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Department of Biochemistry and Biotechnology



*“Environmental fate and interactions
of veterinary antibiotics with the soil microbial community:
resistance, biodegradation or ecotoxicity?”*

A thesis submitted by

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*Environmental fate and interactions
of veterinary antibiotics with the soil
microbial community:
resistance, biodegradation or ecotoxicity?*

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*Στην Ελπίδα που γεννήθηκε και περπάτησε
μαζί με το διδακτορικό αυτό
αλλά και σε όλα τα όμορφα εκείνα
που ξεκινάνε με την ολοκλήρωσή του.*

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List of abbreviations

VAs	Veterinary Antibiotics
TIA	Tiamulin
TLM	Tilmicosin
SMX	Sulfamethoxazole
AMR	Antimicrobial Resistance
ARGs	Antibiotic Resistance Genes
HGT	Horizontal Gene Transfer
PNR	Potential Nitrification Rate
AOM	Ammonia Oxidizing Microorganisms
AOB	Ammonia Oxidizing Bacteria
AOA	Ammonia Oxidizing Archaea
SFO	Single First Order
HS	Hockey Stick
FOMC	First Order Multi-Compartment
DFOP	Double First Order in Parallel
VFAs	Volatile Fatty Acids
TS	Total Solids
VS	Volatile Solids
ASVs	Amplicon Sequence Variants

Summary

Veterinary antibiotics (VAs) are integral to intensive livestock farming. Global VA consumption comprises approximately 75% of the total antibiotic production superseding the amounts used for the treatment of human infections. After administration, VAs vastly remain unmetabolized and are excreted from the animal through urine and faeces. Hence, soil organic fertilization with animal excreta, as manures, derived from intensive farming leads to systematic exposure of agricultural soils to VA residues and poses a threat to the environment and the public health due to potential: (i) VA toxicity on the soil microbial communities, affecting significant ecosystem functions and services; (ii) the dispersal of antibiotic resistance to microbial community members of soil and associated environments (e.g. plants). Furthermore, the continuous exposure of arable fields to VAs might lead to the enhancement of microbial growth-linked biodegradation, despite the inherent antimicrobial activity of VAs. Despite the copious efforts of the research community, current knowledge on the exposure route of VAs from livestock farms to agricultural settings and the impact of VAs on the microbial communities of soil and associated environments is still scarce, while the potential evolution of growth-linked biodegradation of VAs in agricultural soils is vastly understudied and limited to a few groups of VAs (e.g. sulfonamides).

For addressing these gaps this thesis aimed to: (i) explore the environmental fate of important for intensive livestock farming VAs like tiamulin (TIA) and tilmicosin (TLM), starting from livestock farm and animal excretion kinetics, to faecal material and eventually to agricultural soils; (ii) investigate the potential outcomes of the interactions of VAs and the soil microbial community (biodegradation, toxicity and antimicrobial resistance (AMR) stimulation), focusing on the two understudied VAs like TIA and TLM, in parallel with the relatively well-studied, regarding its soil behavior, VA like sulfamethoxazole (SMX); (iii) determine the efficiency of bioaugmentation using a TIA-degrading bacterial strain as a mitigation method for the decontamination of

contaminated faecal material, comparatively to the commonly employed treatments of stockpiling and anaerobic digestion.

Firstly, we studied the persistence of TIA and TLM along their route from pig administration to fecal excretion and to agricultural soils. We administered TIA and TLM to pigs orally, through feed or water, and assessed: (i) the concentration of the VA residues; (ii) the effect of the administration methods on the excretion kinetics and the temporal excretion pattern of VAs; (iii) the persistence of the VA residues in the manures during downstream treatments of stockpiling and anaerobic digestion; (iv) the VA persistence in soils when applied directly or through VA-fortified manures. VA residues in animal excreta were quantified at levels of 0.55–5.99 mg kg⁻¹ for TIA and 4.27–749.6 mg kg⁻¹ for TLM. Different administration routes (feed/water) lead to different excretion patterns and residual levels for the two VAs. Both VAs showed high persistence during stockpiling (DT₉₀= 19.4–119.3 days for TIA and 77.9–165 days for TLM) and anaerobic digestion (DT₉₀ >365 days). Both antibiotics were very persistent when applied to soils with TLM being the most persistent. Fumigation or incorporation via manure increased their persistence suggesting microbial degradation and enhanced sorption respectively. Overall, the use of TIA- and TLM-contaminated feces as manures is expected to lead to VAs dispersal with unexplored consequences for the environment and human health.

We further investigated the consequences of the regular exposure of soils to VAs with particular focus on the interactions of the soil microbial community with the tested VAs. To address this, we employed a soil microcosm experiment where we repeatedly treated two soils with different pH and VA dissipation capacity (fast vs slow as determined in our earlier work) with the studied VAs (TIA, TLM and SMX), either directly or through fortified manures. This approach resulted in accelerated dissipation of TIA only in the “fast” soil and only upon direct application of the antibiotic. Whereas repeated application resulted in either no changes in the dissipation of SMX, and

retardation of the dissipation of TLM. Ecotoxicological measurements showed that TIA and SMX inhibited potential nitrification rates (PNR), and the abundance of ammonia-oxidizing microorganism (AOM), while TLM presence lead to an overall increase. The presence of VA residues in soil imposes strong structural effects on the community of total prokaryotes and AOM, unlike the fungal and protists communities which were mostly responsive to manure incorporation rather than VAs. SMX stimulated sulfonamide resistance, while manure stimulated antibiotic resistance genes (ARGs) and horizontal gene transfer (HGT). Finally, we were able to identify opportunistic pathogens like *Clostridia*, *Burkholderia-Caballeronia-Paraburkholderia*, and *Nocardioidea* as potential ARG reservoirs in soil. Overall, our findings provided unprecedented evidence about the effects of understudied VAs on soil microbiota and highlight risks posed by the application of VA-contaminated manures in agricultural soils.

Finally, in the last experimental section of the thesis, we assessed the performance of bioaugmentation as a remediation method for reducing the TIA content of faecal material and hence preventing the environmental dispersal of TIA residues. We tested the efficiency of bioaugmentation in pig faecal material fortified with TIA at two concentration levels (representative of the range obtained according to the initial experiment), 5 and 50 mg kg⁻¹, using a previously isolated *Sphingomonas* strain, able to degrade TIA in liquid cultures. For achieving our goals we compared bioