprising typical manure-associated pathogens as well as hospital-related pathogens (i.e., *Salmonella*, *Campylobacter*, *Listeria*, *Yersinia*, and *Clostridium*, ESKAPE pathogens).

3.3.9 Statistical analysis

One-way analysis of variance (ANOVA) was conducted to detect differences in the bacteria and ARG diversity indexes and in ARG abundance based on qPCR and ResCap data across different sample types and time points. The ANOVA tests were followed by TukeyHSD post-hoc analysis, and homogeneity of variance was confirmed with Levene's test. Data normality was confirmed with Shapiro-Wilk's method, and when normality was not achieved, group comparison was performed using the equivalent non-parametric test (Kruskal-Wallis). A significance threshold of $p < 0.05$ was considered to be statistically relevant for all ANOVA, except when mentioned otherwise. These analyses were performed with R version 3.6.3 (R Core Team, 2020) and RStudio (Version 1.2.5033; https://www.rstudio.com/). Used software packages consisted of *reshape* (Wickham, 2007) and *tidyverse* (Wickham et al., 2019), a set of packages designed for data cleaning, trimming, and visualization; of *PMCMRplus* (Thorsten, 2020), and *car* (Fox and Weisberg, 2019) for ANOVA and Levene's test.

3.4 Results

3.4.1 Diversity of bacterial populations in manure, soil, and water

Chao1 patterns showed that the soil samples had a greater richness of bacterial ASVs than manure (Figure 1, $p < 0.01$), while water samples showed lower bacterial taxa richness than manure ($p < 0.01$), but higher evenness ($p < 0.01$; Supplementary File 1, Figure 5). The application of manure did not affect the estimated total number of ASVs found in manured soils ($p = 0.78$; Figure 1) nor in the adjacent watercourses ($p = 0.15$) as compared to soil and water before manure application, respectively.

The NMDS ordination based on Bray-Curtis dissimilarity revealed the clustering of samples based on soil textures (PERMANOVA, $p < 0.01$ in both soil and water samples) and farm identity (Figure 1; PERMANOVA, $p < 0.01$ in both soil and water samples). No significant effect of manuring or time-points after manuring was found. The effect of soil texture and farm explained 36% and 48%, and 16% and 24% of the variation in soil and water samples, respectively.

In soil communities, *Bacteroidetes* (19.9 – 43.5%), *Proteobacteria* (20.7 – 38.4%), *Verrucomicrobia* (6.4 – 17.3%), *Acidobacteria* (3.8 – 15.8%), *Actinobacteria* (2.8 – 10.4%) were the most abundant phyla, whereas in manure communities *Bacteroidetes* (37.0 – 62.4%), *Firmicutes* (18.0 – 36.3%), *Proteobacteria* (1.5 – 40.2%), *Spirochaetes* (1.7 – 15.6%), *Tenericutes* (0.5 – 8.1%) were dominant (Supplementary File 1, Figure 4). In water, the abundant bacterial communities were *Proteobacteria* (4.0 – 68.6%), *Bacteroidetes* (0.0 – 60.3%), *Cyanobacteria* (0.0 – 92.1%), *Actinobacteria* (0.0 – 21.2%), and *Verrucomicrobia* (0.0 – 20.6%).

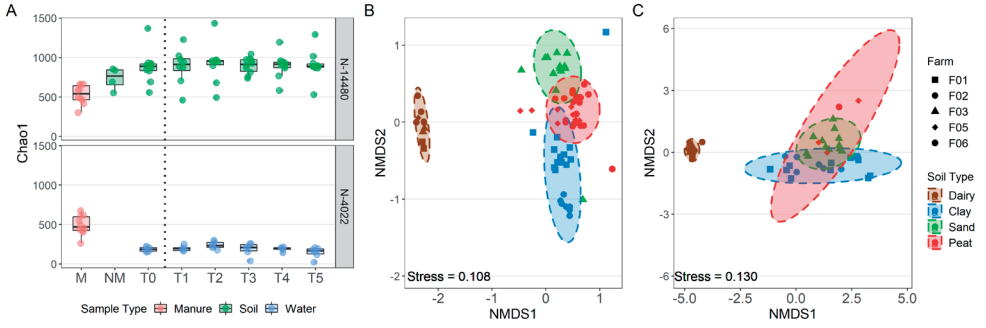


Figure. 1. No changes in microbiome diversity (Chao1) after manure application across sample types (A). Manure samples (M) as well as never-manured soils (NM), and samples before (T0) and after (T1 – T5) manure application are presented under the corresponding rarefaction depth. Non-metric multidimensional scaling (NMDS) plots illustrating Bray-Curtis dissimilarity matrices evidence the clustering of soil (B) and water (C) microbiome samples by farm and soil texture, regardless of having manure applied. Other richness indexes (Observed, Shannon, and Pielou's evenness) can be found in the suppl. material.

3.4.2 Tracing manure-derived bacteria in farm soils

Although manure application did not result in changes in the overall community composition, the abundance of specific taxa was changed shortly after manure application. The differential abundance analysis showed that from the 30 most abundant families, seven families had their abundances significantly increased after manure application (from T0 to T2; $p < 0.01$; Figure 2). Typical environmental bacteria were among the most abundant taxa (e.g., *Burkholderiaceae*, *Chitinophagaceae*, and *Flavobacteriaceae*), but some families (e.g., *Dysgonomonadaceae* and *Ruminococcaceae*) were very abundant in manure and in soils directly after manuring (Figure 2 and 3). Other families, such as

Pseudomonadaceae, were significantly increased in manured soils, even though they were not abundant in manure (Figures 2 and 3).

Manure application resulted in the direct introduction of 26 – 136 ASVs to farm soils, as observed by the overlap of manure and manured-soil ASVs, corresponding to eight families (incl. *Dysgonomonadaceae*; Supplementary File 1, Figure 6). From these, three families were increased shortly after manure application ($p < 0.01$; Figures 2 and 3) and showed their highest relative abundance within the first week after manure application, regardless of the farm. No manure-derived bacteria were detected in water samples after manure application.

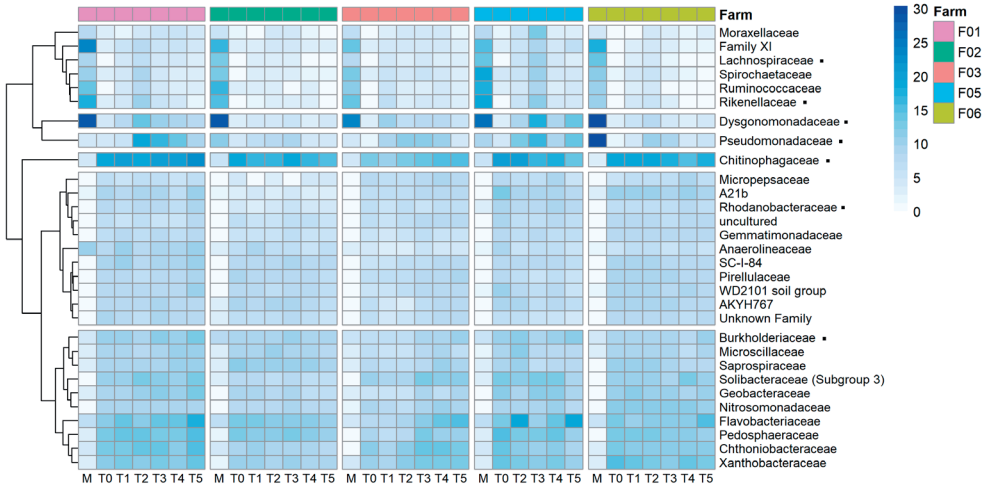


Figure 2. Heatmap demonstrating the 30 most abundant families in manure and soil samples. Manure resulted in the transfer of manure-derived bacteria, but also in native soil bacteria enrichment. Differential abundance analysis based on the negative binomial distribution (DESeq2) showed that marked families (black dot) were significantly changed ($p < 0.01$) between soil before receiving manure (T0) and soil four days after receiving manure (T2). Other time points (T1 – T5) are also displayed. For better visualization, the cubic root of the ASV counts is shown.

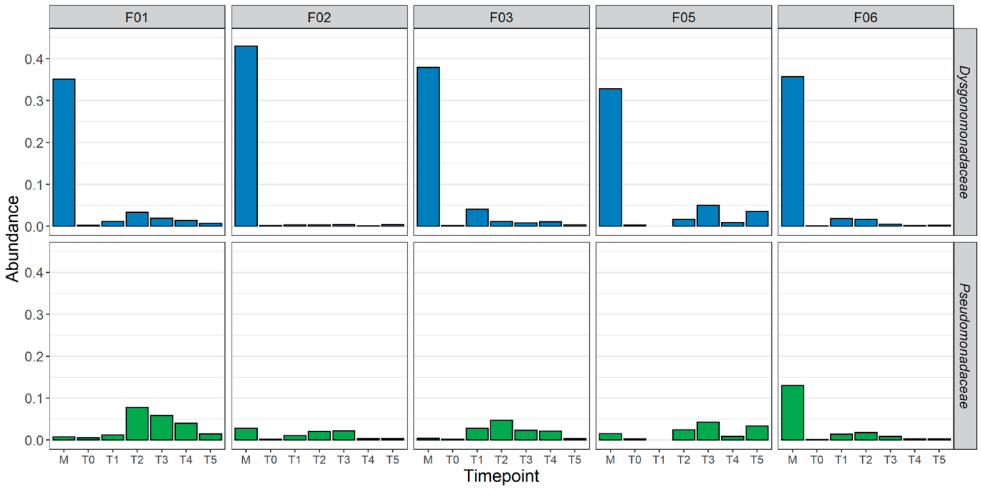


Figure 3. Bar chart demonstrating the relative abundance of Dysgonomonadaceae and Pseudomonadaceae, evidencing their abundance peak in soils shortly after manure application, in the different farms. Their relative abundance is shown in manure samples (M), and soil samples before (T0) and after (T1 – T5) manure application.

3.4.3 Resistome diversity in manure, soil, and water

Manure application resulted in significantly increased ARG diversity in soil and water samples measured four days after the application of manure (T2) and in soils three weeks after manuring (T5; Figure 4; $p < 0.01$). Regardless of the soil characteristics of each farm, the resistome diversity of both soil and water samples was increased by the application of manure, with the strongest changes apparent four days after manuring (T2). At T5 (21 days after manuring), diversity was still significantly increased in soils, but not in water.

The NMDS ordination based on Bray-Curtis dissimilarities revealed that the resistome composition was significantly influenced by manure application and days after manuring in soils (PERMANOVA, $p = 0.001$; Figure 4), but not in water samples ($p = 0.011$). Instead, the resistome profile in water samples was mainly shaped by the soil texture ($p = 0.001$; $r^2 = 0.209$). The effect of manure application was stronger in soils and could explain 51% and 14% of the variation in soil and water samples, respectively.

Among manure samples, resistance to tetracyclines was most abundant (-4.27 ± 0.18 log FPKM/*rrs*; Supplementary File 1, Figure 7), followed by resistance to aminoglycosides (-4.54 ± 0.27), and macrolides (-4.51 ± 0.19). The resistome of both never-manured soil (NM) and soils before manure application (T0) was quite similar, being dominated by resistance to trimethoprim (-4.29 ± 0.35 log FPKM/*rrs*), quinolones (-4.42 ± 0.16), and macrolides (-4.76 ± 0.17). However, after the application of manure, the soil resistome shifted and became dominated by resistance to aminoglycosides (-4.23 ± 0.29 log FPKM/*rrs*), tetracyclines

(-4.59 ± 0.17), and trimethoprim (-4.86 ± 0.36). A similar shift occurred between water samples before and after manure application, with resistance to aminoglycosides and tetracyclines becoming more abundant after manure application (Supplementary File 1, Figure 7).

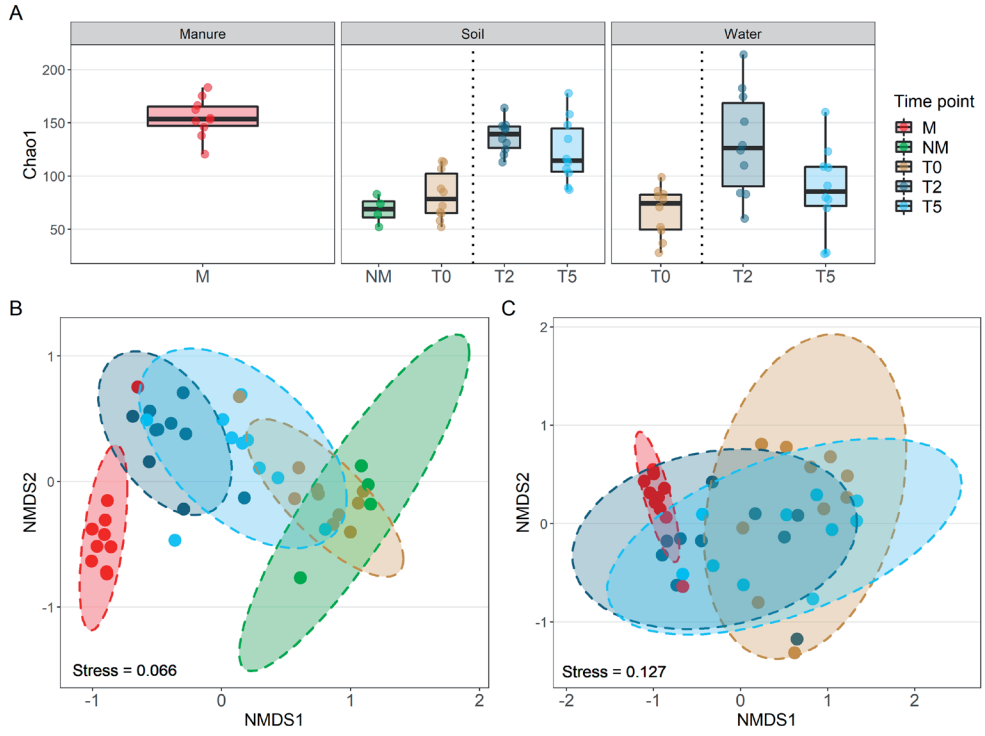


Figure 4. Increase of resistome diversity (Chao1) after manure application across sample types (A). Non-metric multidimensional scaling (NMDS) plots depicting Bray-Curtis dissimilarity matrices demonstrate the clustering of soil (B) and water (C) resistome samples by time points, regardless of the farm and soil texture. Other richness indexes (Observed, Shannon, and Pielou's evenness) can be found in the suppl. Material (Supplementary File 1, Fig. 8).

3.4.4 Manure-induced ARG changes

The differential ARG abundance analysis revealed that depending on the type of gene, the abundance of some ARGs increased after manure application, while others decreased (Figure 5 and S9; $p < 0.01$). For example, genes belonging to the clusters represented by *aph(6)-Ib* or *erm(B)* were among the most abundant ARGs in manure, and their abundance increased after manure application in both soil and water samples. In contrast, *dfrB3* and *oqxB* were among the most abundant ARGs in soils before manure application and decreased in soils after manure application.

In soils, manuring resulted in a significant increase in abundance of *aph(6)-Ib* and *erm(B)* by roughly 1 log, from 8.13 ± 0.77 and 7.71 ± 0.20 log FPKM/kg to 9.13 ± 0.23 and 8.59 ± 0.32 log FPKM/kg respectively ($p < 0.01$; Figure 6). The abundance of *dfrB3* decreased from 9.24 ± 0.24 to 8.43 ± 0.43 log FPKM/kg ($p < 0.01$; Figure 6). In water samples, manuring resulted in the significant increase of *aph(6)-Ib* and *erm(B)* abundance by roughly 2 logs, from 4.53 ± 0.7 and 3.85 ± 0.60 log FPKM/L to 6.16 ± 0.67 and 5.75 ± 0.71 log FPKM/L, respectively ($p < 0.01$; Figure 6), but the abundance of *dfrB3* remained stable at roughly 5 log FPKM/L (Figure 6).

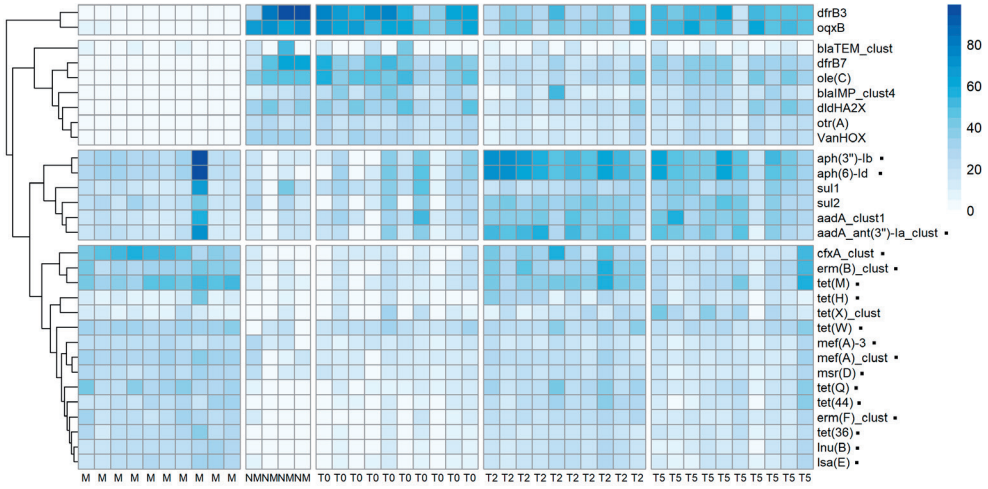


Figure 5. Heatmap demonstrating the 30 most abundant ARG clusters in soil samples, evidencing different ARG patterns, and how manure (M) resulted in the abundance change of certain ARGs. Differential abundance analysis (DESeq2) showed that the marked ARG clusters (black dot) were significantly increased ($p < 0.01$) between soil before receiving manure (T0) and soil four days after receiving manure (T2). Never-manured (NM) and 21 days after receiving manure (T5) are also displayed. For better visualization, the cubic root of ARG abundance is shown. ARG clusters in water samples are depicted in Supplementary File 1, Fig. 9.

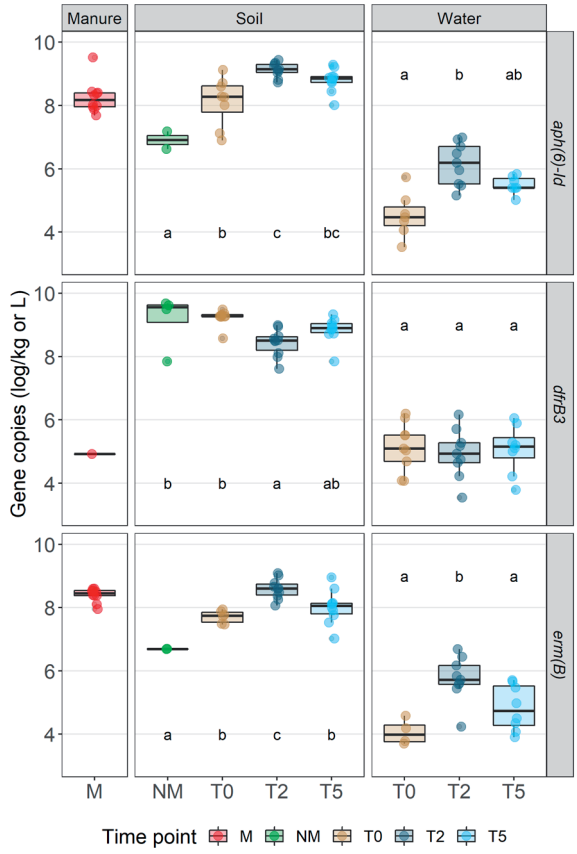


Figure 6. ResCap-based absolute abundance estimates of aph(6)-Id, dfrB3,and erm(B)gene clusters, representing the main trends found in gene abundance changes. The time points represent manure (M), never-manured soils (NM), before (T0), four-days after (T2), and 21-days after (T5) manure application to soil and water samples. Group differences were determined using ANOVA ($p < 0.05$).

3.4.5 Comparison between ARG: qPCR vs. ResCap

In general, when compared to qPCR results, ResCap led to a slight underestimation of the gene abundance, depending mainly on sample type (-2.26 ± 0.22, -0.65 ± 0.47, and -0.58 ± 0.93), but also on the gene (either *sul1*, *erm(B)*, or *tet(W)*; Supplementary File 1, Figure 12). The gene abundances measured by qPCR were positively correlated to the ones measured with ResCap for *sul1* ($r = 0.83$, $p < 0.01$), *erm(B)* ($r = 0.89$, $p < 0.01$), and *tet(W)* ($r = 0.85$, $p < 0.01$), even though an ideal 1:1 correspondence between the two datasets was not found. The qPCR results used for comparison were obtained from the same DNA extracts described here, prior to pooling, and were previously published elsewhere (Macedo et al., 2020).

3.4.6 Bacteriome and resistome correlation (Procrustes)

Based on Procrustes and PROTEST significance analysis comparing the ARG composition and bacterial composition for each sample type of the first two axis from NMDS ordinations, the resistome and bacteriome did not correlate in manure ($p = 0.578$, permutations = 999), soil ($p = 0.046$, permutations = 999), or water samples ($p = 0.132$, permutations = 999).

3.5 Discussion

In this study, we used qPCR (for *rrs*), high throughput 16S rRNA gene sequencing (for bacteriome), and targeted metagenomics (ResCap, for resistome) to evaluate the impact of manure application on the bacteriome and resistome of manured soils and nearby watercourses. The results confirmed the hypothesis that manure application introduced both bacteria and ARG to soils and that after three weeks, their abundance tended to decrease to levels found before manuring. The effects of manuring were more evident and longer lasting in the resistome than in the bacteriome of agricultural soil.

3.5.1 Manure enriches AMR-relevant bacteria in farm soils

In general, the dominant phyla of manure and soil bacterial communities in this study were characteristic of bacteriomes described in other studies (Han et al., 2018; Lopatto et al., 2019; Riber et al., 2014; M. Wang et al., 2018). Manure, soil, and water alpha diversities and *phylum* composition were constant across the sampling campaign, showing that the application of manure did not affect the overall bacteriome diversity of soil and water samples.

Results similar to the ones described in the present study were reported in other field studies of manured soils where tillage has been performed (Lopatto et al., 2019; Riber et al., 2014; W.-Y. Xie et al., 2018), but not in manured-soil microcosm studies (Han et al., 2018; M. Wang et al., 2018). In the latter, soil diversity decreased after receiving manure, regardless of its animal source. Possible reasons for the divergent outcome of these studies lie in differences in the experimental time frame, the diverse inherent soil characteristics, and weather conditions between studies. In this study, the soil had higher bacterial richness than manure, which could explain why manure application did not contribute to an overall diversity increase, and the location where the samples were collected (farm identity), followed by soil texture, were the main drivers shaping the bacterial community. Soil communities are known to be affected by soil texture (Blau et al., 2018; Girvan et al., 2003) and also by edaphic factors (Lauber et al., 2008). Still, four out of the eight families that were introduced to soils by manure amendment had a significantly higher abundance after manuring ($p < 0.01$), as shown by the overlap of manure and manured-soil ASVs and *DESeq2* analyses. These families belonged either to *Firmicutes* or *Bacteroidetes*, which are

the most common phyla found in manure (Ding et al., 2014; Wichmann et al., 2014), and have been recognized as important ARG hosts in soils (Forsberg et al., 2014; Han et al., 2018; Leclercq et al., 2016). Typically, manure-derived bacteria are not well adapted to survive in soils (Bech et al., 2014; Franz et al., 2014; Heuer et al., 2008), and consequently, tend to decrease shortly after introduction in soils. Indeed, in this study, these families were hardly detected three weeks after manure application.

At the same time, *Pseudomonadaceae* and *Moraxellaceae* were presumably enriched after manure application. As manure is commonly applied to soils for its nutrient content, the growth of certain bacterial groups is stimulated (Goldfarb et al., 2011). In general, γ-Proteobacteria, particularly *Pseudomonas*, take part in the primary succession after a disturbance in the bacterial community of soil and aquatic environments (Becerra-Castro et al., 2016; Song et al., 2017; Vadstein et al., 2018). It has been recently shown that manure application to soils can enrich antimicrobial-resistant bacteria (Ding et al., 2014; Hu et al., 2016; Udikovic-Kolic et al., 2014), and both *Acinetobacter* and *Pseudomonas* have been associated with ARG persistence in manure-treated soils (Leclercq et al., 2016). Whether and which of the ARG that were enriched in this study were located on Pseudomonas has to be evaluated by further studies. Here, the relative abundance of *Pseudomonadaceae* decreased over time, reaching levels similar to the ones found before manure application, showing that the manure input had only a short-term effect on the relative abundance of this family. The findings of the current study corroborate previous studies reporting manure enrichment of bacteria relevant to the spread of AMR.

In general, water samples were affected to a minor extent by manure application than soils. Depending on the soil NPK requirements and local legislation, 30 m^3/ha of manure (slurry) is typically applied to soils, corresponding to approximately 40 g/kg soil (Ding et al., 2014). From this, and further depending on multiple factors (e.g., climate, soil texture), it would be expected that only a small fraction of manure bacteria would leach into the water ditches (Bech et al., 2014). Despite manure displaying higher bacterial diversity than water, its contribution to the bacteriome structure of water samples was not detectable, which can explain why no manure-derived ASVs were found in water samples.

3.5.2 Manure provokes an intense short-term increase of ARGs

Overall, resistance to aminoglycosides and tetracyclines were abundant throughout the different samples, but particularly more in manure and manured soils and waters. Resistance to tetracyclines and aminoglycosides is commonly reported in manured soil studies (Chen et al., 2019; Han et al., 2018; Zhang et al., 2019, 2017). As tetracyclines are commonly administered to farm animals, resistance to this antimicrobial class was expected. However, aminoglycosides are not so widely used on a national scale in the Netherlands (de Greef et al., 2019). Previously, (Muurinen et al., 2017) showed that some

aadA genes were increased by manure storage. Considering that all manure samples used in this study were stored below the stables before being applied in the fields, this could partly explain the high abundance of aminoglycoside resistance. However, resistance to aminoglycosides has been strongly correlated to mobile genetic elements (i.e., integrases and transposases) (Zhang et al., 2017), and has been positively associated with corresponding antibiotics used in poultry farms (Luiken et al., 2019). Because neither farm antimicrobial consumption data was collected, nor mobile genetic elements were measured, it is not possible to determine which aspect was more important in this case.

In soils, manure application resulted in an intense short-term increase of ARGs (after four days), which could still be observed after 21 days. The combination of results from this study with the previously published findings (Macedo et al., 2020) suggests that most ARGs will probably recover to abundances found before manure application in a period of roughly 40 days. Elsewhere, a similar increase in ARG content immediately after application of cow manure was observed in a field study where tillage was not performed (Muurinen et al., 2017) and in two microcosm studies (Han et al., 2018; Zhang et al., 2017). However, the temporal succession in some of those studies seems to occur slower than in this study, as there the overall number of ARGs after 40 days was still significantly higher than before manure application. Notably, Zhang et al. (2017) reported a period of 20 days for ARG numbers to decline back to numbers before amendment. Furthermore, the present study corroborates the previous findings that the fate of AMR depends extensively on ARG identity, as seen for genes that were very abundant in manure and not in soils before manure application (increased after manuring; e.g., *aph(6)-Id*, *erm(B)*, *cfxA*, *tet(W)*, or *tet(M)*), and for the ones that were abundant in soils without manure but not in manure (decreased after manuring; e.g., *dfrB3*, *oqxB*, *otr(A)*, or *ole(C)*). In water samples, manure application resulted in the increase of ARGs (after four days), after which their diversity was decreased to levels before manure application. There is a lack of studies focusing on the overall ARG diversity in surface water streams adjacent to manured fields, over time. The findings of this study are consistent with the ones shown by Muurinen et al. (2017), where the number of ARGs in ditch waters two and six weeks after manure application was similar to the numbers found prior to manuring.

Moreover, in this study, manure application was the main variable driving resistome diversity in soils, explaining 51% of the NMDS distribution. Previously, in a multivariate analysis study, Muurinen et al. (2017) also found that the manure amendment was among the variables that significantly explained ARG variation in soils. However, long-term studies indicate that bacterial composition and soil properties have the strongest role in shaping ARG profiles in manured soils (Guo et al., 2018; Wan-Ying Xie et al., 2018), which contradict the observations from the current study. Not only did soil properties (here represented by the soil texture) not show a significant correlation with the resistome structure, but also

no statistically relevant association between the resistome and bacterial composition was found. Although not common, the lack of correlation between resistome and bacteriome and soil properties has also been reported in a long-term manured-soil study (Cheng et al., 2019). While soil environments are known for being a source of ARG diversity, regardless of having a history of manure application (D'Costa et al., 2011; Nesme and Simonet, 2015), here, the ARG diversity observed in never-manured soil and in samples before receiving manure was well below the ARG diversity observed in manure. The input of ARGs from manure was intense both in abundance and diversity and led to a steep increase of ARG levels, thus shaping the overall resistome structure. While manure strongly affected the resistome, the direct contribution to the bacterial community was rather low, thus explaining why no correlation was found between the resistome and the bacteriome. Compared to the existing microbiota, the manure amendment only added a fraction of additional bacteria to soil, as *rrs* abundance was constant before and after manuring in soil and water samples (Supplementary File 1, Figure 10). Considering the amount of manure applied, it corresponded roughly to adding 1 – 10% bacteria to the ones already present in soils, apparently not enough to shift the overall community composition.

3.5.3 ResCap is a promising tool to determine the ARG fate in the environment

Generally, the data obtained from ResCap showed a strong positive correlation with the qPCR-obtained quantifications (Supplementary File 1, Figure 11 and 12). However, differences in the absolute agreement were apparent between sample types. Manure samples showed a stronger underestimation when compared to qPCR (roughly -2 log units), and the water samples showed high variability in abundance differences between ResCap and qPCR data (standard deviation varied 0.874 – 0.914 log units). The reasons for these differences are not entirely clear, and one can only speculate. As metagenome shotgun uses longer intact DNA and qPCR uses short fragments for detection, the extent of DNA fragmentation might contribute to the observed difference between values. Additionally, different hybridization or PCR efficiencies between sample types could have interfered.

ResCap is reported to achieve better recovery of target genes than MGSS and to greatly enhance the sensitivity and specificity of metagenomic methods (Lanza et al., 2018), increasing the number of mapped reads from 1 in 1000 read-pairs (Munk et al., 2018) up to 200-fold (1 in 5). Here, a total of 475 ARG clusters were found in the 74 samples analyzed, including 79, 65, and 47 gene clusters that confer resistance primarily to aminoglycosides, beta-lactams, and tetracyclines, respectively. By introducing a probe-hybridization step, data quality and reproducibility increase as it reduces the variance in target coverage, delivering a better cost-effective approach (Mamanova et al., 2010). However, low efficiencies in the hybridization step, or signal overloads, may affect the

outcome. Additionally, as it requires enzyme-based steps as does qPCR (e.g., amplification during library preparation), inhibition might also bias the results. At the same time, qPCR is considered the method of choice for gene quantification but is not free from potential pitfalls. Final quantification is affected by protocol-related issues (e.g., choice of primers, and their concentration, mastermix), equipment used, among others (Bustin et al., 2009; Rocha et al., 2018; Travis et al., 2011). Additionally, it can only measure a limited number of genes, even in a high-throughput setup, never delivering a complete picture of the sample ARG landscape. In terms of errors, a variability of <3-fold in absolute units, corresponding to circa 0.5 log units, was found in an inter-laboratory comparison using qPCR data (Travis et al., 2011), which the authors considered "small in biological terms."

The choice to evaluate the impact of anthropogenic activities, namely manure application, in the environment is challenging and requires compromising between accuracy and amount of information. When compared to qPCR (Supplementary File 1, Figure 12), the results obtained with ResCap showed an average difference of <10-fold (1 log unit) in overall soil and water samples. Although this difference is considered biologically relevant in absolute terms, when placed in a context of highly polluted environments, such as manured soils (roughly 10^{10} total ARG copies/g; (Han et al., 2018)), or wastewater streams (roughly 10^{8} total ARG copies/mL; (Quintela-Baluja et al., 2019)), it may not be so relevant because the background ARG level in those scenarios is already relatively high. The semi-quantitative aspect of metagenomics, which ResCap also features, is of great value. It should not be disregarded solely based on the loss of accuracy but rather be validated in further methodological studies. ResCap has been previously validated and shown to provide better results than metagenomic shotgun sequencing (Lanza et al., 2018). Here, we applied this technology to a new context and concluded that ResCap can simultaneously generate results on many resistance genes while still giving at least semi-quantitative results. Therefore, one should be careful in presenting the results, as factors such as sample ARG abundance and presence of impurities in the DNA extracts may significantly affect the ResCap-based quantification.

3.5.4 Implications on-farm management

Traditional farming practices were followed while this study was being conducted. Manure was stored below the stables or in silos, during winter, and it was applied during manuring season (spring/summer) multiple times, as farmers commonly do. While the effects of manuring in the resistome of agricultural soil were transient, an intense increase of ARGs occurred simultaneously with the enrichment of native soil bacteria. Therefore, solutions to minimize the input of ARGs into soil should be further investigated. The application of anaerobic digestion treatments of animal waste holds potential because it allows farmers to recover the nutrients and produce biogas. While manure treatment is efficient in removing fecal indicator pathogens and some ARG-carrying bacteria (Iwasaki

et al., 2019; Pandey et al., 2015), some ARGs persist after the treatment (Huang et al., 2019). Nevertheless, because of the costs of implementing and maintaining treatment structures, the majority of the farms applies manure directly in soils, without any treatment.

3.6 Conclusions

In this field study, both soil bacteriome and resistome showed strong resilience to the input of manure over a timeframe of 21 days. Manure application resulted in the input of manure-derived bacteria that did not affect the overall community composition. After being introduced to soils, these bacteria decreased, in a period of three weeks. However, manure application also resulted in the enrichment of fast-growing bacteria, namely *Pseudomonadaceae*. On the other hand, the resistome displayed a temporal shift with an intense increase of ARG diversity and abundance after manure application, but these effects were temporal. It was shown that the fate of ARG depends on the gene identity, and that both bacteriome and resistome structures were shaped by different factors, where the bacterial community did not show a significant relationship with ARG abundance.
This study also highlighted that targeted-metagenomic techniques, such as ResCap to enrich the resistome, provide an excellent tool to explore and assess the fate of AMR in the environment, as they provide the complete picture of the resistome landscape at an affordable price. Additionally, when coupled with 16S rRNA gene quantifications, it reliably correlated with standard methods used for gene quantification (qPCR). However, caution is advised when analyzing the results as sample-related bias may be prone to occur (e.g., inhibition).

3.7 Acknowledgments

The authors gratefully acknowledge the support of the farmers who voluntarily accepted to participate and allowed sample collection; the veterinarians Bas Landerman and Maaike van den Berg for helping to recruit and persuade the participants; Bianca de Vries for promoting communication with the farmers; the former students Harm de Haan, Esther Meinderts, and Lydia Speijker for their assistance in collecting samples and qPCR measurements; and Frank Harders for his invaluable help in library preparation and sequencing of the targeted metagenomics.

3.8 Funding

This work was performed in the cooperation framework of Wetsus, European Centre of excellence for sustainable water technology (www.wetsus.nl). Wetsus is co-funded by the Dutch Ministry of Economic Affairs and Ministry of Infrastructure and Environment, the European Union Regional Development Fund, the Province of Fryslân, and the Northern Netherlands Provinces. This work has also received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie [grant agreement No. 665874]. The authors like to thank the participants of the research theme Source Separated Sanitation for the fruitful discussions and their financial support.

3.9 Supplementary Information

Supplementary data can be found online at
https://doi.org/10.1016/j.scitotenv.2021.145399.

3.10 References

Becerra-Castro, C., Macedo, G., Silva, A.M.T., Manaia, C.M., Nunes, O.C., 2016. Proteobacteria become predominant during regrowth after water disinfection. Sci. Total Environ. 573, 313–323. https://doi.org/10.1016/j.scitotenv.2016.08.054

Bech, T.B., Rosenbom, A.E., Kjaer, J., Amin, M.G.M., Olsen, P., Jacobsen, C.S., 2014. Factors influencing the survival and leaching of tetracycline-resistant bacteria and Escherichia coli through structured agricultural fields. Agric. Ecosyst. Environ. 195, 10–17. https://doi.org/10.1016/j.agee.2014.05.017

Blau, K., Casadevall, L., Wolters, B., Van den Meersche, T., Kreuzig, R., Smalla, K., Jechalke, S., 2018. Soil texture-depending effects of doxycycline and streptomycin applied with manure on the bacterial community composition and resistome. FEMS Microbiol. Ecol. 94, 1–11. https://doi.org/10.1093/femsec/fix145

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A.M., Chase, J., Cope, E.K., Da Silva, R., Diener, C., Dorrestein, P.C., Douglas, G.M., Durall, D.M., Duvallet, C., Edwardson, C.F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J.M., Gibbons, S.M., Gibson, D.L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G.A., Janssen, S., Jarmusch, A.K., Jiang, L., Kaehler, B.D., Kang, K. Bin, Keefe, C.R., Keim, P., Kelley, S.T., Knights, D., Koester, I., Kosciolek, T., Kreps, J., Langille, M.G.I., Lee, J., Ley, R., Liu, Y.X., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B.D., McDonald, D., McIver, L.J., Melnik, A. V., Metcalf, J.L., Morgan, S.C., Morton, J.T., Naimey, A.T., Navas-Molina, J.A., Nothias, L.F., Orchanian, S.B., Pearson, T., Peoples, S.L., Petras, D., Preuss, M.L., Pruesse, E., Rasmussen, L.B., Rivers, A., Robeson, M.S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S.J., Spear, J.R., Swafford, A.D., Thompson, L.R., Torres, P.J., Trinh, P., Tripathi, A., Turnbaugh, P.J., Ul-Hasan, S., van der Hooft, J.J.J., Vargas, F., Vázquez-Baeza, Y., Vogtmann, E., von Hippel, M., Walters, W., Wan, Y., Wang, M., Warren, J., Weber, K.C., Williamson, C.H.D., Willis, A.D., Xu, Z.Z., Zaneveld, J.R., Zhang, Y., Zhu, Q., Knight, R., Caporaso, J.G., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. https://doi.org/10.1038/s41587-019-0209-9

Brankatschk, R., Bodenhausen, N., Zeyer, J., Burgmann, H., 2012. Simple absolute quantification method correcting for quantitative PCR efficiency variations for microbial community samples. Appl. Environ. Microbiol. 78, 4481–4489. https://doi.org/10.1128/AEM.07878-11

Bushnell, B., 2013. BBMap download | SourceForge.net [WWW Document]. URL https://sourceforge.net/projects/bbmap/ (accessed 7.20.20).

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clin. Chem. 55, 611–622. https://doi.org/10.1373/clinchem.2008.112797

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J.A., Smith, G., Knight, R., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J. 6, 1621–1624. https://doi.org/10.1038/ismej.2012.8

Chen, Z., Zhang, W., Yang, L., Stedtfeld, R.D., Peng, A., Gu, C., Boyd, S.A., Li, H., 2019. Antibiotic resistance genes and bacterial communities in cornfield and pasture soils receiving swine and dairy manures. Environ. Pollut. 248, 947–957. https://doi.org/10.1016/j.envpol.2019.02.093

Cheng, J.-H., Tang, X.-Y., Cui, J.-F., 2019. Effect of long-term manure slurry application on the occurrence of antibiotic resistance genes in arable purple soil (entisol). Sci. Total Environ. 647, 853–861. https://doi.org/10.1016/j.scitotenv.2018.08.028

D'Costa, V.M., King, C.E., Kalan, L., Morar, M., Sung, W.W.L., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G.B., Poinar, H.N., Wright, G.D., 2011. Antibiotic resistance is ancient. Nature 477, 457–461. https://doi.org/10.1038/nature10388

Davis, N.M., Proctor, Di.M., Holmes, S.P., Relman, D.A., Callahan, B.J., 2018. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome 6, 226. https://doi.org/10.1186/s40168-018-0605-2

de Greef, S., Mouton, J., Schoffelen, A., Verduin, C., 2019. NethMap 2019: Consumption of antimicrobial agents and antimicrobial resistance among medically important bacteria in the Netherlands / MARAN 2019: Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands in 2018 251.

Ding, G., Radl, V., Schloter-Hai, B., Jechalke, S., Heuer, H., Smalla, K., Schloter, M., 2014. Dynamics of Soil Bacterial Communities in Response to Repeated Application of Manure Containing Sulfadiazine. PLoS One 9, e92958. https://doi.org/10.1371/journal.pone.0092958

Fang, H., Han, L., Zhang, H., Long, Z., Cai, L., Yu, Y., 2018. Dissemination of antibiotic resistance genes and human pathogenic bacteria from a pig feedlot to the surrounding stream and agricultural soils. J. Hazard. Mater. 357, 53–62. https://doi.org/10.1016/j.jhazmat.2018.05.066

Fierer, N., 2017. Embracing the unknown: Disentangling the complexities of the soil microbiome. Nat. Rev. Microbiol. https://doi.org/10.1038/nrmicro.2017.87

Forsberg, K.J., Patel, S., Gibson, M.K., Lauber, C.L., Knight, R., Fierer, N., Dantas, G., 2014. Bacterial phylogeny structures soil resistomes across habitats. Nature 509, 612–616. https://doi.org/10.1038/nature13377

Fox, J., Weisberg, S., 2019. An R Companion to Applied Regression. Sage.

Franz, E., Schijven, J., de Roda Husman, A.M., Blaak, H., 2014. Meta-Regression Analysis of Commensal and Pathogenic Escherichia coli Survival in Soil and Water. Environ. Sci. Technol. 48, 6763–6771. https://doi.org/10.1021/es501677c

Girvan, M.S., Bullimore, J., Pretty, J.N., Osborn, A.M., Ball, A.S., 2003. Soil type is the primary determinant of the composition of the total and active bacterial communities in arable soils. Appl. Environ. Microbiol. 69, 1800–1809. https://doi.org/10.1128/AEM.69.3.1800–1809.2003

Goldfarb, K.C., Karaoz, U., Hanson, C.A., Santee, C.A., Bradford, M.A., Treseder, K.K., Wallenstein, M.D., Brodie, E.L., 2011. Differential growth responses of soil bacterial taxa to carbon substrates of varying chemical recalcitrance. Front. Microbiol. 2. https://doi.org/10.3389/fmicb.2011.00094

Guo, T., Lou, C., Zhai, W., Tang, X., Hashmi, M.Z., Murtaza, R., Li, Y., Liu, X., Xu, J., 2018. Increased occurrence of heavy metals, antibiotics and resistance genes in surface soil after long-term application of manure. Sci. Total Environ. 635, 995–1003. https://doi.org/10.1016/j.scitotenv.2018.04.194

Guron, G.K.P., Arango-Argoty, G., Zhang, L., Pruden, A., Ponder, M.A., 2019. Effects of Dairy Manure-Based Amendments and Soil Texture on Lettuce- and Radish-Associated Microbiota and Resistomes. mSphere 4, 1–14. https://doi.org/10.1128/mSphere.00239-19

Han, X.-M.M., Hu, H.-W.W., Chen, Q.-L.L., Yang, L.-Y.Y., Li, H.-L.L., Zhu, Y.-G.G., Li, X.-Z.Z., Ma, Y.-B.B., 2018. Antibiotic resistance genes and associated bacterial communities in agricultural soils amended with different sources of animal manures. Soil Biol. Biochem. 126, 91–102. https://doi.org/10.1016/j.soilbio.2018.08.018

He, L.-Y., He, L.-K., Liu, Y.-S., Zhang, M., Zhao, J.-L., Zhang, Q.-Q., Ying, G.-G., 2019. Microbial diversity and antibiotic resistome in swine farm environments. Sci. Total Environ. 685, 197–207. https://doi.org/10.1016/j.scitotenv.2019.05.369

Heuer, H., Focks, A., Lamshöft, M., Smalla, K., Matthies, M., Spiteller, M., 2008. Fate of sulfadiazine administered to pigs and its quantitative effect on the dynamics of bacterial resistance genes in manure and manured soil. Soil Biol. Biochem. 40, 1892–1900. https://doi.org/10.1016/j.soilbio.2008.03.014

Hong, P.Y.P.-Y., Yannarell, A.C., Dai, Q., Ekizoglu, M., Mackie, R.I., 2013. Monitoring the Perturbation of Soil and Groundwater Microbial Communities Due to Pig Production Activities. Appl. Environ. Microbiol. 79, 2620–2629. https://doi.org/10.1128/AEM.03760-12

Hu, H.W., Han, X.-M.M., Shi, X.Z., Wang, J.T., Han, L.L., Chen, D., He, J.-Z.Z., 2016. Temporal changes of antibiotic-resistance genes and bacterial communities in two contrasting soils treated with cattle manure. FEMS Microbiol. Ecol. 92, 1–13. https://doi.org/10.1093/femsec/fiv169

Huang, X., Zheng, J., Tian, S., Liu, C., Liu, L., Wei, L., Fan, H., Zhang, T., Wang, L., Zhu, G., Xu, K., 2019. Higher Temperatures Do Not Always Achieve Better Antibiotic Resistance Gene Removal in Anaerobic Digestion of Swine Manure. Appl. Environ. Microbiol. 85, 1–12. https://doi.org/10.1128/AEM.02878-18

Huang, Y., Niu, B., Gao, Y., Fu, L., Li, W., 2010. CD-HIT Suite: a web server for clustering and comparing biological sequences. Bioinformatics 26, 680–682. https://doi.org/10.1093/bioinformatics/btq003

Iwasaki, M., Miyake, M., Maseda, H., Qi, G., Pan, Z., Ihara, I., Umetsu, K., 2019. Thermophilic anaerobic digestion is an effective treatment for reducing cefazolin-resistant bacteria and ESBL-producers

in dairy manure. J. Mater. Cycles Waste Manag. 21, 293–299. https://doi.org/10.1007/s10163-018-0789-3

Jechalke, S., Focks, A., Rosendahl, I., Groeneweg, J., Siemens, J., Heuer, H., Smalla, K., 2014a. Structural and functional response of the soil bacterial community to application of manure from difloxacin-treated pigs. FEMS Microbiol. Ecol. 87, 78–88. https://doi.org/10.1111/1574-6941.12191

Jechalke, S., Heuer, H., Siemens, J., Amelung, W., Smalla, K., 2014b. Fate and effects of veterinary antibiotics in soil. Trends Microbiol. 22, 536–545. https://doi.org/10.1016/j.tim.2014.05.005

Kaiser, K., Wemheuer, B., Korolkow, V., Wemheuer, F., Nacke, H., Schöning, I., Schrumpf, M., Daniel, R., 2016. Driving forces of soil bacterial community structure, diversity, and function in temperate grasslands and forests. Sci. Rep. 6. https://doi.org/10.1038/srep33696

Knudsen, B.E., Bergmark, L., Munk, P., Lukjancenko, O., Priemé, A., Aarestrup, F.M., Pamp, S.J., 2016. Impact of Sample Type and DNA Isolation Procedure on Genomic Inference of Microbiome Composition. mSystems 1. https://doi.org/10.1128/mSystems.00095-16

Lanza, V.F., Baquero, F., Martínez, J.L., Ramos-Ruíz, R., González-Zorn, B., Andremont, A., Sánchez-Valenzuela, A., Ehrlich, S.D., Kennedy, S., Ruppé, E., van Schaik, W., Willems, R.J., de la Cruz, F., Coque, T.M., 2018. In-depth resistome analysis by targeted metagenomics. Microbiome 6, 11. https://doi.org/10.1186/s40168-017-0387-y

Lauber, C.L., Strickland, M.S., Bradford, M.A., Fierer, N., 2008. The influence of soil properties on the structure of bacterial and fungal communities across land-use types. Soil Biol. Biochem. 40, 2407–2415. https://doi.org/10.1016/j.soilbio.2008.05.021

Leclercq, S.O., Wang, C., Sui, Z., Wu, H., Zhu, B., Deng, Y., Feng, J., 2016. A multiplayer game: species of Clostridium , Acinetobacter , and Pseudomonas are responsible for the persistence of antibiotic resistance genes in manure-treated soils. Environ. Microbiol. 18, 3494–3508. https://doi.org/10.1111/1462-2920.13337

Lopatto, E., Choi, J., Colina, A., Ma, L., Howe, A., Hinsa-Leasure, S., 2019. Characterizing the soil microbiome and quantifying antibiotic resistance gene dynamics in agricultural soil following swine CAFO manure application. PLoS One 14, e0220770. https://doi.org/10.1371/journal.pone.0220770

Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550. https://doi.org/10.1186/s13059-014-0550-8

Luiken, R.E.C., Van Gompel, L., Munk, P., Sarrazin, S., Joosten, P., Dorado-García, A., Borup Hansen, R., Knudsen, B.E., Bossers, A., Wagenaar, J.A., Aarestrup, F.M., Dewulf, J., Mevius, D.J., Heederik, D.J.J., Smit, L.A.M., Schmitt, H., Graveland, H., VanEssen, A., Gonzalez-Zorn, B., Moyano, G., Sanders, P., Chauvin, C., David, J., Battisti, A., Caprioli, A., Blaha, T., Wadepohl, K., Brandt, M., Hald, T., Ribeiro Duarte, A.S., Wasyl, D., Skarzyńska, M., Zajac, M., Daskalov, H., Saatkamp, H.W., Stärk, K.D.C., 2019. Associations between antimicrobial use and the faecal resistome on broiler farms from nine European countries. J. Antimicrob. Chemother. 74, 2596–2604. https://doi.org/10.1093/jac/dkz235

Lynch, M.D.J., Neufeld, J.D., 2015. Ecology and exploration of the rare biosphere. Nat. Rev. Microbiol. 13, 217–229. https://doi.org/10.1038/nrmicro3400

Macedo, G., Hernandez-Leal, L., van der Maas, P., Heederik, D., Mevius, D., Schmitt, H., 2020. The impact of manure and soil texture on antimicrobial resistance gene levels in farmlands and adjacent ditches. Sci. Total Environ. 737, 139563. https://doi.org/10.1016/j.scitotenv.2020.139563

Mamanova, L., Coffey, A.J., Scott, C.E., Kozarewa, I., Turner, E.H., Kumar, A., Howard, E., Shendure, J., Turner, D.J., 2010. Target-enrichment strategies for next-generation sequencing. Nat. Methods 7, 111–118. https://doi.org/10.1038/nmeth.1419

Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal 17, 10. https://doi.org/10.14806/ej.17.1.200

McMurdie, P.J., Holmes, S., 2013. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS One 8, e61217. https://doi.org/10.1371/journal.pone.0061217

Munk, P., Knudsen, B.E., Lukjancenko, O., Duarte, A.S.R., Van Gompel, L., Luiken, R.E.C., Smit, L.A.M., Schmitt, H., Garcia, A.D., Hansen, R.B., Petersen, T.N., Bossers, A., Ruppé, E., Lund, O., Hald, T., Pamp, S.J., Vigre, H., Heederik, D., Wagenaar, J.A., Mevius, D., Aarestrup, F.M., 2018. Abundance and diversity of the faecal resistome in slaughter pigs and broilers in nine European countries. Nat. Microbiol. 3, 898–908. https://doi.org/10.1038/s41564-018-0192-9

Muurinen, J., Stedtfeld, R., Karkman, A., Pärnänen, K., Tiedje, J., Virta, M., 2017. Influence of Manure Application on the Environmental Resistome under Finnish Agricultural Practice with Restricted Antibiotic Use. Environ. Sci. Technol. 51, 5989–5999. https://doi.org/10.1021/acs.est.7b00551

Nesme, J., Simonet, P., 2015. The soil resistome: a critical review on antibiotic resistance origins, ecology and dissemination potential in telluric bacteria. Environ. Microbiol. 17, 913–930. https://doi.org/10.1111/1462-2920.12631

Nõlvak, H., Truu, M., Kanger, K., Tampere, M., Espenberg, M., Loit, E., Raave, H., Truu, J., 2016. Inorganic and organic fertilizers impact the abundance and proportion of antibiotic resistance and integron-integrase genes in agricultural grassland soil. Sci. Total Environ. 562, 678–689. https://doi.org/10.1016/j.scitotenv.2016.04.035

Noyes, N.R., Yang, X., Linke, L.M., Magnuson, R.J., Cook, S.R., Zaheer, R., Yang, H., Woerner, D.R., Geornaras, I., McArt, J.A., Gow, S.P., Ruiz, J., Jones, K.L., Boucher, C.A., McAllister, T.A., Belk, K.E., Morley, P.S., 2016. Characterization of the resistome in manure, soil and wastewater from dairy and beef production systems. Sci. Rep. 6, 24645. https://doi.org/10.1038/srep24645

Oksanen, J., Blanchet, G.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., 2019. vegan: Community Ecology Package.

Pan, Y., Cassman, N., de Hollander, M., Mendes, L.W., Korevaar, H., Geerts, R.H.E.M., van Veen, J.A., Kuramae, E.E., 2014. Impact of long-term N, P, K, and NPK fertilization on the composition and potential functions of the bacterial community in grassland soil. FEMS Microbiol. Ecol. 90, 195–205. https://doi.org/10.1111/1574-6941.12384

Pandey, P.K., Biswas, S., Vaddella, V.K., Soupir, M.L., 2015. Escherichia coli persistence kinetics in dairy manure at moderate, mesophilic, and thermophilic temperatures under aerobic and anaerobic environments. Bioprocess Biosyst. Eng. 38, 457–467. https://doi.org/10.1007/s00449-014-1285-3

Pedregosa, F., Varoquaux, G., Gramfort, A., Michel, V., Thirion, B., Grisel, O., Blondel, M., Prettenhofer, P., Weiss, R., Dubourg, V., Vanderplas, J., Passos, A., Cournapeau, D., Brucher, M., Perrot, M., Duchesnay, É., 2011. Scikit-learn: Machine learning in Python. J. Mach. Learn. Res. 12, 2825–2830.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. Nucleic Acids Res. 41, D590–D596. https://doi.org/10.1093/nar/gks1219

Quince, C., Lanzen, A., Davenport, R.J., Turnbaugh, P.J., 2011. Removing Noise From Pyrosequenced Amplicons. BMC Bioinformatics 12, 38. https://doi.org/10.1186/1471-2105-12-38

Quintela-Baluja, M., Abouelnaga, M., Romalde, J., Su, J.-Q., Yu, Y., Gomez-Lopez, M., Smets, B., Zhu, Y.-G., Graham, D.W., 2019. Spatial ecology of a wastewater network defines the antibiotic resistance genes in downstream receiving waters. Water Res. 162, 347–357. https://doi.org/10.1016/j.watres.2019.06.075

R Core Team, 2020. R: A Language and Environment for Statistical Computing.

Riber, L., Poulsen, P.H.B., Al-Soud, W.A., Skov Hansen, L.B., Bergmark, L., Brejnrod, A., Norman, A., Hansen, L.H., Magid, J., Sørensen, S.J., 2014. Exploring the immediate and long-term impact on bacterial communities in soil amended with animal and urban organic waste fertilizers using pyrosequencing and screening for horizontal transfer of antibiotic resistance. FEMS Microbiol. Ecol. 90, 206–224. https://doi.org/10.1111/1574-6941.12403

Rocha, J., Cacace, D., Kampouris, I., Guilloteau, H., Jäger, T., Marano, R.B.M., Karaolia, P., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Berendonk, T.U., Schwartz, T., 2018. Inter-laboratory calibration of quantitative analyses of antibiotic resistance genes. J. Environ. Chem. Eng. 0–1. https://doi.org/10.1016/j.jece.2018.02.022

Song, H.K., Song, W., Kim, M., Tripathi, B.M., Kim, H., Jablonski, P., Adams, J.M., 2017. Bacterial strategies along nutrient and time gradients, revealed by metagenomic analysis of laboratory microcosms. FEMS Microbiol. Ecol. 93. https://doi.org/10.1093/femsec/fix114

Thanner, S., Drissner, D., Walsh, F., 2016. Antimicrobial Resistance in Agriculture. MBio 7, 1–7. https://doi.org/10.1128/mBio.02227-15

Thorsten, T., 2020. PMCMRplus: Calculate Pairwise Multiple Comparisons of Mean Rank Sums Extended.

Travis, E.R., Gaze, W.H., Pontiroli, A., Sweeney, F.P., Porter, D., Mason, S., Keeling, M.J.C., Jones, R.M., Sawyer, J., Aranaz, A., Rizaldos, E., Cork, J., Delahay, R.J., Wilson, G.J., Hewinson, R.G., Courtenay, O., Wellington, E.M., 2011. An Inter-Laboratory validation of a real time PCR assay to measure host excretion of bacterial pathogens, particularly of Mycobacterium bovis. PLoS One 6. https://doi.org/10.1371/journal.pone.0027369

Udikovic-Kolic, N., Wichmann, F., Broderick, N.A., Handelsman, J., 2014. Bloom of resident antibiotic-resistant bacteria in soil following manure fertilization. Proc. Natl. Acad. Sci. U. S. A. 111, 15202–7. https://doi.org/10.1073/pnas.1409836111

Vadstein, O., Attramadal, K.J.K., Bakke, I., Olsen, Y., 2018. K-Selection as Microbial Community Management Strategy: A Method for Improved Viability of Larvae in Aquaculture. Front. Microbiol. 9. https://doi.org/10.3389/fmicb.2018.02730

van Veen, J.A., van Overbeek, L.S., van Elsas, J.D., 1997. Fate and activity of microorganisms introduced into soil. Microbiol. Mol. Biol. Rev. 61, 121–135. https://doi.org/10.1128/.61.2.121-135.1997

Wang, M., Liu, P., Xiong, W., Zhou, Q., Wangxiao, J., Zeng, Z., Sun, Y., 2018. Fate of potential indicator antimicrobial resistance genes (ARGs) and bacterial community diversity in simulated manure-soil microcosms. Ecotoxicol. Environ. Saf. 147, 817–823. https://doi.org/10.1016/j.ecoenv.2017.09.055

Wichmann, F., Udikovic-Kolic, N., Andrew, S., Handelsman, J., 2014. Diverse Antibiotic Resistance Genes in Dairy Cow Manure. MBio 5, 263. https://doi.org/10.1128/mBio.01017-13

Wickham, H., 2007. Reshaping Data with the reshape Package. J. Stat. Softw. 21, 1--20.

Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L., François, R., Grolemund, G., Hayes, A., Henry, L., Hester, J., Kuhn, M., Pedersen, T., Miller, E., Bache, S., Müller, K., Ooms, J., Robinson, D., Seidel, D., Spinu, V., Takahashi, K., Vaughan, D., Wilke, C., Woo, K., Yutani, H., 2019. Welcome to the Tidyverse. J. Open Source Softw. 4, 1686. https://doi.org/10.21105/joss.01686

Xie, W.-Y., Shen, Q., Zhao, F.J., 2018. Antibiotics and antibiotic resistance from animal manures to soil: a review. Eur. J. Soil Sci. 69, 181–195. https://doi.org/10.1111/ejss.12494

Xie, Wan-Ying, Yuan, S.-T., Xu, M.-G., Yang, X.-P., Shen, Q.-R., Zhang, W.-W., Su, J.-Q., Zhao, F.-J., 2018. Long-term effects of manure and chemical fertilizers on soil antibiotic resistome. Soil Biol. Biochem. 122, 111–119. https://doi.org/10.1016/j.soilbio.2018.04.009

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., Aarestrup, F.M., Larsen, M.V., 2012. Identification of acquired antimicrobial resistance genes. J. Antimicrob. Chemother. 67, 2640–2644. https://doi.org/10.1093/jac/dks261

Zhang, Y.-J., Hu, H.-W., Chen, Q.-L., Singh, B.K., Yan, H., Chen, D., He, J.-Z., 2019. Transfer of antibiotic resistance from manure-amended soils to vegetable microbiomes. Environ. Int. 130, 104912. https://doi.org/10.1016/j.envint.2019.104912

Zhang, Y.-J.J., Hu, H.-W.W., Gou, M., Wang, J.-T.T., Chen, D., He, J.-Z.Z., 2017. Temporal succession of soil antibiotic resistance genes following application of swine, cattle and poultry manures spiked with or without antibiotics. Environ. Pollut. 231, 1621–1632. https://doi.org/10.1016/j.envpol.2017.09.074

CHAPTER 4

"One of history's few iron laws is that luxuries tend to become necessities and to spawn new obligations."
Yuval Noah Harari
Sapiens: A Brief History of Humankind, 2011

Thermophilic anaerobic digestion of cattle and swine manure removed *Escherichia coli*, but not sulfite-reducing *Clostridia* or antimicrobial resistance genes

Gonçalo Macedo[1,2], Chris Schott[2,3], Yede van der Zee[2,4], Lucia Hernandez-Leal[2], Peter van der Maas[4], Heike Schmitt[1,2,5]

[1] Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands
[2] Wetsus, European Centre of Excellence for Sustainable Water Technology, Oostergoweg 9, 8911 MA Leeuwarden, The Netherlands
[3] Wageningen University, 6700AA Wageningen, The Netherlands
[4] Van Hall Larenstein, University of Applied Sciences, Agora 1, 8901 BV Leeuwarden, The Netherlands
[5] National Institute for Public Health and the Environment (RIVM), Antonie van Leeuwenhoeklaan 9, 3721 MA Bilthoven, The Netherlands

4.1 Abstract

Anaerobic digestion of ARG-containing animal waste bears the possibility to recover energy and nutrients. Hybrid up-flow anaerobic sludge bed (UASB) thermophilic digesters may increase phosphorus recovery, but their ARG removal capacity from manure is not yet clear. Therefore, this study quantified the abundances of indicator bacteria *Escherichia coli* and sulfite-reducing *Clostridia* (SRC) and the antimicrobial resistance genes (ARGs) *sul1*, *erm(B)*, and *tet(W)* to evaluate the removal capacity of lab-scale thermophilic anaerobic digestors containing the liquid fraction of either cattle or swine manure. The results confirmed that an extensive removal was achieved for *E. coli* in both manure types (to below quantification limit, which was 4.7 log CFU/kg ww). SRC removal was only observed in cattle manure (roughly 0.7 logs), and no ARG removal was evident in both manure types. This work highlights the need to consider other parameters besides nutrient recovery, such as AMR, when developing innovative technology to handle animal waste.

4.2 Introduction

As the human population is on the rise worldwide, so is the demand for food-animal products. This intensifies livestock farming and increases manure production. In the Netherlands alone, over 76 million tons of animal manure are produced every year, most of which is applied untreated on farmlands (WUR, 2019). Manure application is used to close agricultural nutrient cycles and fertilize crops and grasslands. However, it also results in the introduction of partially-metabolized antibiotics (Du and Liu, 2012; Jechalke et al., 2014), antibiotic-resistant bacteria (ARB), and antibiotic resistance genes (ARGs) (Chee-Sanford et al., 2009; Cook et al., 2014; He et al., 2016; Heuer et al., 2011). Antimicrobial resistance (AMR) has been considered as one of the significant challenges to global public health. Thus, exploring new solutions to mitigate antimicrobial resistance in the environment are becoming increasingly relevant.

The application of anaerobic digestion treatments of animal waste, particularly from cattle and swine, has been used because it allows the recovery of nutrients and biogas production, which can be directly used at farms. Anaerobic treatments performed under mesophilic conditions (35 ± 2 °C) can remove ARB, but the extent of the removal can be limited (Beneragama et al., 2013; Resende et al., 2014). Recent publications showed that mesophilic anaerobic treatments do not remove ARGs, as sometimes increases were noted after treatment (Pu et al., 2018; Zhang et al., 2019, 2017). Operating anaerobic reactors under thermophilic conditions (55 ± 2 °C) proved not only to be more efficient than mesophilic treatments in degrading organic matter and yielding higher methane production (Moset et al., 2015), but also in removing fecal indicator pathogens (Iwasaki et al., 2019; Pandey et al., 2015; Pandey and Soupir, 2011), and ARGs (Sun et al., 2016) from manure, even though some ARGs might persist after the treatment (Huang et al., 2019). Due to the high maintenance costs and potential lower treatment stability derived from the higher temperatures, farmers typically operate anaerobic digesters at lower temperatures (Youngquist et al., 2016).

Manure separation technology can also be used to achieve a phosphate-rich compacted solid fraction and a nitrogen-rich liquid fraction (Gebrezgabher et al., 2015). As an additional treatment for the liquid fraction, hybrid up-flow anaerobic sludge bed (UASB) thermophilic digesters could also provide high P-recoveries. This P-recovery approach has been successfully demonstrated in black water (Cunha et al., 2018; Tervahauta et al., 2014), but it is not clear how these reactors perform in removing ARGs from manure. Therefore, this study aimed to determine the bacterial and ARG removal efficiency of a lab-scale thermophilic anaerobic digestor from swine and cattle manure. The reactor performances were evaluated based on qPCR results and enumeration of indicator bacteria (*Escherichia coli* and spores of sulfite-reducing *Clostridia*).

4.3 Methodology

4.3.1 Manure characteristics and sample collection

For the lab-scale thermophilic reactors, manure was collected from a dairy farm composed of 200 cows and from a pig farm growing 4400 fattener pigs. Both farms are located in the Netherlands. Between July and October 2019, a total of three manure batches were collected from each farm, sieved (200 µm), and loaded into four previously characterized up-flow anaerobic sludge blanket (UASB) reactors (Cunha et al., 2019). Each reactor was operated continuously, with interval feeding every two hours, and treated either cattle or swine manure (i.e., two reactors per manure type). Calcium was added to one reactor of each manure type (Ca^{2+}; 0.57 g Ca^{2+}/day and 3.72 g Ca^{2+}/day to cow and pig reactor, respectively). Each reactor had an effective working volume of 45 L, continuously heated at 55 °C. The reactors containing cattle manure had a hydraulic retention time (HRT) of 50 days, while for pig reactors, the HRT was of 30 days. The sludge retention time (SRT) was higher than the HRT in both manure types. Their values can be found in the supplemental information, together with the other physical-chemical parameters of the reactors (Supplementary Table 1).

Upon collection of new manure batches, samples from the raw manure (original), sieve-retained (cake), and sieved through (sieved, also referred as liquid fraction) were collected and processed within 24 hours after collection. The manure's liquid fraction was then stored at 4 °C until being loaded into the reactors (average of 18 days, between 8 and 30 days). Samples from immediately before entering the reactors (influent), sludge, and after treatment (effluent) were collected weekly (14 weeks) and processed within 6 hours after collection. Aliquots were stored at -20 °C before DNA extraction and chemical analysis.

4.3.2 Enumeration of *E. coli* and sulfite-reducing *Clostridia* (SRC)

For the enumeration of total and third-generation cephalosporin resistant *E. coli*, samples were diluted from 10^{-1} to 10^{-4} in sterile saline solution (0.85% m/v) and filtered through 0.45 µm-pore sized mixed cellulose ester S-Pak filters (Merck-Millipore, USA). Then, the filters were placed on Tryptone Bile X-glucuronide (TBX) agar media (Oxoid, UK), and TBX plates containing cefotaxime (CTX; EWC Diagnostics, Netherlands), in duplicate. The plates were incubated at 37 °C, for 4 hours, then at 44 °C, for 12-20 hours, and after incubation, the blue/green colonies typical for *E. coli* were counted. Ten randomly selected blue/green colonies were plated on fresh TBX plates for presumptive confirmation of the results. Based on the ratio of positive confirmation, the total colony-forming unit (CFU) counts were adjusted.

The enumeration of spores of SRC was done according to ISO guidelines (ISO 15213:2003). The samples were heated at 75 ± 5 °C, for 15 minutes and immediately cooled on ice

for 5 minutes. Then, dilution series of 10^{-1} to 10^{-4} were prepared in sterile saline solution (0.85% m/v), filtered through 0.22 µm-pore sized mixed cellulose ester S-Pak filters (Merck-Millipore, USA), and plated on iron-sulfate agar (ISA; Condalab, Spain), in duplicate. The plates were incubated in anaerobic conditions (Anaerocult; Merck-Millipore) at 37 °C, for 24 – 48 hours, and then black colonies were counted.

All bacterial enumerations were calculated according to ISO guidelines (ISO 8199:2018). Briefly, the final bacterial concentration (CFU/kg or L) results from the sum of the total CFU obtained in a sample divided by the total amount of the same sample tested. The limit of quantification (LOQ) was calculated by assuming a count of 10 CFU in the least diluted sample.

4.3.3 DNA extraction and quantification

The DNA extracts were obtained from 200 mg of cake and sludge samples and 400 mg of original, liquid fraction, influent, and effluent using the QIAamp DNA Stool Mini Kit (QIAGEN; Hilden, Germany). The DNA extraction proceeded according to the manufacturer's instructions, with an additional bead-beating step adjusted to the Precellys Evolution (Bertin Instruments; Montigny-le-Bretonneux, France) for enhanced cell lysis. DNA quantification was performed using the Quantus Fluorometer system (Promega; Madison, WI, USA) according to the manufacturer's protocol. The purity of the extracts was verified with NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA).

4.3.4 ARG quantification by qPCR

The amplification of the 16S rRNA genes (*rrs*) and selected ARGs (*sul1, erm(B)*, and *tet(W)*) has been described previously (Macedo 2020). Briefly, the absolute abundance of these genes was quantified by qPCR in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad; Hercules, CA, USA), and each qPCR run tested with a standard curve ranging between 10^3 – 10^7, or 10^1 – 10^7 target copies of standardized gene fragments (gBlocks; IDT technologies, Belgium) for *rrs,* or ARGs, respectively, and non-template controls. More information regarding primers used and reaction chemical composition can be found in Supplementary Table 2. Cut-off values were calculated based on the lowest quantity of genes included in the calibration curve of all plates, and all calibration curves had a signal intensity of >2 threshold cycle (C_t) difference to the non-template controls. The average of the highest C_t obtained from this point across all plates was taken, and the standard deviation was added to it.

All assays were performed on the diluted DNA extracts, in duplicate, and the results were inspected to ensure that each duplicate fell within 1 C_t. The qPCR amplification efficiencies ranged between 91% and 100%, and the melting curves were performed to confirm the amplicon specificity, starting at 65 °C with successive increments of 0.5 °C, up to 95 °C. The

quantifications occurred for each original extract, in duplicates, following the Standard Curve method described elsewhere (Brankatschk et al., 2012).

4.3.5 Statistical analysis

One-way analysis of variance (ANOVA) was conducted to detect differences in the bacteria and ARG abundances. The ANOVA tests were followed by TukeyHSD post-hoc analysis, and homogeneity of variance was confirmed with Levene's test. Data normality was confirmed with Shapiro-Wilk's method, and when normality was not achieved, group comparison was performed using the equivalent non-parametric test (Kruskal-Wallis). A significance threshold of $p < 0.05$ was considered statistically relevant for all ANOVA, except when mentioned otherwise. These analyses were performed with R version 3.6.3 (R Core Team, 2020) and RStudio (Version 1.2.5033; https://www.rstudio.com/). Used software packages consisted of *reshape* (Wickham, 2007) and *tidyverse* (Wickham et al., 2019), a set of packages designed for data cleaning, trimming, and visualization; of *PMCMRplus* (Thorsten, 2020), and *car* (Fox and Weisberg, 2019) for ANOVA and Levene's test.

4.4 Results

4.4.1 Bacteria and ARG abundances in the reactors

Overall, the reactor performed similarly for both manure types in terms of bacterial removal. *E. coli* significantly decreased their abundance after treatment in both manure types, and the SRC spores decreased in cattle but not pig manure. No ESBL-containing *E. coli* was detected in any sample.

In reactors with cattle manure, *E. coli* declined from 6.07 ± 0.38 log CFU/kg to levels below the limit of quantification (LOQ, which was 4.7 log CFU/kg ww; Figure 1) and SRC spores from 6.57 ± 0.30 to 5.80 ± 0.24 log CFU/kg ($p < 0.05$). In reactors with pig manure, *E. coli* declined from 6.15 ± 1.14 log CFU/kg to levels below the LOQ (Figure 1), while SRC spores remained similar (from 5.88 ± 0.53 to 5.66 ± 0.23 log CFU/kg). Throughout the manure separation process, from the original raw manure to the liquid fraction, the abundance of *E. coli* and SRC was relatively stable (Figure 1 and Supplementary Table 3). Notably, cow manure storage at 4 °C resulted in a slight increase of SRC in influent (roughly 0.7 logs; $p = 0.060$).

The ARG abundance during the different stages was relatively stable, with no significant changes being recorded in reactors with bovine manure ($p < 0.05$). However, in the pig reactors, *sul1* abundances were reduced in sludge samples (Figure 1; $p < 0.05$), and *tet(W)* abundances were increased in effluent when compared to influent and sludge ($p < 0.05$). The relative abundance of *ermB* varied considerably during the sampling, with values

ranging roughly 3 logs between the minimum and maximum values. The bla_{TEM} gene was consistently below the detection limit in all the tested samples, and, for this reason, it was excluded from the analysis.

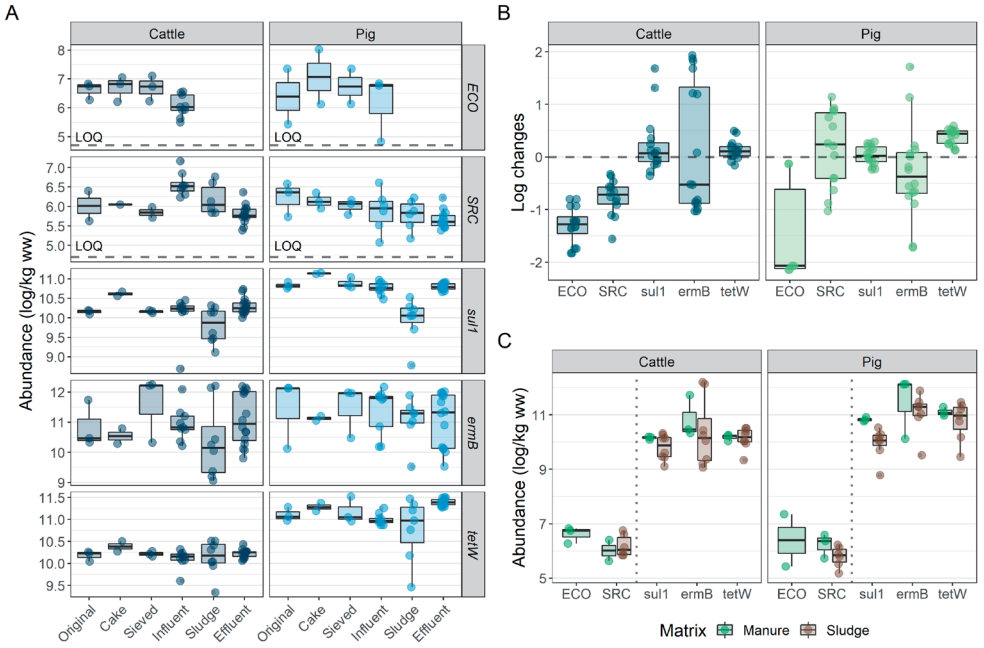


Figure 1. Relative abundance of different indicators in cattle and swine manure. The boxplots with scatter points indicate the abundances at different treatment stages (A), the removal capacity of the reactor (influent *vs.* effluent; B), and abundances between raw manure and reactor sludge (C). *E. coli* (ECO) was extensively removed, while sulfite-reducing *Clostridia* (SRC) declined slightly (only in cow). While no changes were noted for *erm(B)* in both manure types, *sul1* decreased in sludge samples, while *tet(W)* increased in the effluent (only in pig reactors). Changes in *E. coli* were calculated by using the limit of quantification (LOQ, which was 4.7 log CFU/kg ww).

4.4.2 Effect of calcium addition and manure storage

Overall, the addition of calcium to the reactors did not result in abundance changes in the reactors, except in the sludge of the pig reactor, where *tet(W)* was less abundant in the reactor with calcium addition than in the one without ($p < 0.05$; Supplementary Figure 1). Calcium addition may affect the gene/microbe abundances as calcium chloride increases the salt content. Additionally, solid retention and particle agglomeration increase with calcium addition which might also affect the removal.

In cow manure, the storage time significantly affected *E. coli* and SRC abundance, but not the ARG abundance (Supplementary Figure 2). A strong negative correlation was found for *E. coli* (correlation: -0.690; decline of 0.031 logs/day; $p = 0.01$), while a strong positive correlation

was found for SRC (correlation: 0.792; increment of 0.030 logs/day; $p < 0.01$). In pig manure, storage time had no visible effects on the abundances of the measured variables.

4.5 Discussion

In this study, CFU counts and qPCR were used to evaluate the performance and ARG and indicator bacteria removal capacity of lab-scale thermophilic anaerobic digestors that used either cattle or swine manure. The results confirmed that removal of at least 1 log (to below LOQ; thus, absolute removal could not be determined) was achieved for *E. coli* in both manure types, but SRC spores were only slightly reduced in cattle manure (roughly 0.5 logs). No ARG removal was evident.

4.5.1 Reactor output contains similar ARG abundance as raw manure

In general, *E. coli* and SRC are common indicators of fecal contamination in the environment. The ARGs used in this study were selected based on their high prevalence in manure (Schmitt et al., 2019) and representativity of the most commonly applied used antimicrobials used in animal health (de Greeff and Mouton, 2018).

Regarding bacteria removal, results similar to the ones described in the present study have been reported. Previous reports showed that *E. coli* was removed entirely from manure digestate after thermophilic anaerobic digestion (Iwasaki et al., 2019; Pandey et al., 2015). Elsewhere, the bacterial community analysis from thermophilic digestates revealed that *Firmicutes*, from which SRC are members, maintain their relative abundance compared to manure before thermophilic digestion (Huang et al., 2019; Sun et al., 2016).

Up to now, only a handful of publications have been found on thermophilic anaerobic digestion of swine or cattle manure. Contrarily to the findings of the present study, others have reported the removal of ARGs after thermophilic anaerobic digestion while maintaining similar HRTs. For example, (Sun et al., 2016) reported that most of the ARGs declined by 1-2 logs after treatment of dairy manure (HRT of 60 days), including *sul1* and *tet(W)* that decreased roughly 1.5 and 0.25 logs, respectively. Huang et al. (2019) reported average log removals of 0.58 - 0.66 of sulfonamide, macrolide, and tetracycline resistance genes in swine manure (HRT of 30 days).

Previously, it has been shown that the microbial community plays a vital role in the fate of ARGs in anaerobic digestion (Guo et al., 2020; Lu et al., 2020; Zhang et al., 2020). Factors such as digestion time and temperature, microbial diversity and abundance, ammonia stress, and mobile genetic elements may influence the ARG abundance and persistence because they also have a direct or indirect impact on the overall bacterial community. Interestingly, operating reactors at thermophilic temperatures does not necessarily result in better ARG removal despite significantly changing the microbial composition (Huang et al., 2019). On the other hand, by completely removing *E. coli*, thermophilic anaerobic digestion effectively reduced cefazolin-resistant bacteria and ESBL-producers in dairy manure (Iwasaki et al., 2019). Despite no ARG removal being observed, the reactor in the present study successfully removed *E. coli*, and it is possible that removal may have occurred in other ARGs that were not targeted. Therefore, more parameters should be studied to obtain a clearer picture of what is happening inside the reactor, namely regarding the total microbial community and other ARGs. Furthermore, additional processing steps should be investigated regarding their impact on ARG removal. For example, the introduction of relatively short thermal treatment stages to mesophilic digestion was shown to enhance the removal of pathogenic microorganisms (Marañón et al., 2006) and some ARGs (Wallace et al., 2018) while avoiding the higher costs derived from continuous high-temperature operations.

4.5.2 Implications on-farm management

On average, one dairy cow produces roughly 70 kg/day of manure, and a fattening pig roughly 3 kg/day (CBS StatLine; https://opendata.cbs.nl/statline/#/CBS/en/), resulting in over 76 million tons of animal manure produced every year in the Netherlands alone (WUR, 2019). Consequently, manure is widely used as fertilizer and applied directly to soils. On the other hand, due to the potential overaccumulation and nutrient leaching to environmental watercourses, European legislation restricts manure application by limiting nutrient emissions to the environment. Given this scenario, it is possible that the treated effluents, which contain lower N and P concentrations, could be applied at higher amounts than raw manure would. For example, the reactors' effluents without calcium addition had roughly 8% less ammonia than the influent, while the calcium reactors had approximately 12-20% less than the influent. P was more thoroughly removed than NH_4, and the total P in effluents were on average 29 to 68% and 53 to 78% less than in cow and pig influents, respectively (Supplementary Table 1). The potential increase of manure application rates to soils is concerning because no differences in ARG abundances were found between the treated effluents and raw manure. Ultimately, this could imply that ARG emissions to the environment would increase.

4.6 Conclusions

In this study, the lab-scale thermophilic reactors performing anaerobic digestion removed *E. coli* from cow and pig manure. Furthermore, the treatment slightly decreased SRC spore abundances in cattle but not in pig manure, and no ARG removal was observed for both manure types. Still, many questions remain to be answered regarding the ARG host and genetic location (e.g., plasmids). Additional studies should be conducted targeting the reactor's microbial community, other ARGs, and mobile genetic elements, as this could help identify which main factors are shaping the ARG hosts, and consequently, the fate of ARGs.

4.7 Acknowledgments

The authors gratefully acknowledge Nike Schiavo and Aisha Pereira for the technical support during sample collection; and Dick Heederik and Dik Mevius for their help reviewing the manuscript.

4.8 Funding

This work was performed in the cooperation framework of Wetsus, European Centre of excellence for sustainable water technology (www.wetsus.nl). Wetsus is co-funded by the Dutch Ministry of Economic Affairs and Ministry of Infrastructure and Environment, the European Union Regional Development Fund, the Province of Fryslân, and the Northern Netherlands Provinces. This work has also received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie [grant agreement No. 665874]. The authors like to thank the participants of the research theme Source Separated Sanitation for the fruitful discussions and their financial support.

4.9 Supplementary Information

Supplementary Table 1. General characteristics of influent and effluent samples represented by the averaged values and correspondent standard deviations. Hydraulic and sludge retention times (HRT and SRT, respectively) are presented in days.

	Cattle			Pig		
	Influent (17)	**Effluent (17)**	**Effluent (18)***	**Influent (18)**	**Effluent (13)**	**Effluent (16)***
HRT (days)	-	50	50	-	30	30
SRT (days)**		136	346		414	594
pH	8.06 ± 0.35	8.81 ± 0.07	8.97 ± 0.11	8.31 ± 0.19	8.92 ± 0.13	8.86 ± 0.11
TS (g/L)	54.33 ± 6.85	40.41 ± 4.73	30.88 ± 2.49	44.15 ± 7.64	14.63 ± 8.12	7.2 ± 2.19
VS (g/L)	36.05 ± 4.69	23.65 ± 3.21	16.75 ± 1.78	28.56 ± 5.07	8.6 ± 5.69	3.57 ± 1.66
TSS (g/L)	36.25 ± 4.89	18.64 ± 6.11	8.55 ± 3.22	57.56 ± 8.41	42.76 ± 4.92	33.55 ± 4.42
VSS (g/L)	26.14 ± 3.68	12.08 ± 4.38	5.23 ± 2.37	33.1 ± 5.3	22.29 ± 3.34	16.3 ± 1.9
COD (mg/L)	62952 ± 10661	39546 ± 7104	27419 ± 4356	55113 ± 8852	40097 ± 6104	33533 ± 3638
TOC (mgC/L)	13719 ± 3496	10252 ± 776	8885 ± 746	8004 ± 1277	13088 ± 1099	11484 ± 1186
TC (mgC/L)	15858 ± 3392	12832 ± 960	11296 ± 841	13749 ± 2032	16592 ± 966	14066 ± 1430
NH_4 (mg/L)	2671 ± 793	2445 ± 327	2132 ± 246	6179 ± 1575	5670 ± 779	5437 ± 930
P (mg/L)	488 ± 38	346 ± 128	158 ± 61	2106 ± 369	983 ± 370	460 ± 93
Ca (mg/L)	1140 ± 100	715 ± 258	405 ± 199	2467 ± 401	982 ± 484	691 ± 113
Mg (mg/L)	672 ± 73	489 ± 122	278 ± 70	1209 ± 243	367 ± 268	202 ± 53

Note: *Ca^{2+} added to reactor (0.57 and 3.72 g/day to cattle and swine reactor, respectively. ** Calculation for SRT: solids in reactor / (solids washed out per day + solids wasted or sampled per day)

Supplementary Table 2. Primers and conditions used in the qPCR assays.

Target	Primer name	Primer sequence (5'- 3')	Amplicon size (bp)	Reference
rrs	q_338F	ACT CCT ACG GGA GGC AGC AG	179	(Fierer et al. 2005)
	q_518R	ATT ACC GCG GCT GCT GG		
sul1	q_sul1-FW	CGC ACC GGA AAC ATC GCT GCA C	163	(Pei et al. 2006)
	q_sul1-RV	TGA AGT TCC GCC GCA AGG CTC G		
erm(B)	q_ermBF	AAA ACT TAC CCG CCA TAC CA	139	(Knapp et al. 2010)
	q_ermBR	TTT GGC GTG TTT CAT TGC TT		
bla_{TEM}	q_TEM-FW	TTC CTG TTT TTG CTC ACC CAG	112	(Bibbal et al. 2007)
	q_TEM-RV	CTC AAG GAT CTT ACC GCT GTT G		
tet(W)	q_tetW-FW	CGG CAG CGC AAA GAG AAC	59	(Walsh et al. 2011)
	q_tetW-RV	CGG GTC AGT ATC CGC AAG TT		

Reaction composition and temperature cycles

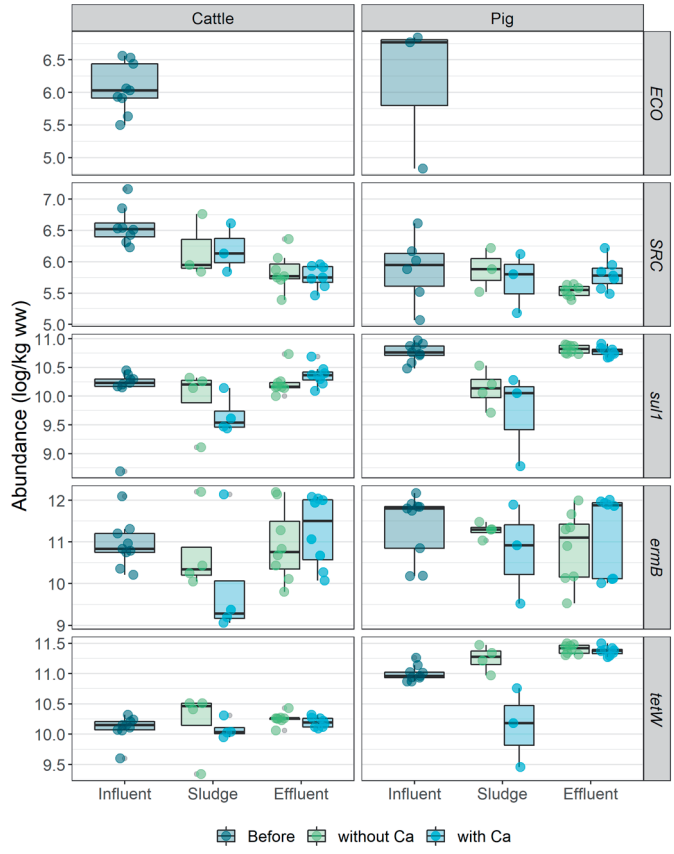
The extracts were quantified in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad; Hercules, CA, USA). Each qPCR reaction contained 1x iQ™ SYBR Green Supermix (Bio-Rad), 300 mM of both forward and reverse primers (except for *ermB* and bla_{TEM} where 400 mM was used), 2 µg of bovine serum albumin (BSA; Thermo Scientific, USA), and 2 µL of 10-fold diluted DNA (1-10 ng), in a final volume of 20 µL. The following thermal-cycling conditions were applied: 95 °C for 10 min (1 cycle); 95 °C for 15 s, 60 °C for 1 min (except for *sul1*, which required 65 °C), in 40 cycles.

Supplementary Table 3. ARG and CFU abundance in manure throughout the treatment stages. Average values and correspondent standard deviations are presented by sample weight (log/kg ww) for CFUs and ARGs.

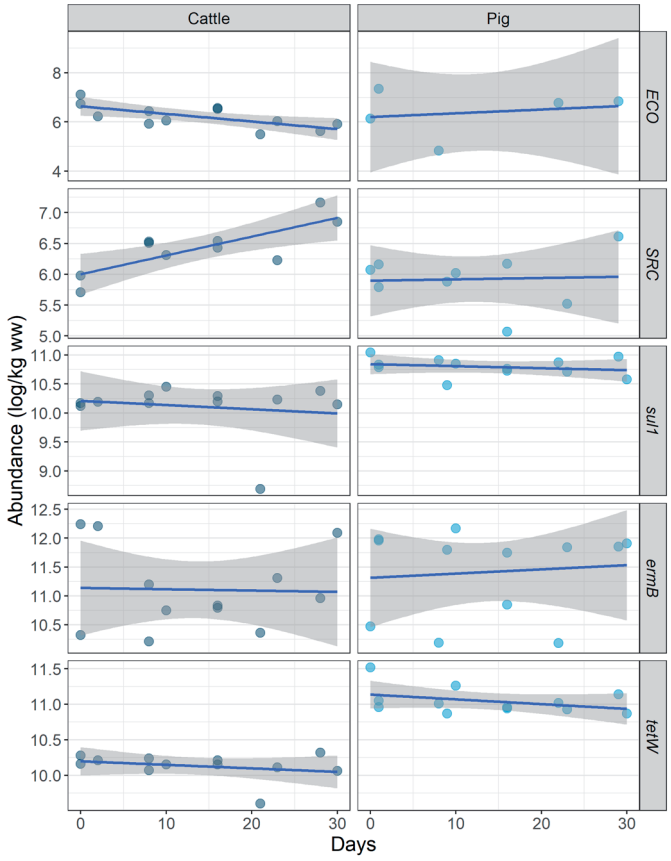
Cattle (n)	**Original (3)**	**Cake (3)**	**Sieved (3)**	**Influent (9)**	**Sludge (4)**	**Effluent (8)**	**Sludge (4)***	**Effluent (8)***
Dry Matter (%)	0,05 ± 0,01	0,11 ± 0,02	0,05 ± 0,01	0,05 ± 0,01	0,08 ± 0,02	0,04 ± 0,01	0,09 ± 0,04	0,03 ± 0,00
ECO	6,62 ± 0,30	6,69 ± 0,43	6,69 ± 0,44	6,07 ± 0,38	< LOQ	< LOQ	< LOQ	< LOQ
SRC	6,02 ± 0,54	6,05 ± NA	5,85 ± 0,19	6,57 ± 0,30	6,18 ± 0,50	5,84 ± 0,30	6,19 ± 0,39	5,76 ± 0,18
rrs	13,02 ± 0,15	9,84 ± 5,92	13,13 ± 0,11	12,87 ± 0,71	12,84 ± 0,69	13,00 ± 0,10	12,54 ± 0,29	12,94 ± 0,07
sul1	10,15 ± 0,06	10,62 ± 0,08	10,16 ± 0,04	10,10 ± 0,54	9,96 ± 0,57	10,23 ± 0,22	9,66 ± 0,33	10,36 ± 0,18
erm(B)	10,84 ± 0,77	10,54 ± 0,36	11,59 ± 1,1	10,94 ± 0,56	10,73 ± 0,99	10,93 ± 0,88	9,94 ± 1,47	11,27 ± 0,85
tet(W)	10,18 ± 0,12	10,38 ± 0,16	10,22 ± 0,06	10,10 ± 0,21	10,19 ± 0,57	10,25 ± 0,10	10,08 ± 0,16	10,20 ± 0,08

Swine (n)	**Original (3)**	**Cake (3)**	**Sieved (3)**	**Influent (9)**	**Sludge (4)**	**Effluent (8)**	**Sludge (4)***	**Effluent (8)***
Dry Matter (%)	0,11 ± 0,07	0,13 ± 0,06	0,06 ± 0,01	0,06 ± 0,02	0,13 ± 0,05	0,04 ± 0,00	0,08 ± 0,05	0,04 ± 0,00
ECO	6,39 ± 1,36	7,07 ± 1,34	6,74 ± 0,86	6,15 ± 1,14	< LOQ	< LOQ	< LOQ	< LOQ
SRC	6,22 ± 0,44	6,14 ± 0,20	6,01 ± 0,19	5,88 ± 0,53	5,87 ± 0,35	5,53 ± 0,10	5,70 ± 0,48	5,80 ± 0,24
rrs	13,22 ± 0,09	13,46 ± 0,13	13,28 ± 0,21	13,15 ± 0,14	12,57 ± 0,45	13,43 ± 0,04	12,17 ± 1,00	13,41 ± 0,06
sul1	10,83 ± 0,08	11,13 ± 0,02	10,89 ± 0,13	10,76 ± 0,16	10,13 ± 0,34	10,82 ± 0,07	9,70 ± 0,81	10,78 ± 0,08
erm(B)	11,46 ± 1,16	11,12 ± 0,11	11,47 ± 0,87	11,39 ± 0,77	11,28 ± 0,19	10,88 ± 0,86	10,78 ± 1,19	11,24 ± 0,96
tet(W)	11,10 ± 0,16	11,28 ± 0,13	11,18 ± 0,30	11,00 ± 0,13	11,25 ± 0,21	11,40 ± 0,08	10,13 ± 0,65	11,37 ± 0,07

Note: *Ca^{2+} added to reactor (0.57 and 3.72 g/day to cattle and swine reactor, respectively.
LOQ: limit of quantification.



Supplementary Figure 1. Comparison of abundances between reactors with (blue) and without (green) calcium addition in samples before entering the reactors (influent), in the sludge beds, and in the reactor effluents.



Supplementary Figure 2. Impact of storage days (at 4 °C) on the abundance of *E. coli*, SRC, *sul1*, *erm(B)*, and *tet(W)* in cow and pig manure.

4.10 References

Beneragama, N., Iwasaki, M., Lateef, S.A., Yamashiro, T., Ihara, I., Umetsu, K., 2013. The survival of multidrug-resistant bacteria in thermophilic and mesophilic anaerobic co-digestion of dairy manure and waste milk. Anim. Sci. J. 84, 426–433. https://doi.org/10.1111/asj.12017

Brankatschk, R., Bodenhausen, N., Zeyer, J., Burgmann, H., 2012. Simple absolute quantification method correcting for quantitative PCR efficiency variations for microbial community samples. Appl. Environ. Microbiol. 78, 4481–4489. https://doi.org/10.1128/AEM.07878-11

Chee-Sanford, J.C., Mackie, R.I., Koike, S., Krapac, I.G., Lin, Y.-F., Yannarell, A.C., Maxwell, S., Aminov, R.I., 2009. Fate and Transport of Antibiotic Residues and Antibiotic Resistance Genes following Land Application of Manure Waste. J. Environ. Qual. 38, 1086. https://doi.org/10.2134/jeq2008.0128

Cook, K.L., Netthisinghe, A.M.P., Gilfillen, R.A., 2014. Detection of Pathogens, Indicators, and Antibiotic Resistance Genes after Land Application of Poultry Litter. J. Environ. Qual. 43, 1546. https://doi.org/10.2134/jeq2013.10.0432

Cunha, J.R., Buisman, C.J.N.P. dr, Zeeman, G.P. dr, Weijden, R.D. van der D.C., 2018. Anaerobic calcium phosphate bio granulation.

Cunha, J.R., Schott, C., van der Weijden, R.D., Leal, L.H., Zeeman, G., Buisman, C., 2019. Recovery of calcium phosphate granules from black water using a hybrid upflow anaerobic sludge bed and gas-lift reactor. Environ. Res. 178, 108671. https://doi.org/10.1016/j.envres.2019.108671

de Greeff, S.C., Mouton, J.W., 2018. Nethmap 2018: Consumption of antimicrobial agents and antimicrobial resistance among medically important bacteria in the Netherlands / Maran 2018: Monitoring of antimicrobial resistance and antibiotic usage in animals in the Netherlands in 2017. https://doi.org/10.21945/RIVM-2018-0046

Du, L., Liu, W., 2012. Occurrence, fate, and ecotoxicity of antibiotics in agro-ecosystems. A review. Agron. Sustain. Dev. 32, 309–327. https://doi.org/10.1007/s13593-011-0062-9

Fahrenfeld, N., Knowlton, K., Krometis, L.A., Hession, W.C., Xia, K., Lipscomb, E., Libuit, K., Green, B.L., Pruden, A., 2014. Effect of Manure Application on Abundance of Antibiotic Resistance Genes and Their Attenuation Rates in Soil: Field-Scale Mass Balance Approach. Environ. Sci. Technol. 48, 2643–2650. https://doi.org/10.1021/es404988k

Fox, J., Weisberg, S., 2019. An R Companion to Applied Regression. Sage.

Gebrezgabher, S.A., Meuwissen, M.P.M., Kruseman, G., Lakner, D., Oude, A.G.J.M., 2015. Factors influencing adoption of manure separation technology in the Netherlands. J. Environ. Manage. 150, 1–8. https://doi.org/10.1016/j.jenvman.2014.10.029

Guo, H.G., Chen, Q.L., Hu, H.W., He, J.Z., 2020. Fate of antibiotic resistance genes during high-solid anaerobic co-digestion of pig manure with lignite. Bioresour. Technol. 303, 122906. https://doi.org/10.1016/j.biortech.2020.122906

He, L.Y., Ying, G.G., Liu, Y.S., Su, H.C., Chen, J., Liu, S.S., Zhao, J.L., 2016. Discharge of swine wastes risks water quality and food safety: Antibiotics and antibiotic resistance genes from swine sources to the receiving environments. Environ. Int. 92–93, 210–219. https://doi.org/10.1016/j.envint.2016.03.023

Heuer, H., Schmitt, H., Smalla, K., 2011. Antibiotic resistance gene spread due to manure application on agricultural fields. Curr. Opin. Microbiol. 14, 236–243. https://doi.org/10.1016/j.mib.2011.04.009

Huang, X., Zheng, J., Tian, S., Liu, C., Liu, L., Wei, L., Fan, H., Zhang, T., Wang, L., Zhu, G., Xu, K., 2019. Higher Temperatures Do Not Always Achieve Better Antibiotic Resistance Gene Removal in Anaerobic Digestion of Swine Manure. Appl. Environ. Microbiol. 85, 1–12. https://doi.org/10.1128/AEM.02878-18

Iwasaki, M., Miyake, M., Maseda, H., Qi, G., Pan, Z., Ihara, I., Umetsu, K., 2019. Thermophilic anaerobic digestion is an effective treatment for reducing cefazolin-resistant bacteria and ESBL-producers in dairy manure. J. Mater. Cycles Waste Manag. 21, 293–299. https://doi.org/10.1007/s10163-018-0789-3

Jechalke, S., Heuer, H., Siemens, J., Amelung, W., Smalla, K., 2014. Fate and effects of veterinary antibiotics in soil. Trends Microbiol. 22, 536–545. https://doi.org/10.1016/j.tim.2014.05.005

Lu, T., Zhang, J., Li, P., Shen, P., Wei, Y., 2020. Enhancement of methane production and antibiotic resistance genes reduction by ferrous chloride during anaerobic digestion of swine manure. Bioresour. Technol. 298, 122519. https://doi.org/10.1016/j.biortech.2019.122519

Macedo, G., Hernandez-Leal, L., van der Maas, P., Heederik, D., Mevius, D., Schmitt, H., 2020. The impact of manure and soil texture on antimicrobial resistance gene levels in farmlands and adjacent ditches. Sci. Total Environ. 737, 139563. https://doi.org/10.1016/j.scitotenv.2020.139563

Marañón, E., Castrillón, L., Fernández, J.J., Fernández, Y., Peláez, A.I., Sánchez, J., 2006. Anaerobic Mesophilic Treatment of Cattle Manure in an Upflow Anaerobic Sludge Blanket Reactor with Prior Pasteurization. J. Air Waste Manage. Assoc. 56, 137–143. https://doi.org/10.1080/10473289.2006.10464448

Moset, V., Poulsen, M., Wahid, R., Højberg, O., Møller, H.B., 2015. Mesophilic versus thermophilic anaerobic digestion of cattle manure: methane productivity and microbial ecology. Microb. Biotechnol. 8, 787–800. https://doi.org/10.1111/1751-7915.12271

Pandey, P.K., Biswas, S., Vaddella, V.K., Soupir, M.L., 2015. Escherichia coli persistence kinetics in dairy manure at moderate, mesophilic, and thermophilic temperatures under aerobic and anaerobic environments. Bioprocess Biosyst. Eng. 38, 457–467. https://doi.org/10.1007/s00449-014-1285-3

Pandey, P.K., Soupir, M.L., 2011. Escherichia coli inactivation kinetics in anaerobic digestion of dairy manure under moderate, mesophilic and thermophilic temperatures. AMB Express 1, 1–10. https://doi.org/10.1186/2191-0855-1-18

Pu, C., Liu, H., Ding, G., Sun, Y., Yu, X., Chen, J., Ren, J., Gong, X., 2018. Impact of direct application of biogas slurry and residue in fields: In situ analysis of antibiotic resistance genes from pig manure to fields. J. Hazard. Mater. 344, 441–449. https://doi.org/10.1016/j.jhazmat.2017.10.031

R Core Team, 2020. R: A Language and Environment for Statistical Computing.

Resende, J.A., Silva, V.L., de Oliveira, T.L.R., de Oliveira Fortunato, S., da Costa Carneiro, J., Otenio, M.H., Diniz, C.G., 2014. Prevalence and persistence of potentially pathogenic and antibiotic resistant bacteria during anaerobic digestion treatment of cattle manure. Bioresour. Technol. 153, 284–291. https://doi.org/10.1016/j.biortech.2013.12.007

Schmitt, H., Blaak, H., Hoeksma, P., van de Schans, M., Kemper, M., de Rijk, S., de RodaHusman, A., 2019. Antibioticaresistente bacteriën, resistentiegenen en antibioticaresiduen in mest. https://doi.org/10.21945/RIVM-2019-0112

Sun, W., Qian, X., Gu, J., Wang, X.-J., Duan, M.-L., 2016. Mechanism and Effect of Temperature on Variations in Antibiotic Resistance Genes during Anaerobic Digestion of Dairy Manure. Sci. Rep. 6, 30237. https://doi.org/10.1038/srep30237

Tervahauta, T., van der Weijden, R.D., Flemming, R.L., Hernández Leal, L., Zeeman, G., Buisman, C.J.N., 2014. Calcium phosphate granulation in anaerobic treatment of black water: A new approach to phosphorus recovery. Water Res. 48, 632–642. https://doi.org/10.1016/j.watres.2013.10.012

Thorsten, T., 2020. PMCMRplus: Calculate Pairwise Multiple Comparisons of Mean Rank Sums Extended.

Travis, E.R., Gaze, W.H., Pontiroli, A., Sweeney, F.P., Porter, D., Mason, S., Keeling, M.J.C., Jones, R.M., Sawyer, J., Aranaz, A., Rizaldos, E., Cork, J., Delahay, R.J., Wilson, G.J., Hewinson, R.G., Courtenay, O., Wellington, E.M., 2011. An Inter-Laboratory validation of a real time PCR assay to measure host excretion of bacterial pathogens, particularly of Mycobacterium bovis. PLoS One 6. https://doi.org/10.1371/journal.pone.0027369

Wallace, J.S., Garner, E., Pruden, A., Aga, D.S., 2018. Occurrence and transformation of veterinary antibiotics and antibiotic resistance genes in dairy manure treated by advanced anaerobic digestion and conventional treatment methods. Environ. Pollut. 236, 764–772. https://doi.org/10.1016/j.envpol.2018.02.024

Wickham, H., 2007. Reshaping Data with the reshape Package. J. Stat. Softw. 21, 1--20.

Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L., François, R., Grolemund, G., Hayes, A., Henry, L., Hester, J., Kuhn, M., Pedersen, T., Miller, E., Bache, S., Müller, K., Ooms, J., Robinson, D., Seidel, D., Spinu, V., Takahashi, K., Vaughan, D., Wilke, C., Woo, K., Yutani, H., 2019. Welcome to the Tidyverse. J. Open Source Softw. 4, 1686. https://doi.org/10.21105/joss.01686

WUR, 2019. Manure Management [WWW Document]. URL https://www.wur.nl/en/article/Manure-Management.htm (accessed 9.18.19).

Youngquist, C.P., Mitchell, S.M., Cogger, C.G., 2016. Fate of Antibiotics and Antibiotic Resistance during Digestion and Composting: A Review. J. Environ. Qual. 45, 537. https://doi.org/10.2134/jeq2015.05.0256

Zhang, J., Buhe, C., Yu, D., Zhong, H., Wei, Y., 2020. Ammonia stress reduces antibiotic efflux but enriches horizontal gene transfer of antibiotic resistance genes in anaerobic digestion. Bioresour. Technol. 295, 122191. https://doi.org/10.1016/j.biortech.2019.122191

Zhang, K., Gu, J., Wang, X., Zhang, X., Hu, T., Zhao, W., 2019. Analysis for microbial denitrification and antibiotic resistance during anaerobic digestion of cattle manure containing antibiotic. Bioresour. Technol. 291, 121803. https://doi.org/10.1016/j.biortech.2019.121803

Zhang, R., Wang, X., Gu, J., Zhang, Y., 2017. Influence of zinc on biogas production and antibiotic resistance gene profiles during anaerobic digestion of swine manure. Bioresour. Technol. 244, 63–70. https://doi.org/10.1016/j.biortech.2017.07.032

CHAPTER 5

"Don't worry. You're just as sane as I am."
J.K. Rowling
Harry Potter and the Order of the Phoenix, 2003

Temperature and nutrient limitations decrease transfer of conjugative IncP-1 plasmid pKJK5 to wild *Escherichia coli* strains

Rebeca Pallares-Vega[1,2,*] Gonçalo Macedo[1,3,*], S. M. Brouwer[4], Lucia Hernandez[1], Peter van der Maas[5], Mark CM van Loosdrecht[2], David G Weissbrodt[2], Dick Heederik[6], Dik Mevius[3,4], Heike Schmitt[6]

[1] Wetsus, European Centre of Excellence for Sustainable Water Technology, Oostergoweg 9, 8911 MA Leeuwarden, The Netherlands
[2] Delft University of Technology, Van der Maasweg 9, 2629, HZ Delft, The Netherlands
[3] Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands
[4] Wageningen Bioveterinary Research, Houtribweg 39, 8221 RA Lelystad, The Netherlands
[5] Van Hall Larenstein, University of Applied Sciences, Agora 1, 8901 BV Leeuwarden, The Netherlands
[6] Institute for Risk Assessment Sciences, Utrecht University, Yalelaan 2, 3584 CM Utrecht, The Netherlands
* These authors have contributed equally to this work and share first authorship

A modified version of this chapter has been published in Frontiers in Microbiology 12 (2021) (https://doi.org/10.3389/fmicb.2021.656250)

5.1 Abstract

Plasmid-mediated dissemination of antibiotic resistance among fecal *Enterobacteriaceae* in natural ecosystems may contribute to the persistence of antibiotic resistance genes in anthropogenically-impacted environments. Plasmid transfer frequencies measured under laboratory conditions might lead to overestimation of plasmid transfer potential in natural ecosystems. This study assessed differences in the conjugative transfer of an IncP plasmid to three natural *E. coli* strains carrying extended-spectrum beta-lactamases, by filter mating. Matings were performed under optimal laboratory conditions (rich LB medium and 37 °C) and environmentally relevant temperatures (25, 15 and 9 °C) or nutrient regimes mimicking environmental conditions and limitations (synthetic wastewater and soil extract). Under optimal nutrient conditions and temperature, two recipients yielded high transfer frequencies (5×10^{-1}) while the conjugation frequency of the third strain was 1000-fold lower. Decreasing mating temperatures to psychrophilic ranges led to lower transfer frequencies, albeit all three strains conjugated under all the tested temperatures. Low nutritive media caused significant decreases in transconjugants (-3 logs for synthetic wastewater; -6 logs for soil extract), where only one of the strains was able to produce detectable transconjugants. Collectively, this study highlights that despite less-than-optimal conditions, fecal organisms may transfer plasmids in the environment, but the transfer of IncP plasmids between microorganisms is limited mainly by low nutrient conditions.

5.2 Introduction

Antimicrobial resistance (AMR) is considered as one of the most significant challenges to global public health (O´Neill, 2016). The spread of antimicrobial resistance genes (ARGs) via horizontal gene transfer (HGT) between bacteria is a growing concern because it facilitates the dissemination of resistance across a wide variety of microorganisms. Understanding the dynamics of plasmid dissemination in the environment is fundamental to contain and mitigate the AMR challenge.

HGT is an effective ecological trait that shapes bacterial evolution (Ochman et al., 2000). Conjugative plasmids are relevant vectors for HGT (Smillie et al., 2010) and dissemination of AMR (Carattoli, 2013). Gut bacteria from both animal and human origin comprise an important source of AMR-conjugative plasmids (Ceccarelli et al., 2019; Hu et al., 2013). Gut bacteria are released into the environment through manure application to agricultural soils and wastewater discharges, ultimately resulting in the introduction of their ARGs, and plasmids in the environment. Despite having limited survivability, once introduced in the environment, gut bacteria might be able to transfer their AMR determinants to the natural bacterial community. *Escherichia coli* is widely accepted as primary indicator of fecal contamination. Although most *E. coli* strains cause only mild infections, their presence is indicative of the potential presence of other more pathogenic organisms which may be relevant for human health.

Monitoring of environmental HGT remains challenging mainly due to cultivation bias (only 1% of indigenous bacteria are estimated to be cultivable (Amann et al., 1995)), but also due to donor-recipient incompatibilities and detection limits of the methodology, which often require a compromise of the experimental design (Pinilla-Redondo et al., 2018; Sørensen et al., 2005). As a result, studies addressing environmental dissemination of AMR plasmids usually apply conditions that are optimal for bacterial transmission, namely high bacterial densities, optimal growth temperatures, and/or high nutrient availability (Bellanger et al., 2014; Jacquiod et al., 2017). Although being relevant for specific scenarios such as mesophilic anaerobic digesters, greenhouses or wastewater in low latitude countries (Al Qarni et al., 2016; Fan et al., 2019), these settings do not reflect the usual average conditions of manured soils, water bodies and wastewater (Abis and Mara, 2006; Barrios-Hernández et al., 2020; Osińska et al., 2020). Such discrepancies in the experimental design might lead to an overestimation of plasmid transfer frequencies and dissemination potential in the environment. Therefore, better insights into how environmental parameters affect plasmid transfer are needed.

The aim of this study was to evaluate *in vitro* the role of environmental factors that could potentially hamper conjugative plasmid transfer from gut bacteria once discharged into

the environment. A conjugative broad host range IncP plasmid was used as vector, and solid-surface filter matings were conducted to study HGT between *Escherichia coli* strains (as both donor and recipients). The transfer was evaluated under different (i) donor-to-recipient cell proportions, (ii) mating temperatures, or (iii) nutritional compositions. The criteria to select the used conditions was based on the presumable main abiotic challenges that gut bacteria face when discharged into the environment, namely nutrient limitations and close-to psychrophilic conditions. The donor-to-recipient cell proportions were tested to assess the limit of the system while aiming for a natural proportion of donor and recipient cells in the mating. By using the same species and a broad-host-range plasmid, potential host-vector and interspecies incompatibilities were discarded as factors. *E. coli* was chosen because it is a relevant opportunistic pathogen for both humans and animals, and it was hypothesized that lower temperatures and lower nutrient concentrations would limit plasmid transfer.

5.3 Materials and Methods

5.3.1 Selection and characterization of strains and plasmids

Three extended-spectrum beta-lactamase (ESBL) carrying *E. coli* strains (09.54, 38.27, and 39.62) isolated from fecal samples of calves or poultry were used as recipients during the mating experiments (Table 1). A genetically engineered *E. coli* strain previously described by Klümper et al. (2015) was selected as donor for the broad-host-range plasmid of the incompatibility group IncP-1. The donor strain (*E. coli* K-12 MG1655::*lacI*^{q}-*pLpp-mCherry-Km*^{R}) is commonly used in dual-labelling fluorescence reporter-gene approaches coupled with fluorescence-activated cell sorting (Pinilla-Redondo et al., 2018) due to the conditionally expressible green fluorescent proteins (GFP) in its IncP-plasmid (pKJK5). The IncP plasmid carries a kanamycin resistance determinant and lacI^{q} repressible promoter upstream the *gfpmut3* gene (Bahl et al., 2007; Klümper et al., 2015; Sengeløv et al., 2001), IncP plasmids are abundant in soils (Willms et al., 2020) as well as wastewater settings (Pallares-Vega et al., 2020), and are known to disseminate among a wide diversity of phylogenetic groups (Popowska and Krawczyk-Balska, 2013). *E. coli* as both donor and recipient was chosen as a model system for bacteria of public health relevance that can potentially move between anthropogenic related and natural environments. Moreover, the use of the same bacterial species and a broad-host-range plasmid minimized potential bias in cross-species conjugation, as well as host-plasmid incompatibilities.

In order to fully characterize the used strains, whole-genome sequencing using paired-end Illumina was performed, as previously described by Rozwandowicz et al. (2020). The annotation of the sequences was performed with Prokka version 1.12 (Seemann, 2014), and plasmid typing was conducted with the online tool PlasmidFinder (Carattoli

et al., 2014) applying an identity cut-off equal or greater than 98%. The annotated sequences are deposited in GenBank, BioProject PRJNA661180 under the accession no. JADPVO000000000.

To identify suitable selective conditions for the identification of transconjugants, the antimicrobial susceptibility profile for each strain was determined by disc diffusion test, according to EUCAST guidelines (EUCAST Disk Diffusion Method for Antimicrobial Susceptibility Testing - version 6.0; available at https://www.eucast.org/). The results were interpreted based on the EUCAST-defined Breakpoints tables for interpretation of MICs and zone diameters (version 8.0) and are summarized in Table S1 in Supplementary Information. Figure 1 displays this study's schematic of the experimental design and procedure.

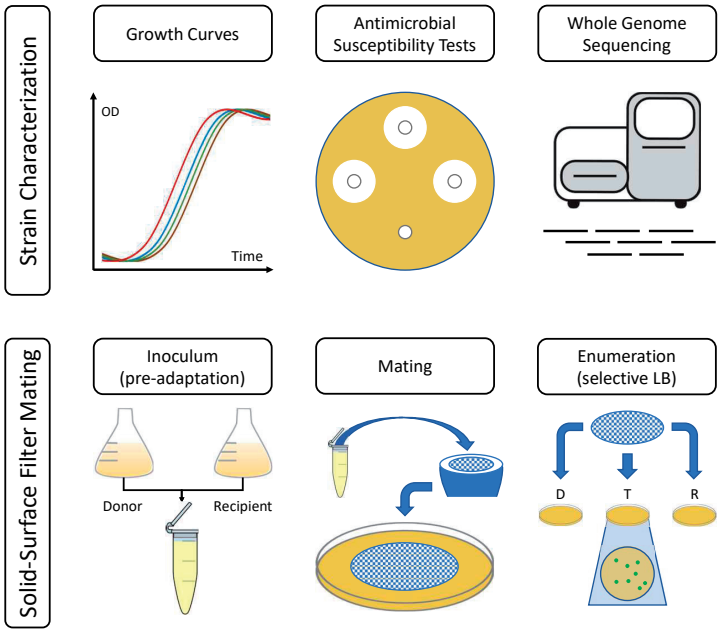


Figure 1. Overview of the procedure to quantify transconjugants. Donors and recipients were grown separately before being mixed, filtered, and incubated for 2 hours, at different temperatures or at different media. In the end, bacteria were recovered, and enumerated, in LB containing antibiotic combinations specific for donors, recipients, or transconjugants.

Table 1. Bacterial strains of *E. coli* used as donor and recipient of broad-host-range IncP-1 plasmid, and their characteristics.

Agent	Role	Origin	Resistance profile	Plasmids	Source
E. coli MG1655::lacI^{q}-pLpp-mCherry-Km^{R}	Donor	Laboratory strain	AMP^{R}, SMX^{R}, KAN^{R}	PKJK5 $P_{A1/04/03}$-gfpmut3 (IncP)	(Klümper et al., 2015)
E. coli 09.54	Recipient	Veal calf	AMP^{R}, CTX^{R}, SMX^{R}, TET^{R}	IncK	This study
E. coli 38.27	Recipient	Poultry	AMP^{R}, CTX^{R}, SMX^{R}, TET^{R}	IncFI, IncH1, IncI1, p0111	This study
E. coli 39.62	Recipient	Poultry	AMP^{R}, CTX^{R}, SMX^{R}, TET^{R}	IncFIB/FII IncK	This study

5.3.2 Culture media and growth curves

Luria-Bertani (LB), synthetic wastewater (SWW), and soil extract (SE) were used as culture media for the filter matings. Pure bacterial cultures were prepared and maintained in LB broth or plates (tryptone 10 g L^{-1}, yeast extract 5 g L^{-1}, sodium chloride 5 g L^{-1}, and agar 15 g L^{-1}) prior to the experiments, and for the selection of donor, recipients, and transconjugants after the matings, the LB plates were enriched with kanamycin (100 µg mL^{-1}; Sigma Aldrich), tetracycline (16 µg mL^{-1}; Sigma Aldrich), and both kanamycin and tetracycline (100 and 16 µg mL^{-1}), respectively.

The SWW aimed to mimic the average conditions and nutrient proportions of conventional domestic wastewater. The composition was based on that of Rottiers and Boeije, 1999, and ISO 11733 guideline, and adjusted to a theoretical COD:N:P concentration and molar ratio close to that of Dutch wastewater (100 : 9.1 : 1.4, Supplementary information Table S2). The SWW solution contained of 0.07 g L^{-1} urea, 0.011 g L^{-1} NH_4Cl, 0.015 g L^{-1} peptone P (Oxoid, UK), 0.015 g L^{-1} Lab Lemco (Oxoid, UK), 0.05 g L^{-1} starch, 0.04 g L^{-1} glycerol that was sterilized by autoclaving. After sterilization, the mix was completed with 0.25 g L^{-1} $NaHCO_3$, 0.12 g L^{-1} skimmed milk powder (Sigma Aldrich, NL), 0.05 g L^{-1} glucose, 0.025 g L^{-1} $FeSO_4$, 0.005 $CaCl_2$, 0.025 g L^{-1} $NaHCO_3$ and 0.02 g L^{-1} $MgHPO_4{\cdot}3H_2O$, 0.016 g L^{-1} L $K_3PO_4{\cdot}H_2O$ (unless indicated otherwise, the components were purchased at VWR, NL). These solutions were separately autoclaved, or filter sterilized prior to their aseptic addition to the final solution. SWW media was finally supplemented with the addition of 0.1% (v/v) of trace metal solution which contained 0.280 g L^{-1} NaEDTA, 0.180 g L^{-1} $ZnCl_2$, 1.144 g L^{-1} H_3BO_3, 0.025 g L^{-1} $CoCl_2{\cdot}6H_2O$, 0.589 g L^{-1} $MnCl_2{\cdot}2H_2O$, 0.120 $CuCl_2{\cdot}2H_2O$, 0.068 g L^{-1} $NiCl_2{\cdot}6H_2O$, 0.025 g L^{-1} $Na_2MoO4{\cdot}5H_2O$, 0.212 g L^{-1} $KCr(SO_4)_2{\cdot}12H_2O$. When needed, agar (15 g L^{-1}) was added for solid media preparation.

Soil samples for SE medium preparation were collected in the late fall of 2019, from a local dairy farm (Friesland, Netherlands) that uses the field for pasture (grassland) and had not been recently subjected to manure application. In total, 7 kg of sandy loam soil were collected from the field and homogenized. The collected soil was air-dried for 3 days and stored in 500 g zip bags at 4°C until being used. The SE media was prepared as described by (Musovic et al., 2010). Briefly, 500 g of dried soil was mixed with 500 mL of demineralized water. Then, the mixture was shaken horizontally, for 3 hours, and left for passive settling of the particles, for 5 hours. After the 5 hours, the supernatant was pipetted and autoclaved (for 15 minutes, at 121 °C) and stored at 4 °C, up to one month. When needed, agar was added as aforementioned.

The general chemical compositions of the LB, SWW, and SE media were determined by ion chromatography (IC), and inductively coupled plasma (ICP-OES). The determination of the chemical oxygen demand (COD), and the total nitrogen was achieved with commercially

available kits (LCK 514 and LCK 338; Hach). The determination of the total organic carbon (TOC) was achieved with Shimadzu TOC-L_{CPH} analyzer. The composition of the different media used is displayed in Table 2.

To quantify the effect of the temperature change in the growth, an inoculum volume of 0.2 % (final volume) of overnight culture of each strain was transferred to fresh LB, and incubated at 9, 15, 25, or 37 °C. The Pathogen Modeling Program (PMP) online model (available at: https://pmp.errc.ars.usda.gov/default.aspx) was used to predict the incubation time range to measure bacterial density. To determine the effect of the nutrient composition, inoculums of 0.2 % (final volume) overnight culture of each strain were transferred to SWW or SE media, and monitored up to three days. The optical density, at 600 nm (OD_{600}), was measured in a UV-Vis Spectrophotometer (Shimadzu Corp). Colony forming units (CFUs) were determined after preparing 10-fold serial dilutions with saline solution (NaCl; 0.85 %), plating in LB agar, and incubating at 37 °C, overnight. Measurements were performed in biological triplicates.

Table 2. Media composition of the culture media used in the matings with either Luria-Bertani (LB), synthetic wastewater (SWW) or soil extract (SE) medium. Legend: total organic carbon (TOC), chemical oxygen demand (COD), total nitrogen (TN), total phosphorus (TP)

(mg L^{-1})	LB		SWW		SE	
Compound	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
TOC	6,820	80	219	1.0	45	-
COD	21,450	2,450	529	37	173	1
TN	2,050	20	48	2	7	0.4
TP	151	1	7.2	0.2	4	0.0
Ca^{2+}	9	1	3.6	0.0	104	1
K^{+}	272	2	11.5	0.1	21	9.9
Mg^{2+}	7	0.1	37.6	0.1	5	0.6
$Fe^{2+/3+}$	0	0.0	4.5	0.0	<0.05	-
S	127	0	38	1	67	2
NH_4^{+}	60	0	6.2	0.0	<0.10	-
NO_3^{-}	4	0.0	<0.10	-	10	0.0
PO_4^{3-}	259	1	>20	-	12	0.0
SO_4^{2-}	96	9	11	0	191	7

5.3.3 Solid surface filter matings: Standard conditions

Conjugation is a process that requires cell proximity and stable spatial conditions during the mating time (ca 3-5 min). Although these conditions can occur in the liquid phase, they are more likely in "surface-like" configurations (Zhong et al., 2010) occurring in soil grains, sludge flocs or biofilms. Bearing this in mind, filter mating was chosen to study the plasmid transfer.

The conjugation assays were performed by mixing 150 µL of fresh culture of the donor and recipient, and vacuum filtered through mixed-cellulose ester filters (0.45 µm; Millipore) in a Millipore filtration system. Prior to mixing, the cultures were grown for approximately 3 h in LB to achieve a density of 10^8 CFU mL^{-1}, as experimentally defined by the growth curves. After filtration, the mixed cultures were transferred to plates containing LB and cells were then incubated at 37 °C. Following the incubation period, the cells were detached from the filter by vortexing in 1 mL of sterile LB broth, for 5 min. Subsequently, serial decimal dilutions were prepared in sterile saline solution, and 100 µL was spread on LB plates containing the previously mentioned antimicrobials. The results were observed after a 24-h incubation period (total counts), at 37 °C, and another 24-h incubation period (colored colonies), at 4 °C. The incubation at 4 °C was performed to enhance the visualization of the GFP protein (Scott et al., 2006) and to count the green colonies, the plates were observed in a blue-light transilluminator (Safe Imager™ 2.0; Invitrogen). To confirm the validity of each assay, matings with only the donor or the recipient were also performed. Each mating was performed in biological triplicates on alternative days.

5.3.4 Solid surface filter matings: Modified conditions

When different proportions of donor-to-recipient ratios (D/R) were tested, the donor cultures harvested until 10^8 CFU mL-1 were serially diluted (10 and 100-fold) in LB and 150 µL was mixed with 150 µL recipient culture to reach the corresponding ratios D/R of 1:10 and 1:100. A total volume of 200 µL of the mixtures were then filtered, and the mating and incubation were performed as aforementioned. The approximate cell density in the filters was 8.9 x 10^6 CFU cm^{-2}. The effect of temperature in transfer frequency was assessed by following the standard condition procedure, but incubating the filters at 25, 15 and 9 °C in LB plates pre-conditioned to the corresponding temperatures. To assess the influence of nutrient availability in the transfer frequency, matings conducted in SWW and SE media were compared to standard nutrient-rich media LB. For SWW matings, donor and recipient cell cultures were pre-adapted to low nutrient conditions by growing them in SWW media (1% overnight inoculum) for approximately 4 h with 180 rpm agitation until a cell density of 10^8 CFU mL-1 was achieved. Then, cell cultures were mixed and filtered as aforementioned in the standard conditions, and filters were placed in SWW agar plates. Plates were incubated at 37 °C for 2 h. For SE matings, no pre-growth from donor nor recipients could be obtained in SE broth, as indicated by the corresponding growth curves (data not shown). Instead, late log phase LB cultures of both donor and recipients ~10^9 were centrifuged and washed twice in saline solution, and the pellet was finally resuspended in 10 mL of SE broth and incubated overnight at 37 °C. Before incubation, an aliquot of the resuspended cells was serially diluted in saline solution, plated in LB and incubated overnight at 37 °C. Following the incubation and based on the cell counts of the suspensions, the cell density of both donor and recipient SE cultures were adjusted to approximately 10^8 CFU mL-1, mixed in 1:1 ratio and filtered as indicated in the standard procedure. Filters were then placed on SE media and incubated

at 37 °C for 24 h. In all modified filter matings, cell recovery and subsequent plating were performed as mentioned in the standard conditions.

5.3.5 Confirmation by PCR

To confirm the strain identity (donor, recipient and transconjugants), five to ten isolates per mating were collected randomly from each of the media containing the antibiotics, and PCR was performed on the crude cell extracts. Reactions targeting the 16S rRNA gene, mCherry, and *gfpmut3* were prepared in 25-µL reactions containing PCR buffer (1x), (Invitrogen, NL) $MgCl_2$ (3.0 mM), (Invitrogen, NL), dNTPs (0.2 mM) (Promega, NL), forward and reverse primers (0.4 µM; Table S3), *Taq* polymerase (1.25 U) (Invitrogen, NL), and 1 µL of DNA. The PCR reactions were carried out in a T100 Thermal Cycler (BioRad), following similar denaturation conditions (95 °C for 30 s), but specific annealing and elongation conditions (57, 55, or 60 °C for 30 s; and 30 – 90 s at 72 °C for the 16S rRNA, *gfpmut3*, and mCherry genes, respectively), in 30 cycles. The specificity of the PCR products was confirmed by visualization in 1.5 % agarose gel stained with ethidium bromide.

5.4 Data Analysis

One-way analysis of variance (ANOVA) was conducted to detect differences in the conjugation frequencies, between strains, temperatures, and culture media. The ANOVA tests were followed by TukeyHSD post-hoc analysis, and homogeneity of variance was confirmed with Levene's test. Data normality was confirmed with Shapiro-Wilk's method, and when normality was not achieved, group comparison was performed using the equivalent non-parametric test (Kruskal-Wallis). A significance score of $p < 0.05$ was considered to be statistically relevant. These analyses were performed with R version 3.5.1 (R Core Team, 2018) and RStudio (Version 1.1.456; https://www.rstudio.com/). Used software packages consisted of *reshape* (Wickham, 2007) and *tidyverse* (Wickham et al., 2019), a set of packages designed for data cleaning, trimming, and visualization; of *Rcmdr* (Fox, 2005), *PMCMRplus* (Thorsten, 2020), and *car* (Fox and Weisberg, 2019) for ANOVA and Levene's test.

5.5 Results

5.5.1 Effect of donor-to-recipient (D/R) ratios

Before the temperature and nutrients assays, the D/R ratios were tested to assess the limit of the system while aiming for a natural proportion of donor and recipient cells in the mating.

Under optimal conditions and 1:1 D/R ratio (37 °C and LB, 8.9 x 10^6 CFU cm^{-2}), two out of three *E. coli* strains (38.27 and 39.62) yielded high transconjugant numbers (10^9 CFUs mL^{-1})

and transfer frequency (5 x 10^{-1}) of IncP-1 plasmids. On the other hand, the mating with strain 09.54 produced 10^6 CFU mL^{-1} (transfer frequency of 10^{-3}). The transfer frequency, measured as the transconjugants-to-donors ratio (T/D), resulted in a slight increase in the 1:10 and 1:100 D/R proportions in comparison with the 1:1 proportion in all strains (except for one replicate of strain 09.54; Figure 2). Contrarily, the transconjugants-to-recipients ratio (T/R) decreased with the different D/R ratios, approximately -0.7 logs and -1.8 logs in the 1:10 and 1:100 proportions, respectively (strains 38.27 and 39.62). A stronger effect of D/R was observed for strain 09.54, where the T/R decreased 1-3 logs and 3-4 logs in the 1:10 and 1:100 proportion, respectively. Similar results were found for the absolute numbers of transconjugants (Figure S1 in Supplementary information). No transconjugants were recovered for one replicate in the mating of the strains 09.54 (1:100; Figure S1). At both 1:10 and 1:100 proportions, transconjugant numbers reached approximately 10^3 CFUs mL^{-1} for at least one of the replicates, which was close to the detection limit (10^2 CFUs mL^{-1}).

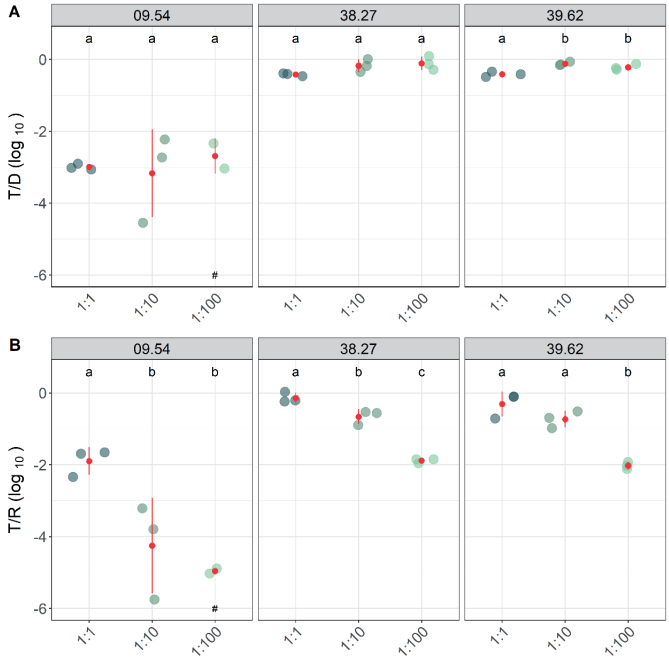


Figure 2: Donor-to-recipient proportions had significant effects on plasmid transfer. Depending on the indicator and strain used, the donor concentration increased or decreased, the transfer frequency. Relative counts of transconjugant-to-donor (T/D; A) and transconjugant-to-recipient (T/R; B) ratios, after 2-h matings performed at three donor-to-recipient ratios (1:1, 1:10, 1:100) are shown together with average and standard deviation values (in red). Different colors depict distinct donor-to-recipient ratios. [a, b, c] Indicate significantly different groups in the transfer frequency between ratios (PostHoc Tukey test, $p < 0.05$), and replicates with no detected transconjugants are highlighted (#).

5.5.2 Role of temperature on conjugative transfer

Conjugation efficiency among ESBL *E. coli* strains was assessed at temperatures ranging from the optimal laboratory (37 °C), room (25 °C) and relevant environmental (15 °C, 9 °C) conditions.

Overall, lower temperatures significantly reduced the number of conjugation events ($p < 0.01$; Figure 3). Both T/D and T/R decreased with decreasing temperatures, with a more pronounced reduction in strain 09.54 than in the other two strains (Figure 3). The highest number of transconjugants was obtained at 37 °C, and at 25 °C, and the number of transconjugants decreased roughly 1 log (strains 38.27 and 39.62) or 2 logs (strain 09.54), depending on the strain. With further temperature reduction, lower transconjugant numbers were observed, and at 9 °C, conjugation still occurred in all tested strains.

The lowest number of transconjugants was obtained at 9 °C for strains 38.27 and 39.62. In strain 09.54, the minimum transconjugant number was already reached at 15 °C and maintained at 9 °C. However, higher variability among replicates was noticeable with strain 09.54 (Figure S2), and one replicate did not yield detectable transconjugants (Figure S2).

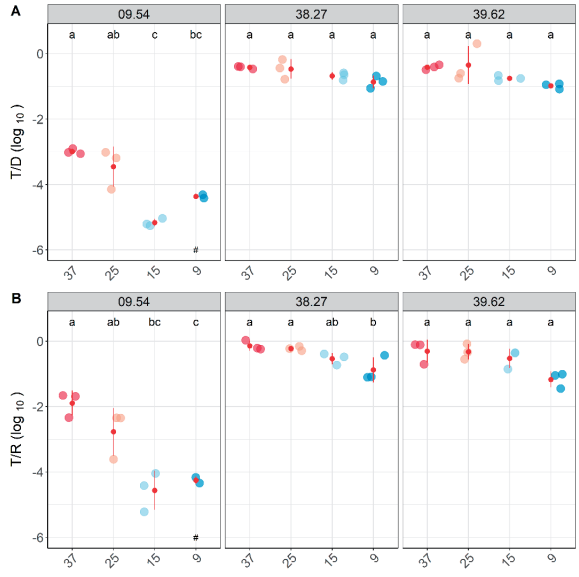


Figure 3: Lower temperature reduced the number of conjugation events. Relative counts of transconjugant-to-donor (T/D; A) and transconjugant-to-recipient (T/R; B) after 2h-matings performed, at diverse temperatures (37 – 9 °C), are shown together with average and standard deviation values (in red). Different colors depict distinct temperatures. [a, b, c] Indicate significantly different groups in the transfer frequency between temperatures (PostHoc Tukey test, $p < 0.05$), and replicates with no detected transconjugants are highlighted (#).

5.5.3 Role of nutrient concentrations on conjugative transfer

Differences in plasmid transfer under diverse nutrient regimes were assessed by comparing conjugation yields and transfer frequencies between rich nutrient media (LB) and common surrogates for natural conditions such as SWW and SE media.

In all tested strains, the decrease in the nutrient concentration of the media resulted in a substantial decrease in conjugation events (Figure 4). In comparison with the matings performed in LB, SWW resulted in the reduction of conjugation events by roughly 2 logs. In SE, a 4-log reduction was observed for strain 39.62 (compared to LB; 4), but no transconjugants were recovered for other strains, despite several attempts.

The decline in transconjugant numbers was particularly severe for strain 09.54, which presented the lower number of transconjugants in LB. Its transconjugants were only recovered in one out of three matings performed in SWW, and when SE was used, a further decrease in the number of transconjugants was observed. While matings with strain 39.62 yielded 1.3 x 10^3 CFUs mL^{-1} transconjugants (3 and 6 logs lower than in SWW and LB, respectively; Figure S3), the strains 09.54 and 38.27 did not produce detectable transconjugants (Figure S3).

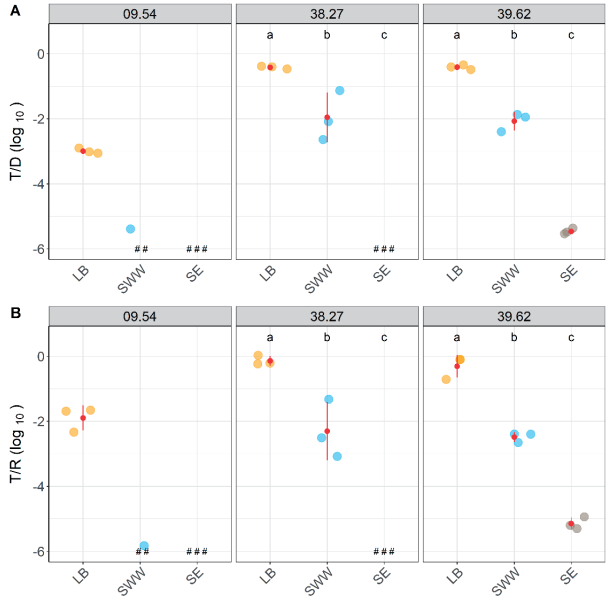


Figure 4: Decrease in nutrient concentration reduced conjugation events. Relative counts of transconjugant-to-donor (T/D; A) and transconjugant-to-recipient (T/R; B) after 2h-matings performed, at diverse nutrient conditions (Luria-Bertani, LB; synthetic wastewater, SWW; and soil extract, SE), are shown together with average and standard deviation values (in red). The replicates with no detected transconjugants are highlighted (#). Different colors depict distinct media. [a, b, c] Indicate significantly different groups of transfer frequency between culture media (PostHoc Tukey test, $p < 0.05$), and replicates with no detected transconjugants are highlighted (#).

5.6 Discussion

The effects of temperature and nutrient abundance during mating of an IncP-1 plasmid were evaluated in three natural ESBL *E. coli* recipient strains by monitoring both total amount of transconjugants and transfer frequencies. The results confirmed that psychrophilic temperatures during mating, as well as nutrient limitation, resulted in the reduction of transfer events. The decrease in the number of transconjugants was more prominent with lower nutrients than with lower temperatures.

5.6.1 Transfer efficiency varied across strains

Under optimal physiological conditions for the growth of the three *E. coli* strains 09.54, 38.27 and 39.62 tested (rich LB medium, higher mesophilic temperature of 37 °C), the conjugative transfer of plasmid significantly differed among the recipients. Two strains showed a high frequency of transfer (5×10^{-1}) while the third (strain 09.54) had 2 logs less. High frequency of transfer is common among IncP plasmids (Thomas and Smith, 1987), and similar transfer frequencies (10^{-2}) have been described before for the pKJK5 plasmid in soil microcosms (Musovic et al., 2006). The difference of transfer frequency among strains from the same species can relate to strain-specific characteristics or repression of silencing systems that either avoid or limits the expression of the new acquire genes in the recipient cell (Frost and Koraimann, 2010). Moreover, the presence of other plasmids in the recipient strains before mating might also influence the transfer efficiency. Enhanced transfer frequency of IncP plasmids towards recipient cells hosting IncF plasmids has been observed (Gama et al., 2017). Although the mechanism of action is not entirely clear, the authors consider that it is not a cooperative process, but more opportunistic use of the IncF transfer machinery by IncP plasmids (Gama et al., 2017). In our experiments, we observed that the two strains with higher transfer frequency contained natural IncF plasmids (among others), whereas 09.54 harboured an IncK plasmid. However, further analysis would be necessary to confirm the role of the co-existing plasmid in the recipient cell.

5.6.2 Reducing input of donors reduced overall transfer frequency

A lower D/R proportion resulted in a decreased number of transconjugants, suggesting that the relative proportion of donors to recipients can limit HGT.

Receiving environmental compartments typically contain high cell densities, for instance, activated sludge usually contains between 10^{9}- 10^{10} CFU mL^{-1} (Manti et al., 2008) and soil contains 10^{6}-10^{8} CFU cm^{-2} (Raynaud and Nunan, 2014). However, exogenous bacteria that enter the system (potential donors) might not be as numerous. Depending on multiple factors, including sewage flows or manure application rates, the potential donors will be a minority in the compartment to which they were introduced.

During conjugation assays, high cell densities (8.9 x 10^6 CFU cm^{-2}) would mirror natural systems. Conversely, the use of D/R ratios lower than 1:1 (i.e., 1:10 and 1:100) would presumably reflect more accurately the conditions found in anthropogenically impacted environments. However, to observe differences in conjugation rates under varied conditions, the number of donors should be sufficient to produce a detectable amount of transconjugants with a wide margin from the limit of detection (3 to 4 logs) in the matings performed under optimal conditions. Goodman et al. (1993) and Rochelle et al. (1989) observed that a minimum of 10^4 CFU cm^{-2} of donors and recipients were necessary to observe transconjugants. Here, conjugation occurred at donor densities as low as 10^4 CFU cm^{-2} yielding a high amount of transconjugants (10^8) for two of the strains (38.27 and 39.62), but not for the third one (strain 09.54). For this last strain, transconjugants were undetectable or close to the limit of detection with initial donor densities of 10^4 or 10^5 CFU cm^{-2} (D/R of 1:100 and 1:10 respectively) Considering that low D/R could prevent the monitoring of conjugation events for at least one of the strains, the subsequent experiments were conducted with a D/R ratio of 1:1. Similar cell densities and ratios have been previously advised to observe changes in conjugal transfer across a range of (presumably) unfavorable conditions (Fernandez-Astorga et al., 1992).

5.6.3 Lower temperature inhibited plasmid transfer, but not entirely

The highest number of transconjugants was obtained at 37 °C, which is also the optimal growth temperature for *E. coli*. However, growth of donors and recipients was observed between their concentrations at the start of the experiment and in the controls (approximately 1 log, in all strains; Figure S2). Together with growth curve data, this suggests that, at 37 °C, part of the transconjugant numbers originated from clonal expansion rather than a new transfer event. Conversely, at other temperatures, the amount of transconjugants observed reflected more accurately the real number of conjugation events, as the 2-h mating time concurred with the lag phase, and, consequently, clonal expansion can assume to be negligible.

Fluctuations in temperature are known to greatly affect the growth and metabolic functions of microorganisms (Trevors et al., 2012). Yet, the effect of a wide range of temperatures on conjugative AMR-related plasmids has seldom been addressed (Bale et al., 1988; Banerjee et al., 2016; Inoue et al., 2005). Although cold conditions are predominantly found around the planet (Rodrigues and Tiedje, 2008) and in relevant environments for AMR spread (Table S4), studies addressing the environmental dissemination of AMR plasmids in microcosms often used rather warm (>25 °C) settings. Warm temperatures (25-30 °C) are also common for *in vitro* studies that focus on either capturing environmental plasmids or addressing the microbial community permissiveness of a given plasmid, because high conjugation rates are required for detecting a high diversity of transconjugants (Jacquiod et al., 2017; Li et al., 2020, 2018).

Conjugation occurred at environmental temperatures (i.e., 15 °C), which are average temperatures found in wastewater and soil worldwide (Table S4), but it also occurred at 9 °C. Typically, most wastewater treatment plants do not operate at temperatures below 9 °C (because of nitrification failure), but in some countries, particularly northern countries, they can operate at temperatures close to 0 °C (Delatolla et al., 2012; Hoang et al., 2014). The use of different strains emphasized that the effect of temperature on the transfer frequency is recipient-dependent, and probably not affected just by chromosomally encoded factors, but also by resident plasmids in the recipient. The different outcomes observed between strains highlights the difficulty of inferring results that can be applicable to all putative recipient strains, even when they belong to the same species.

5.6.4 Lower nutrient composition hindered conjugation

A stronger effect on the transfer frequency was observed in matings performed with lower nutrient concentrations, where the frequency of conjugation was proportional to the nutrient richness of the culture media (LB > SWW > SE). In some cases, it was not possible to recover transconjugants in SE. Some authors suggest that plasmid transfer is related to cell growth and does not occur in non-growing cells (Kohyama and Suzuki, 2019; Seoane et al., 2011), others consider that it happens after cell division and right before entering a non-growing phase (Headd and Bradford, 2020). We observed conjugation in SE media for at least one of the conjugation pairs, despite cell growth was not observed for either donor nor recipients in this media.

Comparatively, the SE and SWW media used in this study contained 40 to 300-fold (SE), and 20- to 40-fold (SWW) lower basic nutrients (C, N, and P) concentrations than the classical nutrient-rich media (LB; Table 2). Conjugation requires energy and cellular resources to occur, and thus, one could expect that low nutrient conditions would hamper plasmid transfer (Goodman et al., 1993). Interestingly, the effect of nutrient deprivation on conjugation is seldom documented. Fernandez-Astorga et al. (1992) addressed the effect of available TOC in liquid media, finding transconjugants even at 1 mg L^{-1} of TOC. Inoue et al. (2005) observed decreasing transconjugants in media with a decreasing amount of dissolved organic carbon (DOC) (6'636 to 21.6 mg L^{-1}), including LB, synthetic, and real wastewater. However, in the two aforementioned studies and elsewhere (Grabow et al., 1975; Headd and Bradford, 2018; MacDonald et al., 1992; O'Morchoe et al., 1988), donor and recipient cells were pre-grown in a nutrient-rich media and then subjected to conjugation in the low nutrient media. Extra energy and nutrients stored in the cells during this pre-growth phase may allow bacteria to undergo conjugation in an earlier stage of the mating, potentially masking the effect of lower nutrition conditions on conjugation (Curtiss et al., 1969). To bypass this bias, Goodman et al. (1993) starved donors and recipients in minimal media (low amount of salts and no carbon source) prior to the conjugation. They found

that, despite the lack of nutrients, conjugation occurred after the donors were starved up to 3 or 20 days, when *E. coli* or *Vibrio* sp. were the donors, respectively. In the current study, when addressing conjugal transfer in low nutrient media, cells were also pre-incubated in the corresponding low-nutrient media (SWW or SE) to avoid the influence of intracellular nutrient reservoirs.

Then again, carbon concentration is likely not the only nutrient that can limit conjugation. In their work, Inoue et al. (2005) observed that transconjugants and transfer rates were 2.5 logs higher in SWW than in 16-fold diluted LB, while both contained similar DOC content (410 mg L^{-1}). Possibly, higher concentration of other nutrients (nitrogen, phosphorus or specific cations) in the SWW allowed an increase in conjugation frequencies and/or clonal expansion of the transconjugants. Pre-growth in media lacking casamino acids delayed *pili* formation after nutritional conditions are restored (Curtiss et al., 1969). As *pili* formation is protein-dependent, N-compounds are required for plasmid transfer. Despite being an essential nutrient, the role of phosphate or inorganic phosphorus deprivation in conjugation has not been explored yet. Phosphorus is known to be a limiting factor of cell growth and metabolism in oligotrophic environments (Smith and Prairie, 2004). In *E. coli*, phosphorus starvation induces a wide range of metabolic changes including cell surface modification and increase of cell adhesion characteristics (adhesins and fimbria), which could affect the interaction between cells and ultimately the conjugation rates. Finally, the concentration of other micronutrients as divalent cations might also influence conjugation. Recently, Sakuda et al. (2018) observed that the addition of divalent cations to low nutrient media (Ca^{2+} and Mg^{2+}) increased the conjugation frequency of IncP-7 plasmids among *Pseudomonas* strains. Yet, the molecular mechanisms of this effect remain unclear.

5.7 Conclusions

When moving from laboratory conditions to environmentally relevant conditions for soils and WWTPs, both lower temperature and lower nutrient concentrations showed to reduce plasmid transfer by bacterial conjugation significantly. The effect lower nutrient concentrations on the number of transconjugants was stronger than the effect of lower temperatures. While nutritional conditions appear critical, the role of single nutrients, such as N and P, is not entirely clear and deserves further follow-up research. Furthermore, the transfer potential was recipient-dependent and varied within ESBL *E. coli* strains of the same species.

To conclude, although abiotic factors can hamper plasmid transfer, measurable conjugation between *E. coli* still occurred under conditions that mimicked those commonly found in

the wastewater and soil environment (9 – 25 °C). Despite conjugation being observed between strains of the same species, this study shows that fecal indicator bacteria were capable of donating plasmids in less-than-optimal contexts, and consequently, can be a source of transferable AMR traits once they reach the environment.

5.8 Acknowledgments

The authors gratefully acknowledge the support of the former students Xavier Gallego y van Seijen, Hana Piková, Gonzalo Monteoliva, Wesley Klaassen, Amaya Gómez, Dimitrios Maroulis, and Jenneke van der Draai for their relentless technical assistance in performing the conjugation essays, and Søren J. Sørensen for kindly donating the donor strain.

5.9 Funding

This work was performed in the cooperation framework of Wetsus, European Centre of excellence for sustainable water technology (www.wetsus.nl). Wetsus is co-funded by the Dutch Ministry of Economic Affairs and Ministry of Infrastructure and Environment, the European Union Regional Development Fund, the Province of Fryslân and the Northern Netherlands Provinces. This work has also received funding from STOWA and from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie [grant agreement No. 665874]. The authors like to thank the participants of the Wetsus research theme Source Separated Sanitation for the fruitful discussions and their financial support.

5.10 Supplementary data

Table S1. Antibiotic susceptibility determined by disc diffusion test, according to EUCAST guidelines. Strains are classified in Resistant (R), Susceptible (S) or Intermediate resistance (I).

Strain	Ampicillin	Cefotaxime	Ciprofloxacin	Gentamicin	Sulfamethoxazole	Tetracycline	Kanamycin	Rifampicin
	AMP (10 ug)	CTX (5 ug)	CIP (5 ug)	GEN (10 ug)	SMX (25 ug)	TET (30 ug)	KN (30 ug)	RF (5 ug)
E. coli MG1655::*lacI*^{q}-*pLpp-mCherry-Km*^{R} / pKJK5::$P_{A1/04/03}$-*gfpmut3*	R	S	S	S	R	S	R	R
09.54	R	R	S	S	R	R	I	R
39.62	R	R	S	S	R	R	S	R
38.27	R	R	S	S	R	R	I	R

Table S2. Average annual concentrations of organic matter (COD, BOD), total nitrogen (TN) and total phosphorus (TP) in the influent wastewater of Dutch WWTPs between 2000-2018. Source CBS: https://opendata.cbs.nl/statline/#/CBS/nl/dataset/7477/table?dl=3DD6. **Abbreviations:** pe– population equivalents, COD – Chemical Oxygen Demand; BOD – Biological Oxygen Demand.

Year	Number of WWTPs	Total capacity (x1.000 pe)	COD (mg L^{-1})	BOD (mg L^{-1})	TN (mg L^{-1})	TP (mg L^{-1})
2000	391	na	470	180	43	7
2001	384	na	461	175	42	7
2002	378	na	477	185	44	7
2003	378	na	550	213	51	9
2004	375	na	506	194	46	8
2005	368	na	525	198	48	8
2006	363	na	520	196	48	8
2007	356	na	471	174	44	7
2008	351	na	503	192	47	8
2009	351	na	536	208	49	8
2010	349	30,365	513	200	46	7
2011	346	30,383	526	206	48	7
2012	343	30,358	505	199	46	7
2013	341	30,364	520	209	48	7
2014	337	30,237	548	218	50	7
2015	334	30,246	516	209	45	7
2016	327	30,122	541	225	49	7
2017	326	29,904	546	232	49	7
2018	323	29,942	593	248	54	7
		Average	517	203	47	7.4
		SD	32	19	3	0.6
		COD:N:P ratio	100		9.1	1.4

Table S3. Primers used for PCR confirmation of strains.

Target	Primer name	Sequence (5´- 3´)	Amplicon size (bp)	Reference
16S rRNA	27F	AGA GTT TGA TCC TGG CTC AG	1465	(Frank et al., 2008)
	1492R	GGT TAC CTT GTT ACG ACT T		
gfpmut3	q_GFPmut3-FW	TCG GTT ATG GTG TTC AAT GC	146	(Norman et al., 2014)
	q_GFPmut3-RV	GAC TTC AGC ACG TGT CTT GTA G		
mCherry	q_mCherry-FW	CCC CGT AAT GCA GAA GAA GA	99	Eurofins Genomics (Vidgren and Gibson, 2018)
	q_mCherry-RV	TTC AGC CTC TGC TTG ATC TC		

Table S4. Temperature range in wastewater and soil in cold countries. Acronyms: WW: Wastewater. NA: Not available

Country	Sampling period		Temperature (°C)		Sample		Reference
	Year	Months	Min	Max	Matrix	Type	
Austria	DS	March April- June -July	7.8	15.5	WW	Sewer	(Kretschmer et al., 2016)
China	2013 2014	January-April-June-October	15	30	WW	NA	(Liu et al., 2016)
Finland	2010 2011	Each season	9	18	WW	NA	(Karkman et al., 2016)
The Netherlands	2017 2018	December to May	9	15	WW	NA	(Barrios-Hernández et al., 2020)
The Netherlands	2009-2011		9	20	WW	NA	(Krzeminski et al., 2012)
Poland	2015	January-April-July-October	8.7	20.9	WW	Influent	(Osińska et al., 2017)
			9.5	21.3	WW	Effluent	
USA (Minnesota)	-	-	10	20	WW	Influent	(Johnston et al., 2019)
Czech Republic	1961-2000	Monthly	-0.5	21.2	Soil	-5 cm	(Pokladníková et al., 2008)
			0.4	19.9	Soil	-20 cm	
China (north)		Monthly average	-2	16	Soil		(Zhou et al., 2015)
Germany (Leibniz)	1894-2019	Monthly average	-7	35	Soil	-5 cm	1
			-9	27	Soil	-20 cm	
The Netherlands	2006-2020	Monthly average	1	21	Soil	-5cm	2
			1	21	Soil	-20 cm	

1 https://www.pik-potsdam.de/services/climate-weather-potsdam/climate-diagrams/ground-temperature
2 http://projects.knmi.nl/cabauw/insitu/observations/soiltemp/

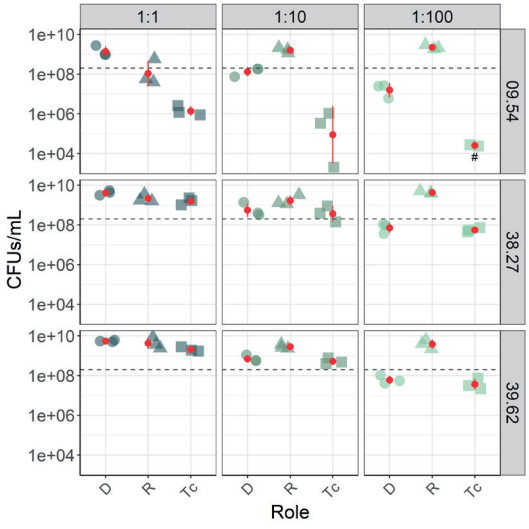


Figure S1. Absolute counts of donors (D), recipients (R), and transconjugants (Tc) after 2h-matings performed with different D/R proportions (1:1, 1:10, 1:100) for each strain. The averages and standard deviations of the matings are displayed in red.

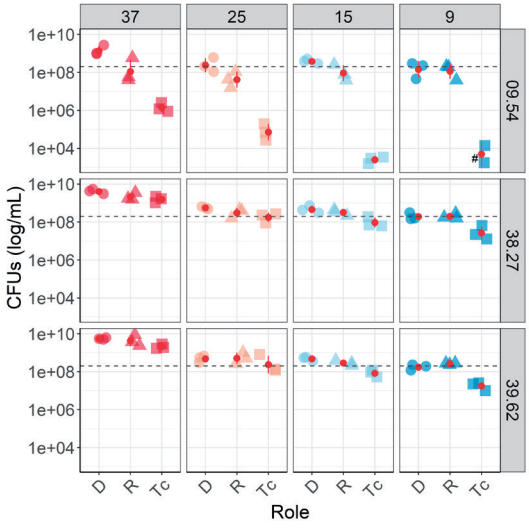


Figure S2. Absolute counts of donors (D), recipients (R), and transconjugants (Tc) after 2h-matings under diverse temperatures. The grey dashed line (2 x 10^8) indicates the approximate original number of cells in the beginning of the mating. "#" stands for the replicates with no detected transconjugants.

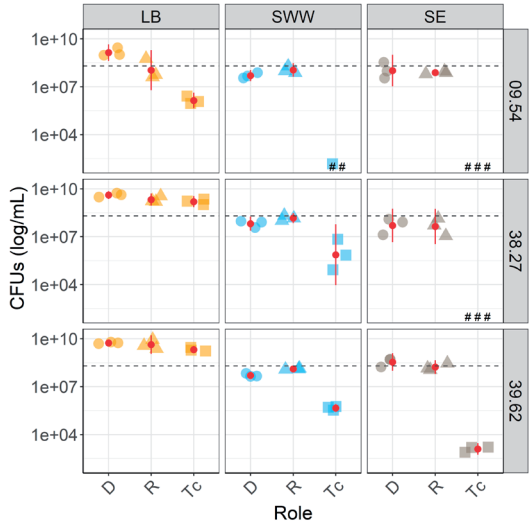


Figure S3. Absolute counts of donor (D), recipient (R) and transconjugants (Tc) after 2h mating in LB, Synthetic wastewater (SWW) and Soil Extract (SE) media. The discontinuous line at $2x10^8$ indicates the approximate original number of cells in the beginning of the mating. "#" stands for the replicates with no detected transconjugants.

5.11 References

Abis, K.L., Mara, D., 2006. Temperature measurement and stratification in facultative waste stabilisation ponds in the UK climate. Environ. Monit. Assess. 114, 35–47. https://doi.org/10.1007/s10661-006-1076-7

Al Qarni, H., Collier, P., O'Keeffe, J., Akunna, J., 2016. Investigating the removal of some pharmaceutical compounds in hospital wastewater treatment plants operating in Saudi Arabia. Environ. Sci. Pollut. Res. 23, 13003–13014. https://doi.org/10.1007/s11356-016-6389-7

Amann, R.I., Ludwig, W., Schleifer, K.-H., 1995. Phylogenetic Identification and In Situ Detection of Individual Microbial Cells without Cultivation. Microbiol. Mol. Biol. Rev. 59, 143–169.

Bahl, M.I., Hansen, L.H., Sørensen, S.J., 2007. Impact of conjugal transfer on the stability of IncP-1 plasmid pKJK5 in bacterial populations. FEMS Microbiol. Lett. 266, 250–256. https://doi.org/10.1111/j.1574-6968.2006.00536.x

Bale, M.J., Fry, J.C., Day, M.J., 1988. Transfer and occurrence of large mercury resistance plasmids in river epilithon. Appl. Environ. Microbiol. 54, 972–978. https://doi.org/10.1128/aem.54.4.972-978.1988

Banerjee, G., Ray, A.K., Kumar, R., 2016. Effect of temperature on lateral gene transfer efficiency of multi-antibiotics resistant bacterium, alcaligenes faecalis. Sains Malaysiana 45, 909–914.

Barrios-Hernández, M.L., Pronk, M., Garcia, H., Boersma, A., Brdjanovic, D., van Loosdrecht, M.C.M., Hooijmans, C.M., 2020. Removal of bacterial and viral indicator organisms in full-scale aerobic granular sludge and conventional activated sludge systems. Water Res. X 6. https://doi.org/10.1016/j.wroa.2019.100040

Bellanger, X., Guilloteau, H., Bonot, S., Merlin, C., 2014. Demonstrating plasmid-based horizontal gene transfer in complex environmental matrices: A practical approach for a critical review. Sci. Total Environ. 493, 872–882. https://doi.org/10.1016/j.scitotenv.2014.06.070

Carattoli, A., 2013. Plasmids and the spread of resistance. Int. J. Med. Microbiol. 303, 298–304. https://doi.org/10.1016/j.ijmm.2013.02.001

Carattoli, A., Zankari, E., Garciá-Fernández, A., Larsen, M.V., Lund, O., Villa, L., Aarestrup, F.M., Hasman, H., 2014. In Silico detection and typing of plasmids using plasmidfinder and plasmid multilocus sequence typing. Antimicrob. Agents Chemother. 58, 3895–3903. https://doi.org/10.1128/AAC.02412-14

Ceccarelli, D., Kant, A., Van Essen-Zandbergen, A., Dierikx, C., Hordijk, J., Wit, B., Mevius, D.J., Veldman, K.T., 2019. Diversity of plasmids and genes encoding resistance to extended spectrum cephalosporins in commensal Escherichia coli From Dutch livestock in 2007-2017. Front. Microbiol. 10, 1–9. https://doi.org/10.3389/fmicb.2019.00076

Curtiss, R., Caro, L.G., Allison, D.P., Stallions, D.R., 1969. Early Stages of Conjugation in Escherichia coli. J. Bacteriol. 100, 1091–1104. https://doi.org/10.1128/JB.100.2.1091-1104.1969

Delatolla, R., Tufenkji, N., Comeau, Y., Gadbois, A., Lamarre, D., Berk, D., 2012. Effects of Long Exposure to Low Temperatures on Nitrifying Biofilm and Biomass in Wastewater Treatment. Water Environ. Res. 84, 328–338. https://doi.org/10.2175/106143012x13354606450924

Fan, X.T., Li, H., Chen, Q.L., Zhang, Y. Sen, Ye, J., Zhu, Y.G., Su, J.Q., 2019. Fate of antibiotic resistant pseudomonas putida and broad host range plasmid in natural soil microcosms. Front. Microbiol. 10, 1–10. https://doi.org/10.3389/fmicb.2019.00194

Fernandez-Astorga, A., Muela, A., Cisterna, R., Iriberri, J., Barcina, I., 1992. Erratum: Biotic and abiotic factors affecting plasmid transfer in Escherichia coli strains (Applied and Environmental Microbiology 58:1 (393)). Appl. Environ. Microbiol. 58, 2708.

Fox, J., 2005. The R Commander: A Basic Statistics Graphical User Interface to R. J. Stat. Softw. 14, 1--42.

Frost, L.S., Koraimann, G., 2010. Regulation of bacterial conjugation: Balancing opportunity with adversity. Future Microbiol. 5, 1057–1071. https://doi.org/10.2217/fmb.10.70

Gama, J.A., Zilhão, R., Dionisio, F., 2017. Conjugation efficiency depends on intra and intercellular interactions between distinct plasmids: Plasmids promote the immigration of other plasmids but repress co-colonizing plasmids. Plasmid 93, 6–16. https://doi.org/10.1016/j.plasmid.2017.08.003

Goodman, A.E., Hild, E., Marshall, K.C., Hermansson, M., 1993. Conjugative plasmid transfer between bacteria under simulated marine oligotrophic conditions. Appl. Environ. Microbiol. 59, 1035–1040. https://doi.org/10.1128/aem.59.4.1035-1040.1993

Grabow, W.O.K., Prozesky, O.W., Burger, J.S., 1975. Behaviour in a river and dam of coliform bacteria with transferable or non-transferable drug resistance. Water Res. 9, 777–782. https://doi.org/10.1016/0043-1354(75)90134-7

Headd, B., Bradford, S.A., 2020. The Conjugation Window in an Escherichia coli K-12 Strain with an IncFII Plasmid. Appl. Environ. Microbiol. 86, 1–18. https://doi.org/10.1128/AEM.00948-20

Headd, B., Bradford, S.A., 2018. Physicochemical factors that favor conjugation of an antibiotic resistant plasmid in non-growing bacterial cultures in the absence and presence of antibiotics. Front. Microbiol. 9, 1–14. https://doi.org/10.3389/fmicb.2018.02122

Hoang, V., Delatolla, R., Abujamel, T., Mottawea, W., Gadbois, A., Laflamme, E., Stintzi, A., 2014. Nitrifying moving bed biofilm reactor (MBBR) biofilm and biomass response to long term exposure to 1°C. Water Res. 49, 215–224. https://doi.org/10.1016/j.watres.2013.11.018

Hu, Y., Yang, X., Qin, J., Lu, N., Cheng, G., Wu, N., Pan, Y., Li, J., Zhu, L., Wang, X., Meng, Z., Zhao, F., Liu, D., Ma, J., Qin, N., Xiang, C., Xiao, Y., Li, L., Yang, H., Wang, Jian, Yang, R., Gao, G.F., Wang, Jun, Zhu, B., 2013. Metagenome-wide analysis of antibiotic resistance genes in a large cohort of human gut microbiota. Nat. Commun. 4. https://doi.org/10.1038/ncomms3151

Inoue, D., Sei, K., Soda, S., Ike, M., Fujita, M., 2005. Potential of predominant activated sludge bacteria as recipients in conjugative plasmid transfer. J. Biosci. Bioeng. 100, 600–605. https://doi.org/10.1263/jbb.100.600

Jacquiod, S., Brejnrod, A., Morberg, S.M., Abu Al-Soud, W., Sørensen, S.J., Riber, L., 2017. Deciphering conjugative plasmid permissiveness in wastewater microbiomes. Mol. Ecol. 26, 3556–3571. https://doi.org/10.1111/mec.14138

Klümper, U., Riber, L., Dechesne, A., Sannazzarro, A., Hansen, L.H., Sørensen, S.J., Smets, B.F., 2015. Broad host range plasmids can invade an unexpectedly diverse fraction of a soil bacterial community. ISME J. 9, 934–945. https://doi.org/10.1038/ismej.2014.191

Kohyama, Y., Suzuki, S., 2019. Conjugative gene transfer between nourished and starved cells of photobacterium damselae ssp. Damselae and escherichia coli. Microbes Environ. 34, 388–392. https://doi.org/10.1264/jsme2.ME19099

Li, L., Dechesne, A., He, Z., Madsen, J.S., Nesme, J., Sørensen, S.J., Smets, B.F., 2018. Estimating the Transfer Range of Plasmids Encoding Antimicrobial Resistance in a Wastewater Treatment Plant Microbial Community. Environ. Sci. Technol. Lett. 5, 260–265. https://doi.org/10.1021/acs.estlett.8b00105

Li, L., Dechesne, A., Madsen, J.S., Nesme, J., Sørensen, S.J., Smets, B.F., 2020. Plasmids persist in a microbial community by providing fitness benefit to multiple phylotypes. ISME J. 14, 1170–1181. https://doi.org/10.1038/s41396-020-0596-4

MacDonald, J.A., Smets, B.F., Rittmann, B.E., 1992. The effects of energy availability on the conjugative-transfer kinetics of plasmid RP4. Water Res. 26, 461–468. https://doi.org/10.1016/0043-1354(92)90046-7

Manti, A., Boi, P., Falcioni, T., Canonico, B., Ventura, A., Sisti, D., Pianetti, A., Balsamo, M., Papa, S., 2008. Bacterial Cell Monitoring in Wastewater Treatment Plants by Flow Cytometry. Water Environ. Res. 80, 346–354. https://doi.org/10.2175/106143007x221418

Musovic, S., Dechesne, A., Sørensen, J., Smets, B.F., 2010. Novel assay to assess permissiveness of a soil microbial community toward receipt of mobile genetic elements. Appl. Environ. Microbiol. 76, 4813–4818. https://doi.org/10.1128/AEM.02713-09

Musovic, S., Oregaard, G., Kroer, N., Sørensen, S.J., 2006. Cultivation-independent examination of horizontal transfer and host range of an IncP-1 plasmid among gram-positive and gram-negative bacteria indigenous to the barley rhizosphere. Appl. Environ. Microbiol. 72, 6687–6692. https://doi.org/10.1128/AEM.00013-06

O'Morchoe, S.B., Ogunseitan, O., Sayler, G.S., Miller, R. V., 1988. Conjugal transfer of R68.45 and FP5 between Pseudomonas aeruginosa strains in a freshwater environment. Appl. Environ. Microbiol. 54, 1923–1929. https://doi.org/10.1128/aem.54.8.1923-1929.1988

O´Neill, J., 2016. TACKLING DRUG-RESISTANT INFECTIONS GLOBALLY: FINAL REPORT AND RECOMMENDATIONS. https://doi.org/10.1088/1751-8113/44/8/085201

Ochman, H., Lawrence, J.G., Groisman, E.A., 2000. Lateral gene transfer and the nature of bacterial innovation. Nature 405, 299–304. https://doi.org/10.1038/35012500

Osińska, A., Korzeniewska, E., Harnisz, M., Felis, E., Bajkacz, S., Jachimowicz, P., Niestępski, S., Konopka, I., 2020. Small-scale wastewater treatment plants as a source of the dissemination of antibiotic resistance genes in the aquatic environment. J. Hazard. Mater. 381, 121221. https://doi.org/10.1016/j.jhazmat.2019.121221

Pallares-Vega, R., Hernandez Leal, L., Fletcher, B.N., Vias-Torres, E., van Loosdrecht, M.C.M., Weissbrodt, D.G., Schmitt, H., 2020. Annual dynamics of antimicrobials and resistance determinants in

flocculent and aerobic granular sludge treatment systems. Water Res. 116752. https://doi.org/10.1016/j.watres.2020.116752

Pinilla-Redondo, R., Cyriaque, V., Jacquiod, S., Sørensen, S.J., Riber, L., 2018. Monitoring plasmid-mediated horizontal gene transfer in microbiomes: recent advances and future perspectives. Plasmid 99, 56–67. https://doi.org/10.1016/j.plasmid.2018.08.002

Popowska, M., Krawczyk-Balska, A., 2013. Broad-host-range IncP-1 plasmids and their resistance potential. Front. Microbiol. 4, 1–8. https://doi.org/10.3389/fmicb.2013.00044

R Core Team, 2018. R: A language and environment for statistical computing. R Found. Stat. Comput. Vienna.

Raynaud, X., Nunan, N., 2014. Spatial ecology of bacteria at the microscale in soil. PLoS One 9. https://doi.org/10.1371/journal.pone.0087217

Rochelle, P.A., Fry, J.C., Day, M.J., 1989. Factors affecting conjugal transfer of plasmids encoding mercury resistance from pure cultures and mixed natural suspensions of epilithic bacteria. J. Gen. Microbiol. 135, 409–424. https://doi.org/10.1099/00221287-135-2-409

Rodrigues, D.F., Tiedje, J.M., 2008. Coping with our cold planet. Appl. Environ. Microbiol. 74, 1677–1686. https://doi.org/10.1128/AEM.02000-07

Rottiers, A., Boeije, G., 1999. Part II: Design and validation of test units. Science (80-.). 38, 711–727.

Rozwandowicz, M., Hordijk, J., Bossers, A., Zomer, A.L., Wagenaar, J.A., Mevius, D.J., Brouwer, M.S.M., 2020. Incompatibility and phylogenetic relationship of I-complex plasmids. Plasmid 109, 102502. https://doi.org/10.1016/j.plasmid.2020.102502

Sakuda, A., Suzuki-Minakuchi, C., Matsui, K., Takahashi, Y., Okada, K., Yamane, H., Shintani, M., Nojiri, H., 2018. Divalent cations increase the conjugation efficiency of the incompatibility P-7 group plasmid pCAR1 among different pseudomonas hosts. Microbiol. (United Kingdom) 164, 20–27. https://doi.org/10.1099/mic.0.000583

Scott, K.P., Mercer, D.K., Glover, L.A., Flint, H.J., 2006. The green fluorescent protein as a visible marker for lactic acid bacteria in complex ecosystems. FEMS Microbiol. Ecol. 26, 219–230. https://doi.org/10.1111/j.1574-6941.1998.tb00507.x

Seemann, T., 2014. Prokka: Rapid prokaryotic genome annotation. Bioinformatics 30, 2068–2069. https://doi.org/10.1093/bioinformatics/btu153

Sengeløv, G., Kristensen, K.J., Sørensen, A.H., Kroer, N., Sørensen, S.J., 2001. Effect of Genomic Location on Horizontal Transfer of a Recombinant Gene Cassette Between Pseudomonas Strains in the Rhizosphere and Spermosphere of Barley Seedlings. Curr. Microbiol. 42, 160–167. https://doi.org/10.1007/s002840010197

Seoane, J., Yankelevich, T., Dechesne, A., Merkey, B., Sternberg, C., Smets, B.F., 2011. An individual-based approach to explain plasmid invasion in bacterial populations. FEMS Microbiol. Ecol. 75, 17–27. https://doi.org/10.1111/j.1574-6941.2010.00994.x

Smillie, C., Garcillán-Barcia, M.P., Francia, M.V., Rocha, E.P.C., de la Cruz, F., 2010. Mobility of plasmids. Microbiol. Mol. Biol. Rev. 74, 434–52. https://doi.org/10.1128/MMBR.00020-10

Smith, E.M., Prairie, Y.T., 2004. Bacterial metabolism and growth efficiency in lakes: The importance of phosphorus availability. Limnol. Oceanogr. 49, 137–147. https://doi.org/10.4319/lo.2004.49.1.0137

Sørensen, S.J., Bailey, M., Hansen, L.H., Kroer, N., Wuertz, S., Sorensen, S., 2005. Studying plasmid horizontal transfer in situ: a critical review. Nat. Rev. Microbiol. 3, 700–710. https://doi.org/10.1038/nrmicro1232

Thomas, C.M., Smith, C.A., 1987. Incompatibility group P plasmids: genetics, evolution, and use in genetic manipulation. Annu. Rev. Microbiol. 41, 77–101. https://doi.org/10.1146/annurev.mi.41.100187.000453

Trevors, J.T., Bej, A.K., Mojib, N., van Elsas, J.D., van Overbeek, L., 2012. Bacterial gene expression at low temperatures. Extremophiles 16, 167–176. https://doi.org/10.1007/s00792-011-0423-y

WHO [WWW Document], 2016. . WHO.

Willms, I.M., Yuan, J., Penone, C., Goldmann, K., Vogt, J., Wubet, T., Schöning, I., Schrumpf, M., Buscot, F., Nacke, H., 2020. Resistance Genes and Mobile Genetic Elements in Land Use. Genes (Basel). 11, 1–18.

Zhong, X., Kro˙l, J.E., Top, E.M., Krone, S.M., 2010. Accounting for mating pair formation in plasmid population dynamics. J. Theor. Biol. 262, 711–719. https://doi.org/10.1016/j.jtbi.2009.10.013

CHAPTER 6

"Tenho o mundo todo
Ao dispor numa vidraça
Só não tenho sítio a que chame casa"
Diabo na Cruz
in "Roque da Casa", Lebre (2018)

Horizontal gene transfer of an IncP1 plasmid to soil bacterial community introduced by *Escherichia coli* through manure amendment under realistic conditions

Gonçalo Macedo[1,2], Asmus Kalckar Olesen[3], Lorrie Maccario[3], Lucia Hernandez-Leal[2],Peter van der Maas[4], Dick Heederik[5], Dik Mevius[1,4], Søren J. Sørensen[3], Heike Schmitt[2,5,7]

[1] Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands
[2] Wetsus, European Centre of Excellence for Sustainable Water Technology, Oostergoweg 9, 8911 MA Leeuwarden, The Netherlands
[3] University of Copenhagen, Copenhagen 2100, Denmark
[4] Van Hall Larenstein, University of Applied Sciences, Agora 1, 8901 BV Leeuwarden, The Netherlands
[5] Institute for Risk Assessment Sciences, Utrecht University, Yalelaan 2, 3584 CM Utrecht, The Netherlands
[6] Wageningen Bioveterinary Research, Houtribweg 39, 8221 RA Lelystad, The Netherlands
[7] National Institute for Public Health and the Environment, Antonie van Leeuwenhoeklaan 9, 3721 MA Bilthoven, The Netherlands

Submitted for publication

6.1 Abstract

Horizontal gene transfer (HGT) is of great concern because it allows for the rapid dissemination of antibiotic resistance genes. This study quantified conjugation of an IncP1 plasmid carried by an *Escherichia coli* host to the indigenous microbial community in agricultural soil after manure application. Furthermore, new plasmid hosts were identified under conditions resembling environmental conditions in microcosms. Conjugation was quantified by plating and flow cytometry. Transconjugants were recovered by fluorescence-activated cell sorting and identified by 16S rRNA gene sequencing. Transconjugants were only observed within the first four days of incubation, and higher temperature enhanced the number of transconjugants detected. Still, transconjugant numbers were in all cases close to the detection limits of this experimental system (1.00 - 2.49 log CFU/g of transconjugants in manured soil). In the pool of recovered transconjugants, we found amplicon sequence variants (ASVs) of genera from soils (*Bacillus* and *Nocardioides*), as well as genera present in manure and soils after manuring (*Comamonas* and *Rahnella*). *Acinetobacter* and *Pseudomonas* were also identified in the transconjugant pool, but their abundance was probably below the detection limit in the microcosms, as it was not possible to track their specific ASVs in the microcosms. This work highlights the importance of environmental conditions and farm practices on the spread of AMR, and it shows that gene transfer is occurring at detectable levels also under realistic conditions in soils.

6.2 Introduction

Antimicrobial resistance (AMR) has been pinpointed as one of the most significant global public health challenges (WHO, 2014). Horizontal gene transfer (HGT) is of particular concern because it drives bacterial evolution (Ochman et al., 2000) and is connected with the rise of AMR (Carattoli, 2013; Dröge et al., 1998; Musovic et al., 2006). Plasmid-mediated gene transfer by conjugation is considered a major HGT mechanism (Norman et al., 2009; Sørensen et al., 2005; Thomas and Nielsen, 2005). Not all plasmids are conjugative or even mobilizable (Smillie et al., 2010), but when they are, they contribute significantly to the communal gene pool (Norman et al., 2009). In agriculture, the application of manure as organic fertilizer to soils results in the introduction of fecal bacteria, their plasmids, and antimicrobial resistance genes (ARGs) (Jechalke et al., 2014; Thanner et al., 2016). Plasmids are known to mediate horizontal gene transfer to soil bacteria (Henschke and Schmidt, 1990; Pukall et al., 1996; Top et al., 1990, 1995).

The advances and limitations of the methods used to address environmental dissemination of AMR-carrying plasmids have been summarized by Pinilla-Redondo et al. (2018) and Rice et al. (2020). Culture-dependent studies often apply optimal bacterial transmission conditions (Bellanger et al., 2014; Jacquiod et al., 2017; Jiao et al., 2017; Soda et al., 2008). Therefore, more recently, culture-independent methods have been developed to link ARGs or plasmids to their hosts (Klümper et al., 2014b; Spencer et al., 2016; Stalder et al., 2019). Traditional microcosm systems are only an approximation of the natural environment, but they allow the manipulation of physicochemical variables and the use of genetically modified microorganisms to monitor HGT accurately. Classical microcosm studies usually adopt a culture-dependent approach that restricts the recipient community to only a few strains, and they use low bacterial diversity settings (e.g., sterile soil) (Krasovsky and Stotzky, 1987; Richaume et al., 1989; Wein-Berg and Stotzky, 1972).

When introduced to soils, fecal bacteria concentrations tend to decline (Franz et al., 2014; van Veen et al., 1997), limiting the timespan for potential ARG transfer to soil bacteria. Less than optimal environmental temperatures (Banerjee et al., 2016) and nutrient limitations (Pukall et al., 1996; Top et al., 1990) often restrict this genetic exchange, rendering it to values below the limits of detection. However, manure application to soil provides nutrients and potential ARG-carrying bacteria, thus creating favourable HGT conditions. The role of plasmids on AMR spread is well studied in human pathogens (Cantón and Coque, 2006; Woerther et al., 2013). However, quantification of transfer events in soils – especially when being manure fertilized – is challenging, and the identity of the new plasmid hosts is often unknown. Recently, the fate of an ARG-carrying plasmid was assessed in greenhouse soil microcosms (Fan et al., 2019), but manure was not added to the soils. Therefore, the role of manure as a source for HGT in soils remains unstudied, especially in conditions that

resemble the ones found in the environment in terms of temperature, incubation period, mating matrix, and recipient community diversity.

The main goal of this study was to quantify the number of conjugation events occurring upon manure application to soils and identify the new host *taxa* under conditions resembling environmental conditions. To achieve this, manured-soil microcosms were spiked with *Escherichia coli* harboring a *gfp*-tagged plasmid. After incubation, the manured-soil microcosm microbiome was extracted with Nycodenz to (i) determine absolute and relative abundance via direct plate counts and flow-cytometry, respectively; and (ii) identify new plasmid hosts via fluorescence-activated cell sorting (FACS) and 16S rRNA gene sequencing.

6.3 Materials and Methods

6.3.1 Donor strain and plasmid characteristics

A genetically engineered *Escherichia coli* MG1655 was used as the donor (Klümper et al., 2015). This strain is chromosomally tagged with a gene cassette encoding constitutive red fluorescence (mCherry) and constitutive *lacI*^{q} production, and it harbored a broad-host-range IncP1 conjugative plasmid pKJK5 (Sengeløv et al., 2001). The pKJK5 plasmid was marked with a *lacI*^{q} repressible promoter upstream the *gfpmut3B* gene encoding for the GFP (Bahl et al., 2007; Klümper et al., 2015), resulting in inhibited *gfpmut3B* expression in the donor strain, but expression when transferred to a new host.

The donor strains were grown aerobically with agitation, at 37 °C, for 3.5 hours from a fresh dilution of an overnight growth culture (defined by a growth curve) in LB medium supplemented with kanamycin (100 µg/mL). The bacterial cells were harvested by centrifugation at 10,000 g for 10 min, and the pellets were washed twice and re-suspended in 0.9% sterile saline solution. The resulting concentration of donor inoculants was confirmed by plating.

To confirm suitable selective conditions for the identification of transconjugants, the antimicrobial susceptibility profile was determined by a disc diffusion test (EUCAST Disk Diffusion Method for Antimicrobial Susceptibility Testing - version 6.0). The results were interpreted based on the EUCAST-defined breakpoints (Breakpoint tables for interpretation of MICs and zone diameters - version 8.0), and they showed that agar containing kanamycin (100 µg/mL), trimethoprim (32 µg/mL), sulfamethoxazole (128 µg/mL), and nystatin (20 µg/mL) would be appropriate to enumerate the donor and recover potential transconjugants.

6.3.2 Manured soil microcosms

Cattle manure and grassland soil samples (loamy texture) were collected at the University Copenhagen campus in Taastrup (Denmark) in September 2019 and stored at 4 °C until used.

Two manured soil microcosm series were prepared in 50-mL tubes containing 15 g of soil (total weight), in four replicates, and were incubated either at 15 or 30 °C, in the dark, up to 21 days. The water holding capacity was adjusted to 60%, and the tubes were not tightly closed to allow gas exchange throughout incubation. To compensate for the weight loss due to evaporation, the microcosms were regularly irrigated with sterile distilled water. The donor strain was spiked to manure so that initial theoretical donor concentrations would correspond to 10^7 CFU/g soil, and the spiked manure was then immediately applied to soils (40 mg/g, fresh weight), which approximately corresponds to a general manure application for arable soils. Destructive sampling occurred before soil amendment (soil, S) and at specific time points after manure application, corresponding to days 1, 4, 7, 14, and 21 (manured soil, MS1 to MS21). Part of each replicate was stored at -20 °C for DNA extraction, and another part was used for Nycodenz extraction of the bacterial communities. Control microcosms, without donor inoculation, were prepared and incubated at the same conditions. Additionally, original manure and soil aliquots were stored at -20 °C for DNA extraction.

6.3.3 Nycodenz extraction and plating

Nycodenz density gradient separation was used to extract the bacterial communities and proceeded as described by Klümper et al. (2014a), with reagent volumes adapted to match the used 5 g of the microcosms. The Nycodenz extracts were stored at near-zero temperatures (on ice and at 4 °C) until used for enumeration or cell sorting.

To enumerate donors and potential transconjugants in the Nycodenz extract, serial dilutions were prepared, and 100 µL of each dilution was plated on LB agar, containing kanamycin (100 µg/mL), trimethoprim (32 µg/mL), sulfamethoxazole (128 µg/mL), and nystatin (20 µg/mL). The plates were incubated at 30 °C, for 24h, and colonies were observed and counted using a Dark Reader Transilluminator (Clare Chemical Research, USA) for GFP excitation; total cells and green fluorescing cells, corresponding to donor and transconjugants, respectively). Because low temperatures favor the maturation of GFP (Scott et al., 2006), the plates were re-counted after a 24-hour incubation at 4 °C; thus, confirming the results obtained directly after incubation. The plating of the microcosm control series confirmed that no background was observable in plates with the mentioned antimicrobials.

6.3.4 Flow cytometry and transconjugant sorting

Cells obtained with Nycodenz extraction were analyzed and sorted using a FACSAria IIIU (BD Biosciences, USA) equipped with the BD FACSDiva software v 8.0.3 (BD Biosciences).

A 70-µm nozzle was used with a sheath pressure of 70 PSI. To detect bacterial cells, the forward scatter (FSC) voltage was increased by ~200 from the CS & T settings. The threshold on both FSC and side scatter (SSC) was lowered to the minimum of 200 in signal height, and the operator was set to "AND." The green fluorescence of GFP was excited by a 488-nm laser (20 mW) and detected using a 530/30-nm bandpass filter. The red fluorescence of mCherry was excited using a 561-nm laser (50 mW) and detected using a 610/20-nm bandpass filter. The channel voltage was increased by ~150 from the CS & T settings. The gating was made so that a double logarithmic bivariate plot with FSC-Area(A) and SSC-A was used to detect events in the bacteria's size and complexity. These events were forwarded to a double logarithmic bivariate plot with green fluorescence intensity and red fluorescence intensity, in which transconjugant events were detected as only green fluorescent and donor cells as red fluorescing. Samples were diluted in PBS until a threshold event rate of ~3000 events/s was obtained. For sorting, the purity precision settings were used.

Due to the low number of overall transconjugants observed, the expected required sorting time would be excessively high (approx. 20h/replicate). Adding to the amount of time needed for sorting, the longer the period spent in sorting, the higher the chance of errors. Therefore, for practical reasons, either 30 or 300 transconjugants (for the 15 or 30 °C microcosm series) were collected from time point MS1 for each microcosm replicate, resulting in a total of 1320 transconjugants collected. In order to maintain a sufficient number of transconjugant cells for subsequent sequencing, the sorted cells were incubated in 10% soil extract for three days, at corresponding microcosm series temperature, and to avoid excessive growth bias. The soil extract was obtained from the same soil used for the microcosm experiments, using a previously described method (Musovic et al., 2010). After the three-day incubation, because no observable signs of growth were noticeable by eye, 10% TSB (tryptone-soy broth) was added to the sorted cells, and they were incubated for one additional day at the same temperature as before. After this period, only the DNA of re-grown transconjugants was extracted for 16S rRNA sequencing. In total, re-grown transconjugants from replicates A and D from the first time point (MS1) were sequenced, but from both 10% soil extract (n = 2) and two from 10% TSB after 10% soil extract (n = 2).

6.3.5 DNA extraction

All DNA extractions were performed with the NucleoSpin Soil kit (Macherey-Nagel; Germany), following the manufacturer's instructions. Total DNA extracts were obtained from 250 mg of manured soil. In contrast, the DNA from the re-grown transconjugants was obtained after concentrating the cells by centrifugation (10,000 g) and resuspension in 250 µL of sterile PBS. DNA quantification and PCR reaction targeting 16S rRNA were used to validate the DNA extractions and to confirm if there was significant growth of transconjugants. Only samples with a clear band at 466 bp compared to PCR negative controls (i.e., DNA extraction from the culture media and MiliQ water control in PCR) were further used for sequencing.

6.3.6 16S rRNA sequencing

Amplicon sequencing libraries were prepared using a two-step PCR, targeting 16S rRNA gene V3-V4 regions. First PCR was performed for 30 cycles using the primers Uni341F (5'-CCTAYGGGRBGCASCAG-3') and Uni806R (5'-GGACTACNNGGGTATCTAAT-3') initially published by Yu et al. (2005) and modified as described by Sundberg et al. (2013). First PCR amplification products were purified using HighPrep PCR clean-up (MagBio Genomics, USA) using a 0.65:1 (beads:PCR reaction) volumetric ratio. A second PCR reaction was performed to add Illumina sequencing adapters and sample-specific dual indexes (IDT Integrated DNA Technologies, USA) using PCRBIO HiFi (PCR Biosystems Ltd., UK) for 15 cycles. The second PCR products were purified with HighPrep PCR Clean-Up System, as described for the first PCR. Sample concentrations were normalized using the SequalPrep Normalization Plate (96) Kit (Thermofisher, USA), following the manufacturer's instructions. The libraries were then pooled and up-concentrated using DNA Clean and Concentrator-5 Kit (Zymo Research, USA). The library pool's concentration was determined using the Quant-iT High-Sensitivity DNA Assay Kit (Life Technologies, USA) and diluted to 4 nM. The library was denatured and sequenced following the manufacturer's instructions on an Illumina MiSeq platform at the Section of Microbiology - University of Copenhagen, using Reagent Kit v3 [2 x 300 cycles] (Illumina, USA).

Cutadapt v.2.3. (Martin, 2011) was used to remove primer sequences used in the first PCR, both on the 5' and the reverse complement on 3' ends, also discarding read pairs for which any of the two primers could not be detected. Reads were further processed for error-correction, merging and amplicon sequence variants (ASVs) generation using DADA2 version 1.10.0 (Callahan et al., 2016) plugin for QIIME2 (Bolyen et al., 2019) with the following parameters: truncL = 280, truncR = 240; trimL = 8, trimR = 8, and otherwise defaults parameters. Each ASV sequence was taxonomically annotated using *q2-feature-classifier classify-sklearn* module trained with SILVA SSU database version 132 (Quast et al., 2013), trimmed for the V3-V4 region only.

Data analysis was performed using *phyloseq* version 1.22.3 (McMurdie and Holmes, 2013) in R statistical software version 3.6.3 (R Core Team, 2020) and RStudio (Version 1.2.5033; https://www.rstudio.com/). Two datasets were created based on the sample's origin. One consisted of the ASVs present in the manured soil microcosms (incl. original soil and manure), and the other of ASVs from the presumable transconjugants (sorted re-grown cells). In the microcosm and transconjugant datasets, ASVs were removed that were not assigned to Bacteria (n = 96 and n = 2, respectively), and assigned to chloroplasts (n = 8,378 and n = 3, respectively) or mitochondria (n = 2,851 and n = 5, respectively). Furthermore, using the *decontam* package (Davis et al., 2018), 14 predicted contaminant ASVs which were linked to blank controls (culture media extraction control, first and second PCR negative controls) were removed from the microcosm dataset, retaining a total of 50,244

ASVs across all samples (2,887,440 reads in total; 72,600 ± 21,575 reads per sample on average). The transconjugant dataset (re-grown sorted cells) was not subjected to analysis with the *decontam* package and contained 25 ASVs across all samples. The occurrence of ASVs in the controls was manually checked (Supplementary Figure 3). Rarefaction curves and library sizes can be found in Supplementary Figure 1. Phylogenetic trees were constructed using *phyloseq*. The raw reads can be accessed under the NCBI Bioproject number PRJNA718741.

6.3.7 Alpha and beta diversities

Samples with less than 20,000 reads were excluded (one soil sample removed). For the overall microcosm bacterial community, alpha diversity indexes (Chao1 richness, Shannon, and Pielou's evenness) were estimated after rarefaction (n = 32,254). The microcosms dataset consisted of 40,959 ASVs, distributed in 30 samples consisting of manure (2,939 ASVs, three samples) and soil samples (38,952 ASVs). Rarefying at 32,254 reads resulted in 9,285 ASVs removed from the dataset. Rarefaction was only performed to estimate the alpha diversity indexes of the microcosm dataset. No rarefaction nor diversity index calculations were performed for the transconjugant dataset.

Beta diversity analysis, using Bray-Curtis dissimilarities, was calculated using the R package *vegan* (Oksanen et al., 2019). The effects of incubation time on microcosms were determined using permutational multivariate analysis of variance (PERMANOVA) and depicted in a non-metric multidimensional scaling (NMDS) ordination plot based on Bray-Curtis distances with 999 permutations. The homogeneity of group dispersion was confirmed by testing for multivariate homogeneity of group dispersions (PERMDISP2).

6.3.8 Statistical analysis

One-way analysis of variance (ANOVA) was conducted to detect differences in bacterial diversity indexes and in cell abundances between temperatures and time points. The ANOVA tests were followed by TukeyHSD post-hoc analysis, and homogeneity of variance was confirmed with Levene's test. Data normality was verified with Shapiro-Wilk's method, and when normality was not achieved, group comparison was performed using the equivalent non-parametric test (Kruskal-Wallis). A significance score of $p < 0.05$ was considered statistically relevant. These analyses were performed with R version 3.6.3 (R Core Team, 2020) and RStudio (Version 1.2.5033; https://www.rstudio.com/). Used software packages consisted of *reshape* (Wickham, 2007) and *tidyverse* (Wickham et al., 2019); a set of packages designed for data cleaning, trimming, and visualization; *PMCMRplus* (Thorsten, 2020), and *car* (Fox and Weisberg, 2019) for ANOVA and Levene's test.

6.4 Results

6.4.1 Impact of manure application on overall microcosm soil diversity

Chao1 patterns showed that soil samples had a greater richness of bacterial ASVs than manure (Figure 1; $p < 0.01$, ANOVA), and application of manure did not increase the estimated total number of ASVs found in manured soils (Figure 1; $p = 0.24$, ANOVA) as compared to the soil before manure application, respectively.

The NMDS ordination based on Bray-Curtis dissimilarity revealed clustering of samples according to time after manure application (Figure 1; PERMANOVA, $p < 0.01$). The effect of the time points explained 56% of the variation in the microcosm samples.

Before manure application, *Proteobacteria* (28.60 ± 0.01%), *Actinobacteria* (19.99 ± 1.32%), and *Acidobacteria* (14.15 ± 0.37%) were the most abundant *phyla* in soils. However, after manuring, *Proteobacteria* (45.60 ± 9.25%), *Bacteroidetes* (16.73 ± 4.64%), and *Firmicutes* (13.00 ± 2.58%) became dominant, as these were highly abundant in manure (Supplementary Figure 2).

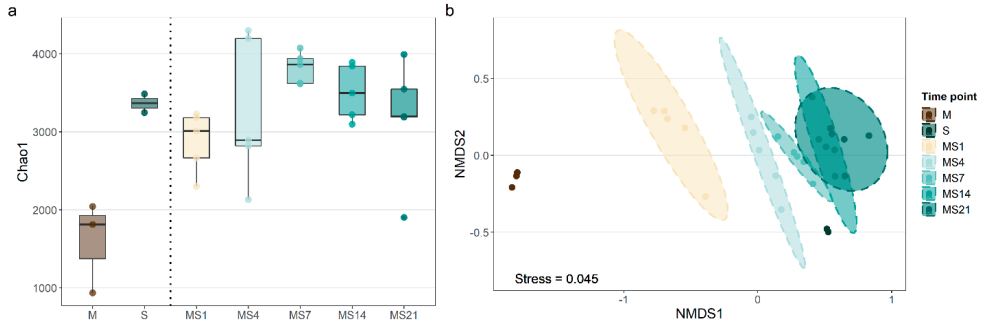


Figure 1. Manure application changed the bacterial community structure. Manure samples (M) had lower bacterial diversity than soil samples (S) and manured soils after days 1, 4, 7, 14, and 21 (MS1 to MS21, respectively) (a). Non-metric multidimensional scaling (NMDS) plots illustrating Bray-Curtis dissimilarity matrices show clustering of the soil bacterial community samples by time after manuring (b), with the strongest shift seen right after manure amendment. The dataset presented in this figure was rarefied, as mentioned earlier. Other alpha diversity indexes can be found in Supplementary Table 3.

6.4.2 Donor and transconjugant abundance in manured soil microcosms

Enumeration of donor and transconjugant by plating showed that transconjugants were only observed within the first four days of incubation (Figure 2). At day one (MS1), low numbers of transconjugants were observed both at 15 and 30 °C (1.00 – 2.49 log CFU/g manured soil), and at day four (MS4), transconjugants were found ($p < 0.05$, ANOVA) only at 30 °C at abundances lower than in MS1 (1.00 – 1.60 log CFU/g manured soil). No transconjugants were detected at MS4 in the 15 °C series.
Higher microcosm incubation temperature resulted in a higher number of transconjugants observed (Figure 2). The detection of transconjugants coincided with the peak of donor cells, whose abundances initially increased in both 15 and 30 °C series ($p < 0.05$), reaching 6.20 – 7.05 log CFU/g manured soil (MS1) and decreased since then ($p < 0.05$) to 2.08 – 2.97 log CFU/g manured soil (MS21) (Figure 2).

Although the majority of bacteria are known to be non-culturable (Amann et al., 1995), the plating was included to enumerate and identify transconjugants directly and to provide absolute concentrations of TC/g soil. The results of flow cytometry resembled the results obtained with plating. The average transconjugant-to-donor ratios (T/D) reached -5.22 ± 0.23 logs (plating) and -4.01 ± 0.16 logs (flow cytometry), at MS1 (Supplementary Table 1 and 2).

The donor recovery, considered as the difference between observed (recovered) and predicted (expected) abundance, was low (-2.20 ± 0.36 logs). Based on initial inoculum concentration (8.90 logs CFU/mL), it was predicted to detect 7.51 log CFU/g after consideration of all dilution and Nycodenz extraction steps, but only 5.31 ± 0.36 log CFU/g were found (Supplementary Table 1).

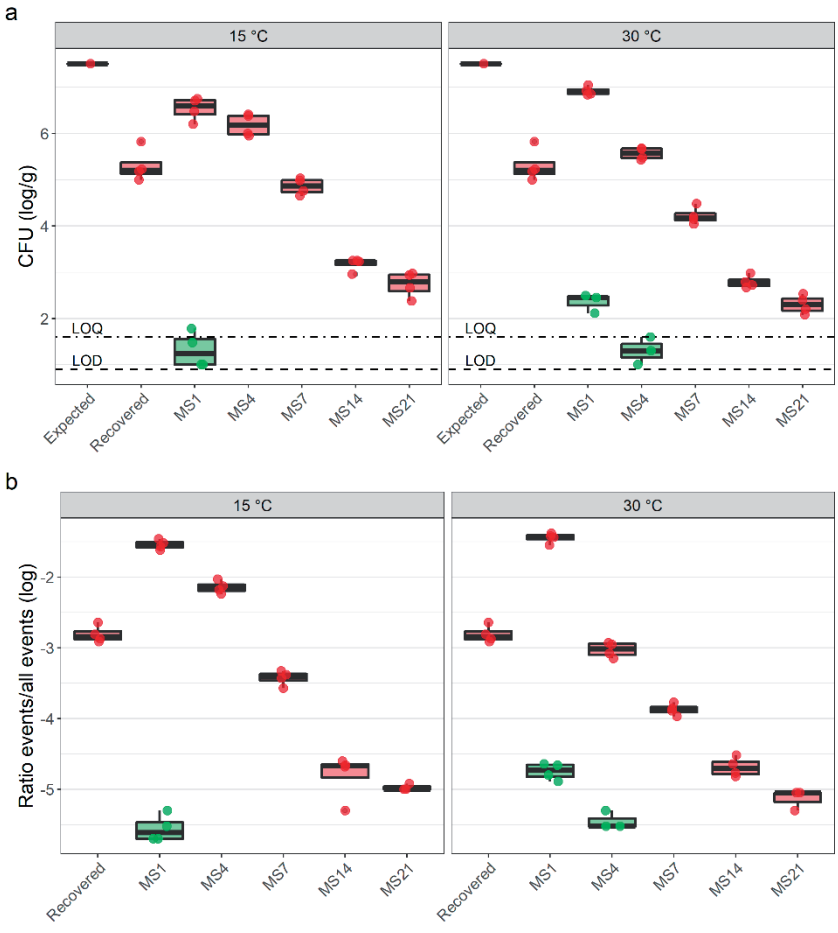


Figure 2. Transconjugants are detected shortly after manure application. Boxplots show the abundance of the donor (red) and transconjugants (green) in manured-soil microcosms determined by plating (a) and flow cytometry (b). Colony-forming units (CFU) of donor and transconjugants were enumerated immediately after manure application (Recovered) and measured after incubation for 1, 4, 7, 14, and 21 days (MS1 – MS21, respectively). Based on initial donor concentrations, 7.51 log CFU/g were spiked (Expected). Flow-cytometry donor and transconjugant counts were normalized by events, and 1 x 10^6 events were quantified per measure.

6.4.3 Phylogenetic analysis of transconjugants

In the transconjugant dataset, 25 ASVs were identified, which represented four major bacteria phyla in a total of 11 families and 11 genera (Figure 3), after excluding the ASVs detected in control samples (Supplementary Figure 3). An overview of the bacterial genera identified in the controls of the transconjugant dataset can be found in Supplementary Figure 4, and the relative abundance of the ASVs found in the controls in microcosms can be found in Supplementary Figure 5.

Both Gram-positive (*Bacillus*) and Gram-negative (*Acinetobacter* and *Comamonas*) were among the most frequently detected genera. From the 25 ASVs detected as transconjugants, two were found in corresponding control soil (*Bacillus* and *Nocardioides*), two others were found in manure (*Comamonas* and *Rahnella*). In contrast, the other 21 were neither detected in control soil nor in manure at any time point or sample. Throughout the experimental timeframe, the genera of *Bacillus* and *Nocardioides* (but not the specific ASV detected in the transconjugants) were found at a low and constant relative abundance (0.036 - 0.073% and 0.002 - 0.026% for *Bacillus* and *Nocardioides*, respectively) in the manured soil microcosms (Figure 3). The genus *Comamonas* was detected in manure and soils after but not before manure application. Once introduced to soils, their relative abundance decreased over time (Figure 3).

While part of the ASVs identified in the transconjugant pools was not found in manure or soils (15 out of 19 ASVs), the genera to which these ASVs corresponded were searched in the microcosm dataset to confirm their probable source. These genera were mainly found in manure and included *Acinetobacter*, *Pseudomonas*, and *Romboutsia* (Figure 3).

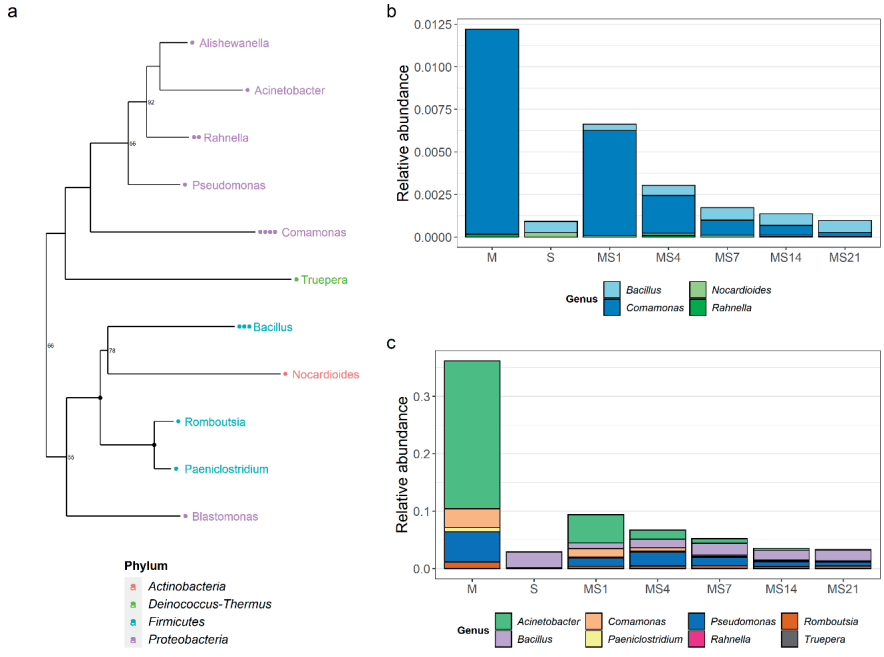


Figure 3. Overview of transconjugant bacterial genera and corresponding relative abundance in the microcosms. The phylogenetic tree shows the transconjugant genera found (a). Bar charts show the replicate-averaged relative abundance of (b) the ASVs of the transconjugants that were also detected in the microcosms, original soil, manure, and relative abundance of (c) the genera of the ASVs identified in the transconjugant pool. The average relative abundance of each genus is depicted in manure samples (M), soils (S), and manured soils at days 1, 4, 7, 14, and 21 (MS1 to MS21, respectively).

6.5 Discussion

In this study, we hypothesized that quantifiable conjugation events with a fecal commensal bacterium (*E. coli*) as the donor would occur in manured soil microcosms and that indigenous soil bacteria would be identified among the *taxa* carrying the transferred plasmid. For being a known fecal source of ARGs that struggles to survive in environmental conditions, *E. coli* is an ideal model host bacterium to study the fate of manure-borne ARGs in the environment.

Nycodenz density gradient centrifugation followed by direct plating and flow-cytometry was used to determine the abundance of conjugation events in manured-soil microcosms. FACS and 16S rRNA gene sequencing assessed the identity and source of the transconjugants (new plasmid hosts). The results confirmed the hypotheses that not only did plasmids in manure bacteria conjugate in a manured soil context, but that native soil bacteria were able to acquire the plasmid.

6.5.1 Manure bacteria conjugate in manured soils

The maximum number of transconjugants in this study corresponded roughly to a transconjugant-to-donor ratio (T/D) of 10^{-4} (transconjugant abundance of 10^2 and 10^3 CFU/g soil), which is similar to ratios found in soils in literature. In early sterile soil studies using *E. coli* as donor, the T/D ratio varied between 10^{-2} and 10^{-4} (transconjugant abundance of 10^2 and 10^3 transconjugant CFU/g soil, respectively) (Krasovsky and Stotzky, 1987; Wein-Berg and Stotzky, 1972), and (Top et al., 1990) reported conjugation ratios of 10^{-4} T/D with an IncP1 plasmid (corresponding to 10^2 transconjugant CFU/g soil) in non-sterile soil, but only when nutrients were added. On the other hand, disparate transconjugant abundances have also been reported. The diversity of experimental set-ups can partly explain the high variability of observed transfer frequencies among studies. Besides the individual donor, recipient, and vector characteristics, most studies were performed under scenarios that do not adequately simulate the complexity found in the environment (e.g., sterile soils, filter mating, nutrient-rich media). Several factors may affect the plasmid transfer frequency in soils, and caution is advised when comparing values between studies. However, despite the variability in observed transconjugant abundance, the findings of the present study, conducted under more complex conditions, are consistent with the findings of the published literature.

The maximum number of transconjugants was obtained shortly after manure application (within the first four days). Similar findings have been reported by (Top et al., 1990), where transconjugants were mainly found shortly after introducing *E. coli* donor strains in soil microcosms. However, depending on the soil type, these results were mainly achieved after the introduction of nutrients. Elsewhere, manure application to soils was responsible

for a 10-fold increment of transconjugants (Götz and Smalla, 1997). Conjugation is known to require energy and cell resources (Goodman et al., 1993; Inoue et al., 2005; Smets et al., 1995), and the findings of the present study corroborate that in the manure-amended soil microcosms, the minimum nutritional requirements for conjugation were met, despite the poor survival of *E. coli* in soils.

In this study, conjugation occurred under more realistic environmental conditions but at similar moderate rates than previously reported experiments. Overall, in the Netherlands alone, over 76 million tons of animal manure are produced every year, most of which is applied untreated on farmlands (WUR, 2019). Manure typically contains 10^5 CFU/g of *E. coli* (Schmitt et al., 2019), which results in the application of roughly 10^{15} *E. coli* CFU to the roughly 1.1 million hectares of grassland (CBS StatLine; https://opendata.cbs.nl/statline/#/CBS/en/). Despite conjugation occurring at low frequencies, the scale of manure application is sufficiently frequent to enable a large number of potential transfer events. However, while IncP1 plasmids are abundant in the environment (Dealtry et al., 2014), they are not so common among *Enterobacteriaceae*, and consequently, are not representative of plasmid families known for their AMR carriages such as IncF, IncI, IncA/C, or IncH (Rozwandowicz et al., 2018). As shown here, environmental conditions and farming practices may promote conditions for conjugation. Manure is commonly applied during the warmer seasons of the year (spring/summer) when the topsoil temperature is also higher (Pepper and Brusseau, 2019). As seen in this study, higher temperatures may result in more transconjugants, and more transconjugants may imply longer plasmid persistence in the bacterial community. Therefore, this study suggests that several requirements and conditions that might facilitate and enhance plasmid persistency and establishment in the soil bacterial community may already be fulfilled.

6.5.2 Manure application leads to plasmid uptake by native soil bacteria

Overall, manure application to soils resulted in the detection of transconjugants, from which several genera were also identified in soil or manure. Among the transconjugant genera, *Bacillus* and *Nocardioides* were traced back to soils with identical ASVs. These genera are ubiquitous and thus commonly found in soils. The presence of members of the order *Bacillales* and the genus *Nocardioides* among transconjugants have been reported in some soil community permissiveness studies (Klümper et al., 2016, 2015), but not in all (Musovic et al., 2014, 2010). Recently, both *Bacillus* and *Nocardioides* have been found in the transconjugant pool of soil microcosms after 5 and 75 days of incubation (Fan et al., 2019), suggesting that maintenance of the acquired plasmid is possible. However, that was not observed in the present study. Although the relative abundance of these two genera remained relatively constant throughout the experimental timeframe, no transconjugants were detected after four days of incubation. Depending on the context, the acquisition of a new plasmid may promote bacterial survival but also reduce the

fitness of the plasmid-carrying host due to an increased metabolic burden (Baltrus, 2013; San Millan and MacLean, 2019). Nevertheless, it is relevant that native soil bacteria can acquire ARG-carrying plasmids from a manure-specific donor, as demonstrated in the current study.

This study used a conjugative IncP1 plasmid, which is considered to be mostly environmental. IncP1 plasmids have been found in high abundances in manure (Binh et al., 2008) and soils (Dealtry et al., 2014), and they were reported to carry genes conferring resistance to multiple antibiotics (e.g., β-lactams, sulfonamides, aminoglycosides, and tetracyclines) (Rozwandowicz et al., 2018). As the name implies, broad-host-range plasmids can be transferred between distinct phylogenetic groups of bacteria, explaining the diversity of bacterial phyla observed among transconjugants. *Acinetobacter* and *Pseudomonas* are common environmental bacteria and have been consistently found in the recovered soil transconjugant pool (Fan et al., 2019; Klümper et al., 2016, 2015; Musovic et al., 2010, 2006). However, in this study, most of the ASVs corresponding to these genera were not further detected in the manured soils. This may be due to abundances below the detection limit. Because the overall number of sorted cells was low and required a re-growth step before sequencing, it is possible that these ASVs were too rare to be detected in the more diverse bacterial community of manure and soil. By tracing the genera in the microcosms, instead of the specific ASVs, it was shown that most of these genera were more abundant in manure than in soil, which was possibly their source.

Due to the low number of transconjugants detected, the transconjugant pool had to be re-grown before sequencing. This re-growth potentially introduced a media bias, and it is possible that other relevant taxa were not detected because of the chosen approach. Nevertheless, it was still shown that manure-introduced plasmids were acquired by native soil bacteria when manure was applied.

6.6 Conclusion

In this microcosm study, *E. coli* representing fecal bacteria successfully transferred a broad host range plasmid to soil and manure bacteria via conjugation. Despite occurring at low frequencies, HGT was observed until the first four days after manure application. Among the new plasmid hosts (transconjugants), *Bacillus* and *Nocardioides* were linked to soils, and *Comamonas* and *Rahnella* were linked to manure. *Acinetobacter* and *Pseudomonas* were identified in the transconjugant pool, but their abundance was probably below the detection limit, as it was not possible to track their specific ASVs in the microcosms.

This study shows that despite constraints posed by environmental conditions such as nutrient and temperature, manure amendment might result in conditions enabling ARG-carrying plasmid transfer from manure to the soil bacterial community. This work highlights the importance of environmental conditions and farm practices in the potential spread of AMR from manure to the wider environment.

6.7 Acknowledgments

The authors gratefully acknowledge Anette Hørdum Løth and Rebeca Pallares-Vega for the technical support and Pieter van Veelen for the supporting role in data analysis.

6.8 Funding

This work was performed in the cooperation framework of Wetsus, European Centre of excellence for sustainable water technology (www.wetsus.nl). Wetsus is co-funded by the Dutch Ministry of Economic Affairs and Ministry of Infrastructure and Environment, the European Union Regional Development Fund, the Province of Fryslân and the Northern Netherlands Provinces. This work has also received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie [grant agreement No. 665874]. The authors like to thank the participants of the research theme Source Separated Sanitation for the fruitful discussions and their financial support.

6.9 Supplementary data

Supplementary Table 1. Donor and transconjugant abundance in soil microcosms, obtained by plating.

Temperature	Time point	Donor		TC		T/D	
		Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
15 °C	Recovered	5.31	0.356				
	MS1	6.54	0.253	1.32	0.384	-5.22	0.227
	MS4	6.18	0.244				
	MS7	4.86	0.180				
	MS14	3.17	0.142				
	MS21	2.74	0.276				
30 °C	Recovered	5.31	0.356				
	MS1	6.92	0.095	2.35	0.209	-4.52	0.248
	MS4	5.56	0.129	1.30	0.300	-4.29	0.427
	MS7	4.22	0.187				
	MS14	2.79	0.136				
	MS21	2.30	0.201				

Notes: colony forming units (log CFUs/g soil) were enumerated for donors and transconjugants (TC) immediately after manure application (Recovered), and measured after incubation at day 1, 4, 7, 14, and 21 (MS1 – MS21, respectively). Transconjugant-to-donor ratios (T/D) were also calculated.

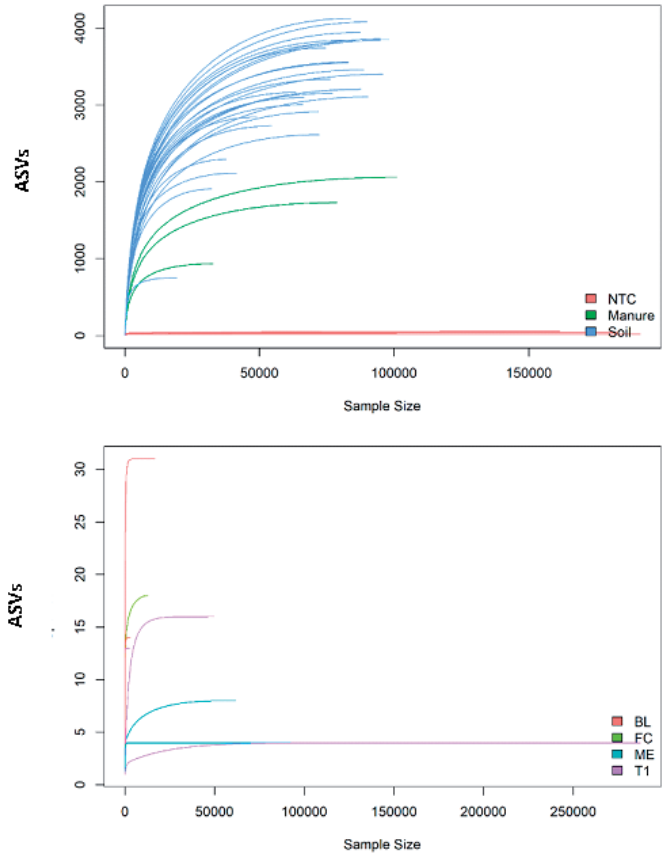
Supplementary Table 2. Donor and transconjugant abundance in soil microcosms, obtained by flow-cytometry.

Temperature	Time point	D/E		T/E		T/D	
		Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
15 °C	Recovered	-2.81	0.119				
	MS1	-1.54	0.069	-5.56	0.19	-4.01	0.158
	MS4	-2.14	0.089				
	MS7	-3.43	0.103				
	MS14	-4.81	0.328				
	MS21	-4.97	0.046				
30 °C	Recovered	-2.81	0.119				
	MS1	-1.45	0.073	-4.75	0.119	-3.30	0.147
	MS4	-3.03	0.105	-5.45	0.127	-2.39	0.181
	MS7	-3.87	0.083				
	MS14	-4.69	0.135				
	MS21	-5.13	0.144				

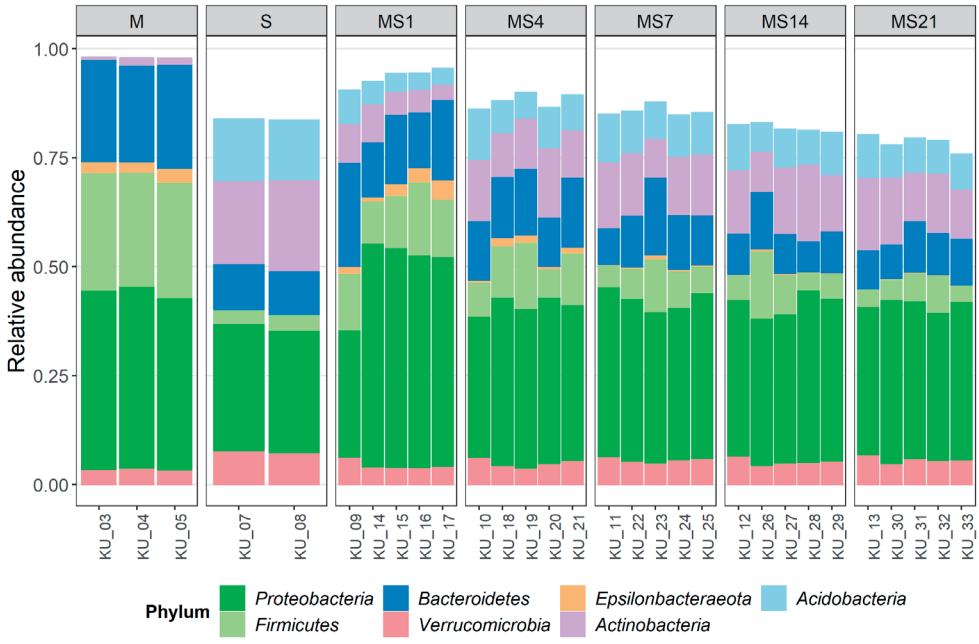
Notes: Donor-to-events (D/E), transconjugant-to-events (T/E), and transconjugant-to-donor (T/D) ratios were calculated based on flow-cytometry data. Counts were normalized by events as, per measurement, 1 x 10^6 events were quantified.

Supplementary Table 3. Averaged alpha diversity indexes, and corresponding standard deviations, of manure (M), soil (S), and manured soils at days 1, 4, 7, 14, and 21 (MS1 to MS21, respectively), grouped by time points. This microcosm series was incubated at 30 °C.

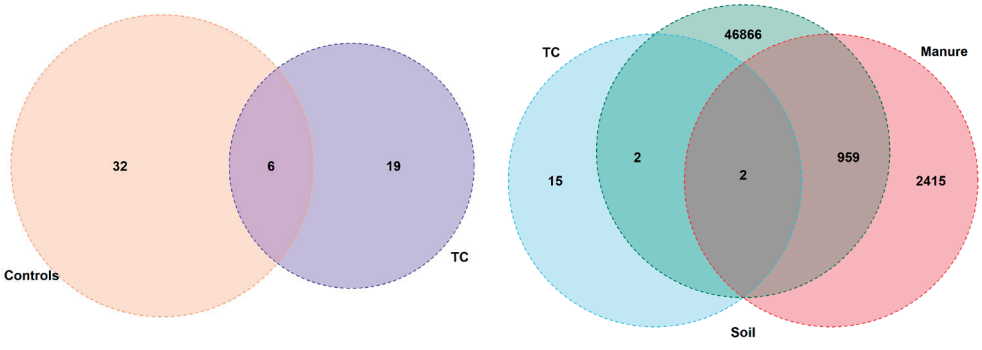
Timepoint	Observed		Chao1		Shannon		Simpson		Evenness	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
M	1420	432	1597	586	5.501	0.234	0.9779	0.0035	0.7618	0.0070
S	2975	54	3366	170	7.217	0.025	0.9986	0.0000	0.9023	0.0011
MS1	2544	229	2877	390	6.041	0.516	0.9498	0.0274	0.7711	0.0719
MS4	2890	611	3267	943	7.084	0.168	0.9980	0.0005	0.8912	0.0143
MS7	3259	126	3821	200	7.202	0.071	0.9981	0.0006	0.8904	0.0067
MS14	3021	236	3509	358	7.121	0.080	0.9983	0.0003	0.8890	0.0096
MS21	2750	506	3164	779	7.024	0.183	0.9980	0.0004	0.8888	0.0048



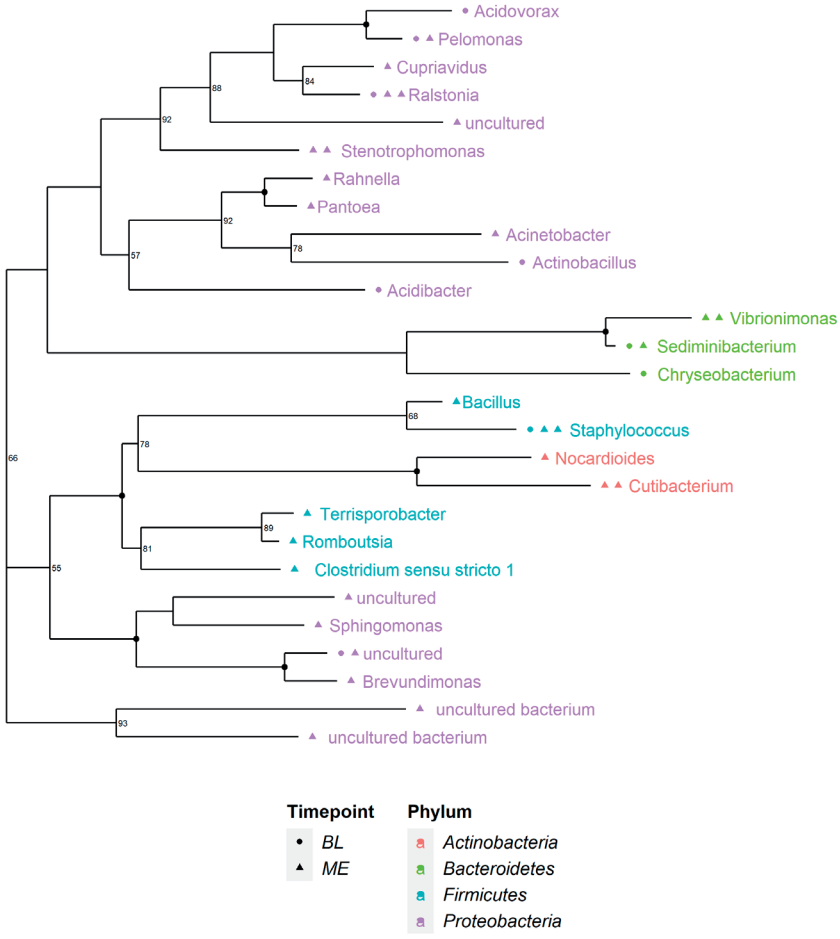
Supplementary Figure 1. Rarefaction curves show high ASV diversity in the microcosms (top), but not in the transconjugant dataset (bottom). In the latter, the ASVs found in samples obtained from re-grown sorted transconjugants (MS1) were compared with the in-line flow cytometry stream (FC) and with the media used for re-growth (ME). In both sequencing runs, blank extractions were included as additional controls (NTC and BL).



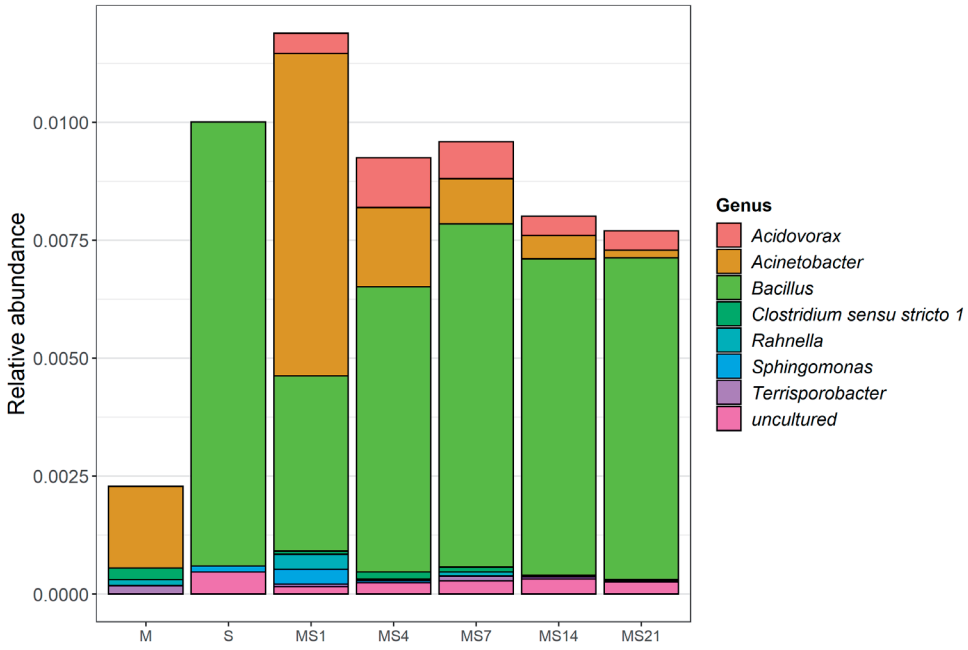
Supplementary Figure 2. The main phyla found in manure became the most abundant in soils after manure application. Bar charts showing the relative abundance in manure samples (M), in soils (S), and in manured soils at days 1, 4, 7, 14, and 21 (MS1 to MS21, respectively).



Supplementary Figure 3. Transconjugant ASVs found in controls were excluded from analysis. Venn diagram showing the number of ASVs found in the control samples and in the samples with transconjugants (TC). The group "controls" refers to a combination of ASVs in the media used for re-growth and the blank extractions.



Supplementary Figure 4. Overview of the bacterial genera found in the controls of the transconjugant dataset. Dendrogram showing the genera found in the controls obtained from the in-line flow-cytometry stream (FC), the media used for re-growth (ME), and the blank extractions (BL). The dendrogram was created using the *phyloseq* package with bootstrap values displayed in the nodes.



Supplementary Figure 5. Relative abundance of control ASVs tracked in the microcosms. Bar plot showing the relative abundance of the genera found in manure samples (M), in soils (S), and in manured soils at days 1, 4, 7, 14, and 21 (MS1 to MS21, respectively).

6.10 References

Amann, R., Ludwig, W., Schleifer, K., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59, 143–169.

Bahl, M.I., Hansen, L.H., Goesmann, A., Sørensen, S.J., 2007. The multiple antibiotic resistance IncP-1 plasmid pKJK5 isolated from a soil environment is phylogenetically divergent from members of the previously established α, β and δ sub-groups. Plasmid 58, 31–43. https://doi.org/10.1016/j.plasmid.2006.11.007

Baltrus, D.A., 2013. Exploring the costs of horizontal gene transfer. Trends Ecol. Evol. 28, 489–495. https://doi.org/10.1016/j.tree.2013.04.002

Banerjee, G., Ray, A.K., Kumar, R., 2016. Effect of temperature on lateral gene transfer efficiency of multi-antibiotics resistant bacterium, alcaligenes faecalis. Sains Malaysiana 45, 909–914.

Bellanger, X., Guilloteau, H., Bonot, S., Merlin, C., 2014. Demonstrating plasmid-based horizontal gene transfer in complex environmental matrices: A practical approach for a critical review. Sci. Total Environ. 493, 872–882. https://doi.org/10.1016/j.scitotenv.2014.06.070

Binh, C.T.T., Heuer, H., Kaupenjohann, M., Smalla, K., 2008. Piggery manure used for soil fertilization is a reservoir for transferable antibiotic resistance plasmids. FEMS Microbiol. Ecol. 66, 25–37. https://doi.org/10.1111/j.1574-6941.2008.00526.x

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A.M., Chase, J., Cope, E.K., Da Silva, R., Diener, C., Dorrestein, P.C., Douglas, G.M., Durall, D.M., Duvallet, C., Edwardson, C.F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J.M., Gibbons, S.M., Gibson, D.L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G.A., Janssen, S., Jarmusch, A.K., Jiang, L., Kaehler, B.D., Kang, K. Bin, Keefe, C.R., Keim, P., Kelley, S.T., Knights, D., Koester, I., Kosciolek, T., Kreps, J., Langille, M.G.I., Lee, J., Ley, R., Liu, Y.X., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B.D., McDonald, D., McIver, L.J., Melnik, A. V., Metcalf, J.L., Morgan, S.C., Morton, J.T., Naimey, A.T., Navas-Molina, J.A., Nothias, L.F., Orchanian, S.B., Pearson, T., Peoples, S.L., Petras, D., Preuss, M.L., Pruesse, E., Rasmussen, L.B., Rivers, A., Robeson, M.S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S.J., Spear, J.R., Swafford, A.D., Thompson, L.R., Torres, P.J., Trinh, P., Tripathi, A., Turnbaugh, P.J., Ul-Hasan, S., van der Hooft, J.J.J., Vargas, F., Vázquez-Baeza, Y., Vogtmann, E., von Hippel, M., Walters, W., Wan, Y., Wang, M., Warren, J., Weber, K.C., Williamson, C.H.D., Willis, A.D., Xu, Z.Z., Zaneveld, J.R., Zhang, Y., Zhu, Q., Knight, R., Caporaso, J.G., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. https://doi.org/10.1038/s41587-019-0209-9

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869

Cantón, R., Coque, T.M., 2006. The CTX-M β-lactamase pandemic. Curr. Opin. Microbiol. https://doi.org/10.1016/j.mib.2006.08.011

Carattoli, A., 2013. Plasmids and the spread of resistance. Int. J. Med. Microbiol. 303, 298–304. https://doi.org/10.1016/j.ijmm.2013.02.001

Davis, N.M., Proctor, Di.M., Holmes, S.P., Relman, D.A., Callahan, B.J., 2018. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome 6, 226. https://doi.org/10.1186/s40168-018-0605-2

Dealtry, S., Ding, G.C., Weichelt, V., Dunon, V., Schlüter, A., Martini, M.C., Del Papa, M.F., Lagares, A., Amos, G.C.A., Wellington, E.M.H., Gaze, W.H., Sipkema, D., Sjöling, S., Springael, D., Heuer, H., Van Elsas, J.D., Thomas, C., Smalla, K., 2014. Cultivation-independent screening revealed hot spots of IncP-1, IncP-7 and IncP-9 plasmid occurrence in different environmental habitats. PLoS One 9, e89922. https://doi.org/10.1371/journal.pone.0089922

Dröge, M., Pühler, A., Selbitschka, W., 1998. Horizontal gene transfer as a biosafety issue: A natural phenomenon of public concern. J. Biotechnol. 64, 75–90. https://doi.org/10.1016/S0168-1656(98)00105-9

Fan, X.T., Li, H., Chen, Q.L., Zhang, Y. Sen, Ye, J., Zhu, Y.G., Su, J.Q., 2019. Fate of antibiotic resistant pseudomonas putida and broad host range plasmid in natural soil microcosms. Front. Microbiol. 10, 1–10. https://doi.org/10.3389/fmicb.2019.00194

Franz, E., Schijven, J., de Roda Husman, A.M., Blaak, H., 2014. Meta-Regression Analysis of Commensal and Pathogenic Escherichia coli Survival in Soil and Water. Environ. Sci. Technol. 48, 6763–6771. https://doi.org/10.1021/es501677c

Goodman, A.E., Hild, E., Marshall, K.C., Hermansson, M., 1993. Conjugative plasmid transfer between bacteria under simulated marine oligotrophic conditions. Appl. Environ. Microbiol. 59, 1035–1040. https://doi.org/10.1128/aem.59.4.1035-1040.1993

Götz, A., Smalla, K., 1997. Manure enhances plasmid mobilization and survival of Pseudomonas putida introduced into field soil. Appl. Environ. Microbiol. 63, 1980–1986. https://doi.org/10.1128/aem.63.5.1980-1986.1997

Henschke, R.B., Schmidt, F.R.J., 1990. Plasmid mobilization from genetically engineered bacteria to members of the indigenous soil microflora in situ. Curr. Microbiol. 20, 105–110. https://doi.org/10.1007/BF02092881

Inoue, D., Sei, K., Soda, S., Ike, M., Fujita, M., 2005. Potential of predominant activated sludge bacteria as recipients in conjugative plasmid transfer. J. Biosci. Bioeng. 100, 600–605. https://doi.org/10.1263/jbb.100.600

Jacquiod, S., Brejnrod, A., Morberg, S.M., Abu Al-Soud, W., Sørensen, S.J., Riber, L., 2017. Deciphering conjugative plasmid permissiveness in wastewater microbiomes. Mol. Ecol. 26, 3556–3571. https://doi.org/10.1111/mec.14138

Jechalke, S., Heuer, H., Siemens, J., Amelung, W., Smalla, K., 2014. Fate and effects of veterinary antibiotics in soil. Trends Microbiol. 22, 536–545. https://doi.org/10.1016/j.tim.2014.05.005

Jiao, Y.-N., Chen, H., Gao, R.-X., Zhu, Y.-G., Rensing, C., 2017. Organic compounds stimulate horizontal transfer of antibiotic resistance genes in mixed wastewater treatment systems. Chemosphere 184, 53–61. https://doi.org/10.1016/j.chemosphere.2017.05.149

Klümper, U., Dechesne, A., Riber, L., Brandt, K.K., Gülay, A., Sørensen, S.J., Smets, B.F., 2016. Metal stressors consistently modulate bacterial conjugal plasmid uptake potential in a phylogenetically conserved manner. ISME J. Accepted, 1–14. https://doi.org/10.1038/ismej.2016.98

Klümper, U., Dechesne, A., Smets, B.F., 2014a. Protocol for Evaluating the Permissiveness of Bacterial Communities Toward Conjugal Plasmids by Quantification and Isolation of Transconjugants. Springer, Berlin, Heidelberg, pp. 275–288. https://doi.org/10.1007/8623_2014_36

Klümper, U., Droumpali, A., Dechesne, A., Smets, B.F., 2014b. Novel assay to measure the plasmid mobilizing potential of mixed microbial communities. Front. Microbiol. 5, 1–9. https://doi.org/10.3389/fmicb.2014.00730

Klümper, U., Riber, L., Dechesne, A., Sannazzarro, A., Hansen, L.H., Sørensen, S.J., Smets, B.F., 2015. Broad host range plasmids can invade an unexpectedly diverse fraction of a soil bacterial community. ISME J. 9, 934–945. https://doi.org/10.1038/ismej.2014.191

Krasovsky, V.N., Stotzky, G., 1987. Conjugation and genetic recombination in Escherichia coli in sterile and nonsterile soil. Soil Biol. Biochem. 19, 631–638. https://doi.org/10.1016/0038-0717(87)90109-X

Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal 17, 10. https://doi.org/10.14806/ej.17.1.200

McMurdie, P.J., Holmes, S., 2013. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS One 8, e61217. https://doi.org/10.1371/journal.pone.0061217

Musovic, S., Dechesne, A., Sorensen, J., Smets, B.F., 2010. Novel Assay To Assess Permissiveness of a Soil Microbial Community toward Receipt of Mobile Genetic Elements. Appl. Environ. Microbiol. 76, 4813–4818. https://doi.org/10.1128/AEM.02713-09

Musovic, S., Klümper, U., Dechesne, A., Magid, J., Smets, B.F., 2014. Long-term manure exposure increases soil bacterial community potential for plasmid uptake. Environ. Microbiol. Rep. 6, 125–130. https://doi.org/10.1111/1758-2229.12138

Musovic, S., Oregaard, G., Kroer, N., Sorensen, S.J., 2006. Cultivation-Independent Examination of Horizontal Transfer and Host Range of an IncP-1 Plasmid among Gram-Positive and Gram-Negative Bacteria Indigenous to the Barley Rhizosphere. Appl. Environ. Microbiol. 72, 6687–6692. https://doi.org/10.1128/AEM.00013-06

Norman, A., Hansen, L.H., Sørensen, S.J., Sorensen, S.J., 2009. Conjugative plasmids: vessels of the communal gene pool. Philos. Trans. R. Soc. B Biol. Sci. 364, 2275–2289. https://doi.org/10.1098/rstb.2009.0037

Ochman, H., Lawrence, J.G., Groisman, E.A., 2000. Lateral gene transfer and the nature of bacterial innovation. Nature 405, 299–304. https://doi.org/10.1038/35012500

Oksanen, J., Blanchet, G.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., 2019. vegan: Community Ecology Package.

Pepper, I.., Brusseau, M.L., 2019. Physical-Chemical Characteristics of Soils and the Subsurface, in: Environmental and Pollution Science. Elsevier, pp. 9–22. https://doi.org/10.1016/B978-0-12-814719-1.00002-1

Pinilla-Redondo, R., Cyriaque, V., Jacquiod, S., Sørensen, S.J., Riber, L., 2018. Monitoring plasmid-mediated horizontal gene transfer in microbiomes: recent advances and future perspectives. Plasmid 99, 56–67. https://doi.org/10.1016/j.plasmid.2018.08.002

Pukall, R., Tschäpe, H., Smalla, K., 1996. Monitoring the spread of broad host and narrow host range plasmids in soil microcosms. FEMS Microbiol. Ecol. 20, 53–66. https://doi.org/10.1016/0168-6496(96)00021-9

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. Nucleic Acids Res. 41, D590–D596. https://doi.org/10.1093/nar/gks1219

R Core Team, 2020. R: A Language and Environment for Statistical Computing.

Rice, E.W., Wang, P., Smith, A.L., Stadler, L.B., 2020. Determining Hosts of Antibiotic Resistance Genes: A Review of Methodological Advances. Environ. Sci. Technol. Lett. acs.estlett.0c00202. https://doi.org/10.1021/acs.estlett.0c00202

Richaume, A., Angle, J.S., Sadowsky, M.J., 1989. Influence of soil variables on in situ plasmid transfer from Escherichia coli to Rhizobium fredii. Appl. Environ. Microbiol. 55, 1730–1734. https://doi.org/10.1128/aem.55.7.1730-1734.1989

Rozwandowicz, M., Brouwer, M.S.M., Fischer, J., Wagenaar, J.A., Gonzalez-Zorn, B., Guerra, B., Mevius, D.J., Hordijk, J., 2018. Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae. J. Antimicrob. Chemother. 73, 1121–1137. https://doi.org/10.1093/jac/dkx488

San Millan, A., MacLean, R.C., 2019. Fitness Costs of Plasmids: A Limit to Plasmid Transmission, in: Microbial Transmission. American Society of Microbiology, pp. 65–79. https://doi.org/10.1128/microbiolspec.MTBP-0016-2017

Schmitt, H., Blaak, H., Hoeksma, P., van de Schans, M., Kemper, M., de Rijk, S., de RodaHusman, A., 2019. Antibioticaresistente bacteriën, resistentiegenen en antibioticaresiduen in mest. https://doi.org/10.21945/RIVM-2019-0112

Scott, K.P., Mercer, D.K., Glover, L.A., Flint, H.J., 2006. The green fluorescent protein as a visible marker for lactic acid bacteria in complex ecosystems. FEMS Microbiol. Ecol. 26, 219–230. https://doi.org/10.1111/j.1574-6941.1998.tb00507.x

Sengeløv, G., Kristensen, K.J., Sørensen, A.H., Kroer, N., Sørensen, S.J., 2001. Effect of Genomic Location on Horizontal Transfer of a Recombinant Gene Cassette Between Pseudomonas Strains in the Rhizosphere and Spermosphere of Barley Seedlings. Curr. Microbiol. 42, 160–167. https://doi.org/10.1007/s002840010197

Smets, B.F., Rittmann, B.E., Stahl, D.A., 1995. Quantification of the effect of substrate concentration on the conjugal transfer rate of the TOL plasmid in short-term batch mating experiments. Lett. Appl. Microbiol. 21, 167–172. https://doi.org/10.1111/j.1472-765X.1995.tb01033.x

Smillie, C., Garcillán-Barcia, M.P., Francia, M.V., Rocha, E.P.C., de la Cruz, F., 2010. Mobility of plasmids. Microbiol. Mol. Biol. Rev. 74, 434–52. https://doi.org/10.1128/MMBR.00020-10

Soda, S., Otsuki, H., Inoue, D., Tsutsui, H., Sei, K., Ike, M., 2008. Transfer of antibiotic multiresistant plasmid RP4 from escherichia coli to activated sludge bacteria. J. Biosci. Bioeng. 106, 292–296. https://doi.org/10.1263/jbb.106.292

Sørensen, S.J., Bailey, M., Hansen, L.H., Kroer, N., Wuertz, S., Sorensen, S., 2005. Studying plasmid horizontal transfer in situ: a critical review. Nat. Rev. Microbiol. 3, 700–710. https://doi.org/10.1038/nrmicro1232

Spencer, S.J., Tamminen, M. V, Preheim, S.P., Guo, M.T., Briggs, A.W., Brito, I.L., A Weitz, D., Pitkänen, L.K., Vigneault, F., Virta, M.Pj., Alm, E.J., 2016. Massively parallel sequencing of single cells by epicPCR links functional genes with phylogenetic markers. ISME J. 10, 427–436. https://doi.org/10.1038/ismej.2015.124

Stalder, T., Press, M.O., Sullivan, S., Liachko, I., Top, E.M., 2019. Linking the resistome and plasmidome to the microbiome. ISME J. 13, 2437–2446. https://doi.org/10.1038/s41396-019-0446-4

Sundberg, C., Al-Soud, W.A., Larsson, M., Alm, E., Yekta, S.S., Svensson, B.H., Sørensen, S.J., Karlsson, A., 2013. 454 pyrosequencing analyses of bacterial and archaeal richness in 21 full-scale biogas digesters. FEMS Microbiol. Ecol. 85, 612–626. https://doi.org/10.1111/1574-6941.12148

Thanner, S., Drissner, D., Walsh, F., 2016. Antimicrobial Resistance in Agriculture. MBio 7, 1–7. https://doi.org/10.1128/mBio.02227-15

Thomas, C.M., Nielsen, K.M., 2005. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nat. Rev. Microbiol. 3, 711–721. https://doi.org/10.1038/nrmicro1234

Top, E., Mergeay, M., Springael, D., Verstraete, W., 1990. Gene escape model: Transfer of heavy metal resistance genes from Escherichia coli to Alcaligenes eutrophus on agar plates and in soil samples. Appl. Environ. Microbiol. 56, 2471–2479. https://doi.org/10.1128/aem.56.8.2471-2479.1990

Top, E.M., De Rore, H., Collard, J.M., Gellens, V., Slobodkina, G., Verstraete, W., Mergeay, M., 1995. Retromobilization of heavy metal resistance genes in unpolluted and heavy metal polluted soil. FEMS Microbiol. Ecol. 18, 191–203. https://doi.org/10.1016/0168-6496(95)00054-7

van Veen, J.A., van Overbeek, L.S., van Elsas, J.D., 1997. Fate and activity of microorganisms introduced into soil. Microbiol. Mol. Biol. Rev. 61, 121–135. https://doi.org/10.1128/.61.2.121-135.1997

Wein-Berg, S.R., Stotzky, G., 1972. Conjugation and genetic recombination of Escherichia coli in soil. Soil Biol. Biochem 4, 171–180.

WHO, 2014. Antimicrobial Resistance: Global Report on Surveillance 2014.

Woerther, P.L., Burdet, C., Chachaty, E., Andremont, A., 2013. Trends in human fecal carriage of extended-spectrum β-lactamases in the community: Toward the globalization of CTX-M. Clin. Microbiol. Rev. https://doi.org/10.1128/CMR.00023-13

WUR, 2019. Manure Management [WWW Document]. URL https://www.wur.nl/en/article/Manure-Management.htm (accessed 9.18.19).

Yu, Y., Lee, C., Kim, J., Hwang, S., 2005. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. Biotechnol. Bioeng. 89, 670–679. https://doi.org/10.1002/bit.20347

CHAPTER 7

"… technology develops cumulatively, rather than in isolated heroic acts, and that it finds most of its uses after it has been invented, rather than being invented to meet a foreseen need."

Jared Diamond

Guns, Germs, and Steel: The Fates of Human Societies, 1997

General Discussion

7.1 Outline

In this thesis, the impact of manure application to agricultural soils on the microbial community and their antimicrobial resistance genes was investigated using two main approaches. Field studies were conducted to identify the main factors that drive antimicrobial resistance genes (ARG) spread from manure to soil and surrounding ditches. Moreover, the dynamics of the microbiome and resistome was studied in manured soils and adjacent water ditches. Simultaneously, laboratory experiments were performed to determine the role of parameters potentially limiting horizontal gene transfer under field-relevant conditions. Simulated microcosms were designed to quantify conjugation and identify the hosts of newly acquired plasmids. Additionally, a new thermophilic anaerobic digestor designed for high phosphate recovery was analyzed regarding its potential in removing ARGs and indicator bacteria. This chapter aims to reflect on and contextualize reported findings in farm practices. Ultimately, this may help define concrete points of action aiming to mitigate AMR spread via manure from livestock farms to the environment.

7.2 Farm context

On average, a dairy cow produces manure roughly at 70 kg/day, and a fattening pig roughly at 3 kg/day (CBS StatLine; https://opendata.cbs.nl/statline/#/CBS/en/). In the Netherlands, these estimates for manure production result in over 76 million tons of animal manure produced every year (WUR, 2019). Consequently, farmers have a very strong incentive to dispose manure, either by applying manure directly to agricultural land or by transfer to land of other farmers or manure treatment.

Current legislations restrict manure application by limiting the amounts of nitrogen and phosphate from all animal manure that farmers may use; by defining how farmers use the manure and the periods in which they can do so; by specifying the conditions for manure transport and storage; and by controlling the number of farm animals by available farmland area (https://business.gov.nl/regulation/animal-manure/). These standards are defined to limit nutrient leaching to environmental water courses, resulting in eutrophication. However, the impact of nutrient amendment on the soil and water resistome is not considered in those standards. Additionally, at present, no standards exist that limit transfer of antibiotic residues or ARGs to soils that result from the use of antimicrobials in livestock production.

7.3 On-farm measures to contain the environmental spread of AMR

7.3.1 Reducing AMU will also reduce the input of ARB and ARGs to soils

It is known that agriculture contributes to the emergence and spread of ARGs in the environment. This contribution starts with animal antimicrobial usage (AMU), where usage is directly linked with the abundance of resistant bacteria in animal's intestines, both at the farm (Luiken et al., 2019; Van Gompel et al., 2019) and national level (de Greef et al., 2019; Levy, 2014; Tang et al., 2017). Partially-metabolized antibiotic residues end up in manure together with resistant bacteria. These are later transported to soils, possibly imposing a selective pressure and increasing the diversity and abundance of ARGs (Jechalke et al., 2014; Thanner et al., 2016). Therefore, by restricting the types of antimicrobials allowed in veterinary practice, as it has been done in practice under Dutch and EU legislation, together with directly reducing the overall AMU in farms, the input of ARGs and resistant bacteria in soil decreases. However, for many reasons, antimicrobials cannot be fully banned (e.g., sick animals require treatment); thus, targeting antimicrobials alone will not lead to the desired reduction of AMR levels. Disease-preventive measures, resulting in prudent AMU application, is currently considered the best policy.

7.3.2 Treating manure before the application will decrease antibiotic and ARG transfer to soils

In chapter 4, a pilot-scale reactor performing thermophilic anaerobic digestion was sampled to quantify its removal capacity of indicator bacteria and selected ARGs. While it was efficient in removing *E. coli*, no ARG removal was evident in both cattle and pig manure. Nevertheless, a diversity of other manure treatment technologies is already available for farmers. From composting to digestion, the efficiency of removal of ARGs depends largely on the treatment, on the manure, but also on the ARG type, where sometimes increase and not removal, is observed (He et al., 2020; Youngquist et al., 2016). The removal of antibiotic residues in manure also depends on treatment conditions, on the animal origin, and the antibiotic class. However, in general, antibiotics have higher removal efficiencies than ARGs (Massé et al., 2014; Spielmeyer, 2018). Currently, no process is considered ideal for general application, and this is probably because livestock waste treatment technologies are not designed to remove ARGs and ARB specifically. Instead, they aim to minimize the loss of nutrients and energy.

Paradoxically, farms tend not to implement manure treatment technologies due to the high initial financial investments required and subsequent high maintenance costs, but this may not be the sole reason. In a survey involving 111 Dutch dairy farms (Gebrezgabher et al., 2015), age and education level of the farmer and farm size were essential variables explaining the likelihood of adoption of manure separation processes. This is a much

simpler technology that is easier to implement and has more direct benefits for the farmer but has little or no effect on the abundance of indicator bacteria or ARGs, as seen in Chapter 4.

Despite the apparent potential farmer's resilience to implement new technologies at farms, the variability observed between studies regarding ARG decrease (and sometimes increase) may discourage efforts to implement waste treatment technologies at farms. Unquestionably, more research is needed to develop or optimize methods that can successfully and stably remove ARGs from manure at acceptable costs. However, farm-specific solutions may be needed, depending on the type and abundance of ARGs that manure contains, which may not be feasible. Manure combines the most relevant horizontal gene transfer (HGT) parameters to occur: the abundance of nutrients and high density and diversity of live bacteria. As ARG presence is not specific for certain *taxon*, manure treatment processes may select bacterial groups that carry these resistance traits or that can acquire them from the other bacteria. Some manure treatment options impose strong selective pressure on the bacterial community, primarily due to anaerobiosis and temperature increase. Ultimately, a complete ARG removal might only be achieved after complete manure sterilization.

7.3.3 Reducing nutrient emissions will prevent the increase in ARG levels and reduce the likelihood of new ARG implementation via HGT

The findings reported throughout this thesis highlight the impact of manure application on soils regarding for the presence of ARGs in the environment. This impact is partly due to the direct transport of ARGs to soil bacteria, but also because manure is rich in nutrients which can enrich the fraction of soil bacteria that harbor ARGs. In chapters 2 and 3, the impact of manure application was measured in a sample of five farms that followed traditional farming practices. It was shown that ARG decay in the environment was strongly influenced by the ARG identity (in soil) and soil texture (in water). Also, the soil bacteriome and resistome showed strong resilience to the input of manure, with the resistome displaying an intensive temporal shift in ARG diversity and abundance after manure application. Simultaneously, despite not affecting the overall bacterial community composition, manure application resulted in the introduction of manure bacteria and the enrichment of fast-growing bacteria.

Manure application to soils stimulates growth of certain bacterial groups (Goldfarb et al., 2011), which creates a disturbance of the overall bacterial community. Fast-growing bacteria (such as γ-Proteobacteria) tend to take part in the primary succession after a disturbance in the bacterial community of soil and aquatic environments (Becerra-Castro et al., 2016; Song et al., 2017; Vadstein et al., 2018). Indeed, it has been shown that manure application to soils can enrich antimicrobial-resistant fast-growing bacteria (Ding et al.,

2014; Hu et al., 2016; Udikovic-Kolic et al., 2014). If such nutrient-based disturbance is kept over time (e.g., multiple manuring rounds, over multiple years), it may result in long-term enrichment of AMR traits in the environment. Multiple long term studies (over ten years) on the impact of soil fertilization of ARG abundance associate the use of manure and inorganic fertilizers with increased ARG levels (Chen et al., 2018; Guo et al., 2018; Rahman et al., 2018; Xie et al., 2018).

In chapter 5, a series of conjugation experiments were performed to assess the impact of temperature and nutrient availability on conjugation events. It was shown that environmental factors, particularly nutrient availability, were responsible for a steep decline in conjugation events. HGT is critical for the spread of AMR, but it is difficult to prevent from occurring because it plays such an important ecological role. Conjugation is generally considered the most relevant mechanism for ARG transfer. Depending on their incompatibility family, mobile plasmids may facilitate transfer to a broad bacterial host range. However, this versatility also comes with a fitness cost that limits plasmid transmission (San Millan and MacLean, 2019), usually referred to as a "metabolic burden." The most apparent consequence of maintaining plasmids is the need for resources (e.g., carbon, nitrogen, phosphorous) and energy to carry out basic cellular processes (Baltrus, 2013). In general, larger genomes imply higher metabolic costs, and conjugative plasmids tend to be quite large (Smillie et al., 2010).

Moreover, in chapter 6, the fate of plasmids over time was assessed in manured soil microcosms. It was shown that ARG transfer between manure and soil bacteria occurred under realistic conditions, but the transconjugants were only observed within the first days after manure application. This short duration of transconjugants may be related with the plasmid fitness cost, which is typically estimated by comparing plasmid-carriers with plasmid-free cells. It has been shown that *de novo* transconjugants had slower growth than lineages that had been replicating for several generations, indicating a plasmid acquisition cost (Prensky et al., 2021). These costs are handicaps, and in the face of such disadvantage, the surrounding environmental conditions may determine the stabilization of newly acquired plasmids in the host. In the end, nutrient-rich manure amendment may provide just the necessary conditions for hosts to maintain their newly acquired plasmids, which may be more difficult in scenarios with lower nutrient availability.

7.4 Method, vision, and meditation – future perspectives

The field of microbiology has always been strongly influenced by methods and technology available because microorganisms cannot be seen by the naked eye, even though their effects on human health can. Historically, whenever a significant methodology breakthrough occurred, a period of scientific discoveries followed. First, microscopic lenses were designed by Anton van Leeuwenhoek (circa 1650s), then artificial culture media were developed by Louis Pasteur (1860), and more recently, with the discovery of molecular methods making use of DNA polymerase by Arthur Kornberg (1959). To date, the majority of diagnostics and experimental designs depend on these concepts and discoveries.

Throughout this thesis, a diverse set of methods and strategies were used to monitor changes in ARG and transconjugant abundance as a response to an environmental disturbance (i.e., manure application). The methodology ranged from more traditional techniques (e.g., plating, conjugation) to molecular-based approaches (e.g., qPCR, sequencing). Strategy wise, the work shown in this thesis started from focusing on individual targets and moved to a broader view on AMR. In a way, this trend can also be seen in published scientific literature.

Earlier studies regarding environmental spread of AMR mainly focused on individual aspects of resistance. They tended to target certain bacterial groups or species (e.g., coliforms, enterococci) that were enumerated and isolated, or a small set of ARGs. These studies were mainly limited by the workload involved, study conduct and logistics, and costs of application of certain analytical methodologies. Only more recently, with the price drop of the high-throughput methods and increase in computational capacity, the conditions that were previously limiting study costs were minimized, and it allowed researchers to focus on what is usually referred as "big data". By the Oxford University Press definition, these are "extremely large data sets that may be analyzed computationally to reveal patterns, trends, and associations, especially relating to human behavior and interactions". This "big data" approach changed the way research is done as it enabled engaging in more holistic views by allowing to add more and more complexity (i.e., more variables and samples) to analytical models. Indeed, studies involving high-throughput qPCR (HT-qPCR) or metagenomics are currently trending in the scientific literature. On the other hand, while the amount of data per sample is "big," the number of samples is still relatively modest. Because statistical analytics become more demanding (i.e., with multiple endpoints to be studied), studies also need to be bigger to avoid all kinds of statistical issues (e.g., multiple testing).

Some technologies show much promise in providing substantial new insights in microbial life, and particularly, in AMR ecology. Interestingly, most of these approaches were initially conceived for clinical purposes and later adapted to environmental studies. For example, the first targeted metagenomics approach was designed for diagnosis of human inherited diseases (Ng et al., 2009) and only later applied to AMR with the ARG enrichment technique ResCap (Lanza et al., 2018). Similarly, HT-qPCR was initially designed to target pathogens and virulence factors (Stedtfeld et al., 2008) and only later adapted to quantify ARGs in environmental samples (Looft et al., 2012; Stedtfeld et al., 2018). However, in ecological terms, it is essential to know "who is doing what," and until now, connecting the "who" with the "what" has been one of the major caveats of high-throughput data. While 16S rRNA sequencing covers the "who," shotgun metagenomics (incl. ResCap) and HT-qPCR aim at the "what".

One of the most appealing approaches that is on the rise is the Chromosome Conformation Capture, also known as Hi-C. Also coming from the medical field, this method was designed to comprehensively detect chromatin interactions in the mammalian nucleus. Essentially, chromatin is crosslinked with formaldehyde, then digested, and re-ligated in such a way that only DNA fragments that are covalently linked together form ligation products (Belton et al., 2012). The ligation products contain the information of not only where they originated from in the genomic sequence but also where they reside, physically, in the 3D organization of the genome. Regarding AMR, this technique allows for the reconstruction of plasmid-host associations and the *in situ* host range identification of ARGs, plasmids, and integrons by physically linking them to their host chromosomes (Stalder et al., 2019). Alternatively, epicPCR (Emulsion, Paired Isolation and Concatenation PCR) provides a simple way to partition bulk reactions into millions of individual reactions, each within a single droplet. Emulsion-based approaches have been used to study human haplotypes from single cells and single-cell immunology (Turchaninova et al., 2013; Turner and Hurles, 2009) because they can link functional genes and phylogenetic markers in uncultured single cells. The potential applications include identifying functional community members, tracing horizontal gene transfer networks and mapping ecological interactions between microbial cells (Spencer et al., 2016).

As stated earlier, Microbiology is a field strongly dependent of method innovation. One significant source of such innovation is the medical field, and much potential is expected from the methods that were mentioned. However, conducting sound research is not about having plenty of data, or using trendy methods. One should always be critical on the research question, the adequate experimental design required to answer the research question, (quality of) sample collection, data analysis and conclusions. It is relevant to mention, as well, that the handling and analysis of such amounts of data is only possible because of the advances in computational processing but also due to complex

mathematical modeling that enables understanding of the data. Without these different elements, the acquired data would be useless.

Future AMR research will need to integrate individual bacterial properties with their ecological context. The questions will be more about "when who is doing what", and to answer it, multiple methods will need to be used to complement the gaps inherent to each method used. Conceptually, this means understanding the ubiquitism of AMR and that it is not eradicable. Instead, it should be manageable to levels that minimize "spillover" events. Additionally, focusing on the vectors of resistance (e.g., plasmids) as entities of independent biology and similar importance as their bacterial hosts will provide key insights in mitigation of AMR spread.

7.5 Final remarks

As mentioned earlier, AMR is currently considered as an issue that requires a One Health approach (Robinson et al., 2016), where human health is associated with animal and environmental health leading to multi-faceted approaches. Nevertheless, anthropogenic activities, in this case livestock production and associated manure application, are at the core of this relationship. Undoubtedly, agricultural farms and their practices are significant variables in AMR spread. Remarkably, livestock production is traditionally seen as a natural activity and the waste/emissions originating from this activity are seen as organic forms of waste that are processed in natural processes. However, there is nothing natural in livestock production. For example, the animal species are highly domesticated and bred for industrial purposes, and the overwhelming production of both products (e.g., meat, milk) and waste (e.g., manure) is not seen in any "natural" system. For soil amendment purposes, manure is regulated in terms of nutrient emissions, but not regarding pathogens or ARGs. While the allowed manure emission values limit nitrate contamination and water eutrophication, they still enrich for soil resident bacteria possibly carrying ARGs and may facilitate ARG implementation via HGT. Therefore, not only legislation should be stricter regarding nutrient emissions, but it also restricts ARG emissions. It is essential to include AMR in governmental policies.

7.6 References

Baltrus, D.A., 2013. Exploring the costs of horizontal gene transfer. Trends Ecol. Evol. 28, 489–495. https://doi.org/10.1016/j.tree.2013.04.002

Becerra-Castro, C., Macedo, G., Silva, A.M.T., Manaia, C.M., Nunes, O.C., 2016. Proteobacteria become predominant during regrowth after water disinfection. Sci. Total Environ. 573, 313–323. https://doi.org/10.1016/j.scitotenv.2016.08.054

Belton, J.-M., McCord, R.P., Gibcus, J.H., Naumova, N., Zhan, Y., Dekker, J., 2012. Hi–C: A comprehensive technique to capture the conformation of genomes. Methods 58, 268–276. https://doi.org/10.1016/j.ymeth.2012.05.001

Chen, Q.L., An, X.L., Zheng, B.X., Ma, Y.B., Su, J.Q., 2018. Long-term organic fertilization increased antibiotic resistome in phyllosphere of maize. Sci. Total Environ. 645, 1230–1237. https://doi.org/10.1016/j.scitotenv.2018.07.260

de Greef, S., Mouton, J., Schoffelen, A., Verduin, C., 2019. NethMap 2019: Consumption of antimicrobial agents and antimicrobial resistance among medically important bacteria in the Netherlands / MARAN 2019: Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands in 2018 251.

Ding, G., Radl, V., Schloter-Hai, B., Jechalke, S., Heuer, H., Smalla, K., Schloter, M., 2014. Dynamics of Soil Bacterial Communities in Response to Repeated Application of Manure Containing Sulfadiazine. PLoS One 9, e92958. https://doi.org/10.1371/journal.pone.0092958

Gebrezgabher, S.A., Meuwissen, M.P.M., Kruseman, G., Lakner, D., Oude, A.G.J.M., 2015. Factors influencing adoption of manure separation technology in the Netherlands. J. Environ. Manage. 150, 1–8. https://doi.org/10.1016/j.jenvman.2014.10.029

Goldfarb, K.C., Karaoz, U., Hanson, C.A., Santee, C.A., Bradford, M.A., Treseder, K.K., Wallenstein, M.D., Brodie, E.L., 2011. Differential growth responses of soil bacterial taxa to carbon substrates of varying chemical recalcitrance. Front. Microbiol. 2. https://doi.org/10.3389/fmicb.2011.00094

Guo, T., Lou, C., Zhai, W., Tang, X., Hashmi, M.Z., Murtaza, R., Li, Y., Liu, X., Xu, J., 2018. Increased occurrence of heavy metals, antibiotics and resistance genes in surface soil after long-term application of manure. Sci. Total Environ. 635, 995–1003. https://doi.org/10.1016/j.scitotenv.2018.04.194

He, Y., Yuan, Q., Mathieu, J., Stadler, L., Senehi, N., Sun, R., Alvarez, P.J.J., 2020. Antibiotic resistance genes from livestock waste: occurrence, dissemination, and treatment. npj Clean Water 3, 4. https://doi.org/10.1038/s41545-020-0051-0

Hu, H.W., Han, X.-M.M., Shi, X.Z., Wang, J.T., Han, L.L., Chen, D., He, J.-Z.Z., 2016. Temporal changes of antibiotic-resistance genes and bacterial communities in two contrasting soils treated with cattle manure. FEMS Microbiol. Ecol. 92, 1–13. https://doi.org/10.1093/femsec/fiv169

Jechalke, S., Heuer, H., Siemens, J., Amelung, W., Smalla, K., 2014. Fate and effects of veterinary antibiotics in soil. Trends Microbiol. 22, 536–545. https://doi.org/10.1016/j.tim.2014.05.005

Lanza, V.F., Baquero, F., Martínez, J.L., Ramos-Ruíz, R., González-Zorn, B., Andremont, A., Sánchez-Valenzuela, A., Ehrlich, S.D., Kennedy, S., Ruppé, E., van Schaik, W., Willems, R.J., de la Cruz, F.,

Coque, T.M., 2018. In-depth resistome analysis by targeted metagenomics. Microbiome 6, 11. https://doi.org/10.1186/s40168-017-0387-y

Levy, S., 2014. Reduced Antibiotic Use in Livestock: How Denmark Tackled Resistance. Environ. Health Perspect. 122. https://doi.org/10.1289/ehp.122-A160

Looft, T., Johnson, T.A., Allen, H.K., Bayles, D.O., Alt, D.P., Stedtfeld, R.D., Sul, W.J., Stedtfeld, T.M., Chai, B., Cole, J.R., Hashsham, S.A., Tiedje, J.M., Stanton, T.B., 2012. In-feed antibiotic effects on the swine intestinal microbiome. Proc. Natl. Acad. Sci. 109, 1691–1696. https://doi.org/10.1073/pnas.1120238109

Luiken, R.E.C., Van Gompel, L., Munk, P., Sarrazin, S., Joosten, P., Dorado-García, A., Borup Hansen, R., Knudsen, B.E., Bossers, A., Wagenaar, J.A., Aarestrup, F.M., Dewulf, J., Mevius, D.J., Heederik, D.J.J., Smit, L.A.M., Schmitt, H., Graveland, H., VanEssen, A., Gonzalez-Zorn, B., Moyano, G., Sanders, P., Chauvin, C., David, J., Battisti, A., Caprioli, A., Blaha, T., Wadepohl, K., Brandt, M., Hald, T., Ribeiro Duarte, A.S., Wasyl, D., Skarzyńska, M., Zajac, M., Daskalov, H., Saatkamp, H.W., Stärk, K.D.C., 2019. Associations between antimicrobial use and the faecal resistome on broiler farms from nine European countries. J. Antimicrob. Chemother. 74, 2596–2604. https://doi.org/10.1093/jac/dkz235

Massé, D., Saady, N., Gilbert, Y., 2014. Potential of Biological Processes to Eliminate Antibiotics in Livestock Manure: An Overview. Animals 4, 146–163. https://doi.org/10.3390/ani4020146

Ng, S.B., Turner, E.H., Robertson, P.D., Flygare, S.D., Bigham, A.W., Lee, C., Shaffer, T., Wong, M., Bhattacharjee, A., Eichler, E.E., Bamshad, M., Nickerson, D.A., Shendure, J., 2009. Targeted capture and massively parallel sequencing of 12 human exomes. Nature 461, 272–276. https://doi.org/10.1038/nature08250

Prensky, H., Gomez-Simmonds, A., Uhlemann, A., Lopatkin, A.J., 2021. Conjugation dynamics depend on both the plasmid acquisition cost and the fitness cost. Mol. Syst. Biol. 17, 1–15. https://doi.org/10.15252/msb.20209913

Rahman, M.M., Shan, J., Yang, P., Shang, X., Xia, Y., Yan, X., 2018. Effects of long-term pig manure application on antibiotics, abundance of antibiotic resistance genes (ARGs), anammox and denitrification rates in paddy soils. Environ. Pollut. 240, 368–377. https://doi.org/10.1016/j.envpol.2018.04.135

Robinson, T.P., Bu, D.P., Carrique-Mas, J., Fèvre, E.M., Gilbert, M., Grace, D., Hay, S.I., Jiwakanon, J., Kakkar, M., Kariuki, S., Laxminarayan, R., Lubroth, J., Magnusson, U., Thi Ngoc, P., Van Boeckel, T.P., Woolhouse, M.E.J., 2016. Antibiotic resistance is the quintessential One Health issue. Trans. R. Soc. Trop. Med. Hyg. https://doi.org/10.1093/trstmh/trw048

San Millan, A., MacLean, R.C., 2019. Fitness Costs of Plasmids: A Limit to Plasmid Transmission, in: Microbial Transmission. American Society of Microbiology, pp. 65–79. https://doi.org/10.1128/microbiolspec.MTBP-0016-2017

Smillie, C., Garcillán-Barcia, M.P., Francia, M.V., Rocha, E.P.C., de la Cruz, F., 2010. Mobility of plasmids. Microbiol. Mol. Biol. Rev. 74, 434–52. https://doi.org/10.1128/MMBR.00020-10

Song, H.K., Song, W., Kim, M., Tripathi, B.M., Kim, H., Jablonski, P., Adams, J.M., 2017. Bacterial strategies along nutrient and time gradients, revealed by metagenomic analysis of laboratory microcosms. FEMS Microbiol. Ecol. 93. https://doi.org/10.1093/femsec/fix114

Spencer, S.J., Tamminen, M. V, Preheim, S.P., Guo, M.T., Briggs, A.W., Brito, I.L., A Weitz, D., Pitkänen, L.K., Vigneault, F., Virta, M.Pj., Alm, E.J., 2016. Massively parallel sequencing of single cells by epicPCR links functional genes with phylogenetic markers. ISME J. 10, 427–436. https://doi.org/10.1038/ismej.2015.124

Spielmeyer, A., 2018. Occurrence and fate of antibiotics in manure during manure treatments: A short review. Sustain. Chem. Pharm. 9, 76–86. https://doi.org/10.1016/j.scp.2018.06.004

Stalder, T., Press, M.O., Sullivan, S., Liachko, I., Top, E.M., 2019. Linking the resistome and plasmidome to the microbiome. ISME J. 13, 2437–2446. https://doi.org/10.1038/s41396-019-0446-4

Stedtfeld, R.D., Baushke, S.W., Tourlousse, D.M., Miller, S.M., Stedtfeld, T.M., Gulari, E., Tiedje, J.M., Hashsham, S.A., 2008. Development and Experimental Validation of a Predictive Threshold Cycle Equation for Quantification of Virulence and Marker Genes by High-Throughput Nanoliter-Volume PCR on the OpenArray Platform. Appl. Environ. Microbiol. 74, 3831–3838. https://doi.org/10.1128/AEM.02743-07

Stedtfeld, R.D., Guo, X., Stedtfeld, T.M., Sheng, H., Williams, M.R., Hauschild, K., Gunturu, S., Tift, L., Wang, F., Howe, A., Chai, B., Yin, D., Cole, J.R., Tiedje, J.M., Hashsham, S.A., 2018. Primer set 2.0 for highly parallel qPCR array targeting antibiotic resistance genes and mobile genetic elements. FEMS Microbiol. Ecol. 94. https://doi.org/10.1093/femsec/fiy130

Tang, K.L., Caffrey, N.P., Nóbrega, D.B., Cork, S.C., Ronksley, P.E., Barkema, H.W., Polachek, A.J., Ganshorn, H., Sharma, N., Kellner, J.D., Ghali, W.A., 2017. Restricting the use of antibiotics in food-producing animals and its associations with antibiotic resistance in food-producing animals and human beings: a systematic review and meta-analysis. Lancet Planet. Heal. 1, e316–e327. https://doi.org/10.1016/S2542-5196(17)30141-9

Thanner, S., Drissner, D., Walsh, F., 2016. Antimicrobial Resistance in Agriculture. MBio 7, 1–7. https://doi.org/10.1128/mBio.02227-15

Turchaninova, M.A., Britanova, O. V., Bolotin, D.A., Shugay, M., Putintseva, E. V., Staroverov, D.B., Sharonov, G., Shcherbo, D., Zvyagin, I. V., Mamedov, I.Z., Linnemann, C., Schumacher, T.N., Chudakov, D.M., 2013. Pairing of T-cell receptor chains via emulsion PCR. Eur. J. Immunol. 43, 2507–2515. https://doi.org/10.1002/eji.201343453

Turner, D.J., Hurles, M.E., 2009. High-throughput haplotype determination over long distances by haplotype fusion PCR and ligation haplotyping. Nat. Protoc. 4, 1771–1783. https://doi.org/10.1038/nprot.2009.184

Udikovic-Kolic, N., Wichmann, F., Broderick, N.A., Handelsman, J., 2014. Bloom of resident antibiotic-resistant bacteria in soil following manure fertilization. Proc. Natl. Acad. Sci. U. S. A. 111, 15202–7. https://doi.org/10.1073/pnas.1409836111

Vadstein, O., Attramadal, K.J.K., Bakke, I., Olsen, Y., 2018. K-Selection as Microbial Community Management Strategy: A Method for Improved Viability of Larvae in Aquaculture. Front. Microbiol. 9. https://doi.org/10.3389/fmicb.2018.02730

Van Gompel, L., Luiken, R.E.C., Sarrazin, S., Munk, P., Knudsen, B.E., Hansen, R.B., Bossers, A., Aarestrup, F.M., Dewulf, J., Wagenaar, J.A., Mevius, D.J., Schmitt, H., Heederik, D.J.J., Dorado-García, A., Smit, L.A.M., Graveland, H., van Essen, A., Gonzalez-Zorn, B., Moyano, G., Sanders, P., Chauvin, C., David, J., Battisti, A., Caprioli, A., Blaha, T., Wadepohl, K., Brandt, M., Hald, T., Duarte, A.S.R., Wasyl, D., Skarżyńska, M., Zając, M., Hoszowski, A., Daskalov, H., Saatkamp, H.W., Stärk, K.D.C., 2019. The antimicrobial resistome in relation to antimicrobial use and biosecurity in pig farming, a metagenome-wide association study in nine European countries. J. Antimicrob. Chemother. 74, 865–876. https://doi.org/10.1093/jac/dky518

WUR, 2019. Manure Management [WWW Document]. URL https://www.wur.nl/en/article/Manure-Management.htm (accessed 9.18.19).

Xie, W.-Y., Yuan, S.-T., Xu, M.-G., Yang, X.-P., Shen, Q.-R., Zhang, W.-W., Su, J.-Q., Zhao, F.-J., 2018. Long-term effects of manure and chemical fertilizers on soil antibiotic resistome. Soil Biol. Biochem. 122, 111–119. https://doi.org/10.1016/j.soilbio.2018.04.009

Youngquist, C.P., Mitchell, S.M., Cogger, C.G., 2016. Fate of Antibiotics and Antibiotic Resistance during Digestion and Composting: A Review. J. Environ. Qual. 45, 537. https://doi.org/10.2134/jeq2015.05.0256

CHAPTER 8

"… we are shaped by the luck of the draw, the circumstances in which we grew up, and the education we received. But we all have two sets of very powerful tools that we completely control: our eyes and our ears. Watching others, listening to their advice, and reading about people are three of the best things I ever did."

Alex Ferguson

Leading: Learning from Life and My Years at Manchester United, 2015

APPENDIX

8.1 Summary

Antimicrobial resistance (AMR) is a growing and significant challenge to global public health. Currently, AMR is approached from a "One Health" perspective, which includes exploring the occurrence of AMR in animals, humans, the environment, and its transmission between. Large amounts of manure are produced from livestock at the farm level, and one of the most common ways to dispose of such high amounts of manure is using it as a soil fertilizer. However, manure application to soils also results in the transport of antibiotic-resistant bacteria (ARB), antibiotic resistance genes (ARGs), and partially metabolized antibiotics, which results in environmental contamination of manured soils and surface water. This thesis investigated the impact of manure application on agricultural soils on the microbial community and their antimicrobial resistance genes.

In **Chapter 2**, the soil texture's role in ARG dynamics in manured soils and surrounding surface waters was evaluated. Six dairy farms with distinct soil textures (clay, sand, and peat) were sampled at different time points after the application of manure, and three representative ARGs *sul1*, *erm(B)*, and *tet(W)* were quantified with qPCR. Manuring initially increased ARG levels in both soils and surface waters, which gradually declined over time. The results demonstrated that rather than showing similar decay dynamics, factors such as the type of ARG and soil texture drive the ARG persistence in the environment.

In **Chapter 3**, high-throughput sequencing and qPCR for 16S rRNA gene fragments and ResCap were used to explore the dynamics of bacteria and ARGs introduced to soils and adjacent water ditches, both at community and individual scale, over three weeks. Overall, the soil bacteriome and resistome showed strong resilience to the input of manure, as manuring did not impact the overall structure of the bacteriome, and its effects on the resistome were transient. Initially, manure application resulted in a substantial increase of ARGs in soils and adjacent waters while not affecting the overall bacterial community composition. Still, specific families increased after manure application, either through the input of manure or through enrichment after manuring. Depending on the type of ARG, manure application resulted mainly in an increase but occasionally also in a decrease of the absolute abundance of ARG clusters. The results showed that the bacteriome and resistome structures are shaped by different factors, where the bacterial community composition could not explain the changes in ARG diversity or abundances. Additionally, it was also highlighted the potential of applying targeted metagenomic techniques, such as ResCap, to study the fate of AMR in the environment.

In **Chapter 4**, the ARG removal potential of a manure treatment process using a newly designed up-flow anaerobic sludge bed reactor operating at thermophilic conditions was examined. The abundances of indicator bacteria *Escherichia coli* and sulfite-reducing

Clostridia (SRC) and the ARGs *sul1*, *erm(B)*, and *tet(W)* were quantified at different stages containing the liquid fraction of either cattle or swine manure. The results confirmed that an extensive removal was achieved for *E. coli* in both manure types, SRC removal was only observed in cattle manure, and no ARG removal was evident in both manure types. This work highlighted the need to consider other parameters besides nutrient recovery, such as AMR when developing innovative technology to handle animal waste, as the abundance of the selected ARGs was not reduced.

In **Chapter 5**, the potential effect of temperature and nutrient availability in the conjugation frequency between *E. coli* strains was assessed. Matings were performed under optimal laboratory conditions and environmentally mimicking temperature or nutrient regimes. Under optimal nutrient conditions and temperature, the recipients yielded the highest transfer frequencies and decreasing the mating temperatures to psychrophilic ranges led to lower conjugation events. Low nutritive media caused more accentuated decreases in transconjugants. This work highlighted that fecal organisms may still be able to transfer plasmids in the environment despite less-than-optimal conditions.

In **Chapter 6**, the fate of plasmids in manured soil microcosms introduced by fecal microorganisms over time was investigated. The transfer of an IncP1 plasmid to the indigenous microbial community in agricultural soil after manure application was quantified by plating and flow cytometry. New plasmid hosts were identified under conditions resembling environmental conditions by fluorescence-activated cell sorting followed by 16S rRNA gene sequencing. Overall, transconjugants were only observed within the first four days of incubation, and higher temperature enhanced the number of transconjugants detected but still close to the detection limits of the experimental system. Among the transconjugant pool, genera whose source was traced from soils and manure were found. These results highlighted the importance of environmental conditions and farm practices on the spread of AMR, and they show that gene transfer occurs at detectable levels and under realistic conditions in soils.

Finally, in **Chapter 7**, the findings reported throughout the thesis were discussed. The results were contextualized from a farm perspective, and action points were suggested to reduce the farm burden in environmental AMR spread. Additionally, insights on the future of AMR research were provided.

8.2 Nederlandse samenvatting

Antimicrobiële resistentie (AMR) is een belangrijke en toenemende uitdaging voor de mondiale volksgezondheid. Momenteel wordt AMR benaderd vanuit een "One Health"-perspectief, wat inhoudt dat het vóórkomen van AMR bij dieren, mensen, het milieu en de overdracht tussen deze reservoirs wordt onderzocht. Door de veestapel worden grote hoeveelheden mest geproduceerd, welke wordt gebruikt als meststof voor de bodem. Het uitrijden van mest op de bodem resulteert echter ook in het transport van antibiotica-resistente bacteriën (ARB), antibiotica-resistentiegenen (ARGs) en gedeeltelijk gemetaboliseerde antibiotica, wat resulteert in milieuverontreiniging van bemeste aardes en oppervlaktewater. In dit proefschrift is onderzocht wat de invloed is van het uitrijden van mest op landbouwgronden op de microbiële gemeenschap en hun antimicrobiële resistentiegenen.

In **Hoofdstuk 2** werd de rol van de bodemtextuur op de ARG dynamiek in bemeste aardes en het omringende oppervlaktewater onderzocht. Zes melkveehouderijen met verschillende grondsoorten (klei, zand, en veen) werden bemonsterd op verschillende tijdstippen na het uitrijden van mest, en drie representatieve ARGs *sul1*, *erm(B)*, en *tet(W)* werden met qPCR gekwantificeerd. De bemesting verhoogde aanvankelijk de ARG-niveaus in zowel de bodem als het oppervlaktewater, na verloop van tijd daalden de niveaus weer geleidelijk. De dynamiek varieerde per grondsoort en gen. De resultaten laten dus zien dat factoren zoals het type ARG en de bodemtextuur de ARG persistentie in het milieu bepalen.

In **Hoofdstuk 3** werd de dynamiek van bacteriën en ARGs onderzocht die in de bodem en aangrenzende wateren werden geïntroduceerd. Daarvoor werden moleculaire technieken, namelijk high-throughput sequencing en qPCR voor 16S rRNA genfragmenten en ResCap, gebruikt zowel op gemeenschaps- als op individuele schaal. De bemesting had geen invloed op de algemene samenstelling van de bacteriëngemeenschap (bacterioom). Toch namen specifieke families toe na mesttoediening, hetzij door de inbreng van mest, hetzij door verrijking na bemesting. Aanvankelijk resulteerde de mestafgifte in een aanzienlijke toename van het aantal ARG's in de bodem en de aangrenzende wateren, maar dit effect was van voorbijgaande aard. Afhankelijk van het type ARG resulteerde mesttoediening voornamelijk in een toename, maar soms ook in een afname van de absolute abundantie van ARG-clusters. De resultaten toonden aan dat de bacterioom- en resistoomstructuren door bemesting in sterk verschillende mate beïnvloed worden, en dat de samenstelling van de bacteriegemeenschap de veranderingen in ARG-diversiteit of -abundanties hier niet kon verklaren. Daarnaast werd ook het potentieel aangetoond van gerichte metagenomische technieken, zoals ResCap, voor het bestuderen van de ontwikkeling van AMR in het milieu.
In **Hoofdstuk 4** werd de mate van verwijdering van ARG van een mestverwerkingsproces onderzocht, namelijk van een nieuw ontworpen up-flow anaerobe slibbed reactor die werkt onder thermofiele condities. De indicatorbacteriën *Escherichia coli* en sulfietreducerende

Clostridia (SRC) en de ARGs *sul1*, *erm(B)*, en *tet(W)* werden gekwantificeerd in verschillende stadia van de reactor in de vloeibare fractie van ofwel runder- of varkensmest. De resultaten toonden aan dat een hoge mate van verwijdering werd bereikt van *E. coli* in beide mestsoorten, terwijl SRC-verwijdering alleen werd waargenomen in rundermest, en voor beide mestsoorten geen ARG-verwijdering gevonden werd. Uit dit werk bleek de noodzaak om bij de ontwikkeling van innovatieve technologie voor de verwerking van dierlijk afval naast de terugwinning van nutriënten ook andere parameters in beschouwing te nemen, zoals AMR, aangezien de concentratie van de geselecteerde ARG's niet verminderde.

In **Hoofdstuk 5** werd het mogelijke effect van temperatuur en beschikbaarheid van nutriënten op de overdracht van genetisch materiaal, oftewel de conjugatiefrequentie, tussen *E. coli*-stammen bestudeerd. Conjugatieproeven werden uitgevoerd onder optimale laboratorium condities en onder regimes die representatief zijn voor milieu-omstandigheden, wat betreft de temperatuur en nutriënten. Onder optimale voedingsomstandigheden en temperatuur werden de hoogste overdrachtsfrequenties gevonden, en het verlagen van de temperatuur leidde tot lagere conjugatiegebeurtenissen. Voedingsarme media veroorzaakten een sterke daling van het aantal transconjuganten. Dit werk heeft aangetoond dat fecale organismen nog steeds plasmiden in het milieu kunnen overdragen, ondanks de suboptimale omstandigheden.

In **Hoofdstuk 6** werd het lot van plasmiden in bemeste aarde onderzocht waarin fecale micro-organismen werden geïntroduceerd. De overdracht van een IncP1 plasmide naar de inheemse microbiële gemeenschap werd gekwantificeerd met behulp van kweektechnieken en flowcytometrie. Onder voor het milieu representatieve omstandigheden werden nieuwe gastheren geïdentificeerd, dit door middel van fluorescentie-geactiveerde celsortering gevolgd door 16S rRNA-gensequencing. Transconjuganten werden alleen waargenomen in de eerste vier dagen van incubatie, en een hogere temperatuur verhoogde het aantal waargenomen transconjuganten, wat desondanks nog steeds dicht bij de detectielimiet van het experimentele systeem lag. In de transconjuganten werden genera aangetroffen waarvan als herkomst de bodem en mest kon worden afgeleid. Deze resultaten benadrukken het belang van bemesting en milieuomstandigheden op de verspreiding van AMR, en ze tonen aan dat genoverdracht op detecteerbare niveaus en onder realistische omstandigheden in de bodem voorkomt.

Tenslotte werden in **Hoofdstuk 7** de bevindingen besproken die in het gehele proefschrift werden gerapporteerd. De resultaten werden gecontextualiseerd vanuit het perspectief van het landbouwbedrijf, en er werden actiepunten voorgesteld om de impact van het landbouwbedrijf voor de verspreiding van AMR in het milieu te verminderen. Daarnaast werden inzichten in de toekomst van AMR onderzoek gegeven.

8.3 Acknowledgments

A Ph.D. is often considered a journey of self-discovery and personal growth. However, this journey is not traveled alone, and it would not be possible for me to complete it without the guidance and support of several people. In this section, their contribution will be acknowledged, and I hope I do not fail to mention anyone. If that is the case, my apologies.

I would like to start by thanking my co-promotors and promotors for their support throughout this Ph.D. **Heike**, you gave me the opportunity to obtain a Ph.D. degree. You inspired me with a completely different supervision strategy than I have always been used to. Thank you for guiding me and encouraging me to do more, particularly when I lacked the faith and vision to continue. **Lucia**, I am thankful for the Portuguese "5-min" conversations. With you, I learned how to adapt my language to less technical terms, and it helped me communicate better. **Dik** and **Dick**, it was an honor to work under your guidance. I am grateful for your sharp critical overview and teaching me to see the bigger picture instead of focusing exclusively on details. **Peter**, our conversations and discussions were always appreciated, and I treasure how you helped me keep the farmer's perspective.

I also want to thank all the Source Separated Sanitation theme participants for the innumerous fruitful discussions and suggestions, particularly to the other PhDs on the AMR research group and Wetsus MicroLab staff. **Ilse**, **Eri**, **Alicia**, **Dana**, **Bianca**, **Aga**, **Bert**, **Inez**, and **Pieter**, thank you for your support, help, and time. Most accomplishments often overlook the importance of simple suggestions and discussions, but while "invisible," these steps are crucial to reach the destination, and your contribution to my work and growth was essential. **Rebeca**, the words are scarce, and they would not do justice to what you mean to me. You are a "refreshing" friend, and I doubt anyone will truly understand what we have been through together. Thank you for being my paranymph.

I am also extremely grateful to my students **Harm**, **Esther**, **Lydia**, **Xavier**, **Adrian**, **Robin**, **Hana**, **Gonzalo**, **Wesley**, **Yede**, and **Jenneke**. Your contribution to this thesis is unquestionable, and each in your own way, you inspired me to become a better researcher and supervisor. I hope you hold these memories in the same regard as I do.

To my beloved officemates and friends **Philipp**, **Terica**, **Wiecher**, **Andrew**, **Gerwin**, **Victor A**, **Thomas**, **Shuyana**, **Swarupa**, **Catarina**, **Ruizhe**, and **Danielle**, we made the kingdom of 1.18C (canteen side + **Wokke**) the best place to be at Wetsus. Our office BBQs (regardless of the weather) are unforgettable and one of the most precious memories of my time at Wetsus. Do not worry; all those gossips and weird conversations are still safe with me!

I also want to thank all my colleagues at Wetsus for the shared terrific experiences. **Hakan**, **Raquel**, **Natascha**, **Sandra**, **Qingdian**, **Janneke D**, **Angel**, **Rita**, **Mariana**, **João**, **Olga**, **Rik**, **Paulina**, **Karine**, … you make Wetsus unique. A lovely and monstrous thank you to the legendary band members **Ricardo**, **Stan**, **Fabian**, **Jorrit**, **Louis**, **Prashanth**, **Sofia**, **Victor T**, **Pau**, **Brahzil**, **Steffen**, **Maarten**, **Wokke**, and *la squadra* **Carlo**, **Ettore**, **Michele**, and **Mariagiorgia**. Those practices and performances were worth every sweat and effort we put in! We rocked the place, and as goes the tradition: I love you, guys!

Besides music, I am passionate about sports. I want to extend my gratitude and thank all the team members of all futsal competitions **Ricardo**, **Chris**, **Carlo**, **Thomas**, **Hector**, **Paraschos**, **Filipe**, **Geert-Jan**, **Angel**, **Cesar**, **Jaap**, **Aziz**, and **Advait**. Despite my competitive temperament, I am very proud of our achievements and for playing alongside you. To my volleyball and Hajraa companions, thank you for your patience, sweat, and passion. I flew half-world to play with you, and I would do it again! **Thomas**, **Hector**, and **Louis**, you made me feel like a captain – "handen in de lucht voor het fuifje"!

Additionally, I would like to thank all my new colleagues of the CoE-Microbiology/AVA, particularly **Mark**, **Marleen**, and **Geert,** for believing in me and helping in my transition to the industry world. I am learning new things every day, and your mentorship has been vital.

I consider myself privileged. New friends and colleagues welcomed me to a new country, but I also had tremendous support from my friends and family in Portugal. **Patrícia**, you are always with me, in my bright and dark days. You are one of the persons I admire the most, and together, we always achieve great things. **Mom**, **Hélder**, **Dad**, **Susana**, thank you for everything you do for me. You taught me the essential things in life and to always aspire to do more. **Rita** and **Mafalda**, thank you for all the bickering, fights, corrections, … and love. You are my sisters, and I owe you much. **Helena**, **Mário**, **Célia**, **Manuel**, and **Mafalda T**, thank you for welcoming me into the family. I will always treasure how you constantly supported me and made me feel as I belong.

Last but not least, my lab peeps, **Ivone**, **Carlos**, and **Sofia**, thank you for chaperoning me in the lab. I learned plenty from you, and I would not be here if it were not for you. **Cristina**, you are deeply missed. To my friends, **Filipa**, **Tiago**, **Filipe**, **Diana**, **Ana**, **Paulo**, **Márcio**, and **Cátia**, I know most of you already since high school, and our dinners and holidays together are always epic. Thank you for being such an important part of my life and helping me survive this Ph.D.

I am a very fortunate and privileged person. I really have an inherited, an adopted, and a chosen family, and I could not do this without you. Thank you!

8.4 About the author

Gonçalo Macedo was born on the 9th of February 1989 in Porto, Portugal. After finishing his degree in Biology at the University of Porto, Gonçalo enrolled at the Catholic University of Portugal (Porto) to pursue an MSc. degree in Applied Microbiology, which he obtained with *magna cum laude* distinction in 2014. During his master thesis, Gonçalo worked on the characterization of AMR-carrying plasmids in *Escherichia coli* strains isolated from wastewater, and soon after his MSc. graduation, he joined the same AMR-research group as a research assistant. There, he worked for two years and participated in national and European projects, including JPI-AMR StARE and the NORMAN network.

In April 2016, Gonçalo moved to the Netherlands to embrace a new chapter in his life and start his doctoral studies in "Infection and immunity" at the Graduate School of Life Sciences, Utrecht University, in a collaborative project with Wetsus, European Center of Excellence for Sustainable Water Technology (Leeuwarden). His doctoral research was focused on the spread of antimicrobial resistance in farmlands, particularly in the effect of manure application to soils and adjacent surface waters, whose results are presented in this thesis. During this research, Gonçalo collaborated with other institutions, including Wageningen Bioveterinary Research Institute (WBVR) and Copenhagen University, where he spent a four-month secondment in 2019.

Currently, Gonçalo is working as a Microbiology specialist at Merck Sharp & Dohme B.V., in Oss.

8.5 List of publications

1) Pallares-Vega, R.; **Macedo, G.**; Brouwer, M. S. M.; Hernandez Leal, L.; van der Maas, P.; van Loosdrecht, M. C. M.; Weissbrodt, D. G.; Heederik, D.; Mevius, D.; Schmitt, H. Temperature and Nutrient Limitations Decrease Transfer of Conjugative IncP-1 Plasmid pKJK5 to Wild Escherichia Coli Strains. Front. Microbiol. **2021**, 12 (July). https://doi.org/10.3389/fmicb.2021.656250.

2) **Macedo, G.**; van Veelen, H. P. J.; Hernandez-Leal, L.; van der Maas, P.; Heederik, D.; Mevius, D.; Bossers, A.; Schmitt, H. Targeted Metagenomics Reveals Inferior Resilience of Farm Soil Resistome Compared to Soil Microbiome after Manure Application. *Sci. Total Environ.* **2021**, *770*, 145399. https://doi.org/10.1016/j.scitotenv.2021.145399.

3) **Macedo, G.**; Hernandez-Leal, L.; van der Maas, P.; Heederik, D.; Mevius, D.; Schmitt, H. The Impact of Manure and Soil Texture on Antimicrobial Resistance Gene Levels in Farmlands and Adjacent Ditches. *Sci. Total Environ.* **2020**, *737*, 139563. https://doi.org/10.1016/j.scitotenv.2020.139563.

4) Sousa, J. M.; **Macedo, G.**; Pedrosa, M.; Becerra-Castro, C.; Castro-Silva, S.; Pereira, M. F. R.; Silva, A. M. T.; Nunes, O. C.; Manaia, C. M. Ozonation and UV254nm Radiation for the Removal of Microorganisms and Antibiotic Resistance Genes from Urban Wastewater. *J. Hazard. Mater.* **2017**, *323* (5), 434–441. https://doi.org/10.1016/j.jhazmat.2016.03.096.

5) Becerra-Castro, C.; **Macedo, G.**; Silva, A. M. T.; Manaia, C. M.; Nunes, O. C. *Proteobacteria* Become Predominant during Regrowth after Water Disinfection. *Sci. Total Environ.* **2016**, *573* (573), 313–323. https://doi.org/10.1016/j.scitotenv.2016.08.054.

6) Moreira, N. F. F.; Sousa, J. M.; **Macedo, G.**; Ribeiro, A. R.; Barreiros, L.; Pedrosa, M.; Faria, J. L.; Pereira, M. F. R.; Castro-Silva, S.; Segundo, M. A.; Manaia, C. M.; Nunes, O. C.; Silva, A. M. T. Photocatalytic Ozonation of Urban Wastewater and Surface Water Using Immobilized TiO_2 with LEDs: Micropollutants, Antibiotic Resistance Genes and Estrogenic Activity. *Water Res.* **2016**, *94*, 10–22. https://doi.org/10.1016/j.watres.2016.02.003.

7) Manaia, C. M.; **Macedo, G.**; Fatta-Kassinos, D.; Nunes, O. C. Antibiotic Resistance in Urban Aquatic Environments: Can It Be Controlled? *Appl. Microbiol. Biotechnol.* **2016**, *100* (4), 1543–1557. https://doi.org/10.1007/s00253-015-7202-0.

8) Nazareth, N.; Magro, F.; Machado, E.; Ribeiro, T. G.; Martinho, A.; Rodrigues, P.; Alves, R.; **Macedo, G. N.**; Gracio, D.; Coelho, R.; Abreu, C.; Appelberg, R.; Dias, C.; Macedo, G.; Bull, T.; Sarmento, A. Prevalence of *Mycobacterium Avium subsp. paratuberculosis* and *Escherichia coli* in Blood Samples from Patients with Inflammatory Bowel Disease. *Med. Microbiol. Immunol.* **2015**, *204* (6), 681–692. https://doi.org/10.1007/s00430-015-0420-3.

9) Varela, A. R.; **Macedo, G. N.**; Nunes, O. C.; Manaia, C. M. Genetic Characterization of Fluoroquinolone Resistant *Escherichia coli* from Urban Streams and Municipal and Hospital Effluents. *FEMS Microbiol. Ecol.* **2015**, *91* (5), fiv015–fiv015. https://doi.org/10.1093/femsec/fiv015.

10) Magro, F.; Santos-Antunes, J.; Albuquerque, A.; Vilas-Boas, F.; **Macedo, G. N.**; Nazareth, N.; Lopes, S.; Sobrinho-Simões, J.; Teixeira, S.; Dias, C. C.; Cabral, J.; Sarmento, A.; Macedo, G. Epstein–Barr Virus in Inflammatory Bowel Disease—Correlation with Different Therapeutic Regimens. *Inflamm. Bowel Dis.* **2013**, *19* (8), 1710–1716. https://doi.org/10.1097/MIB.0b013e318281f31c.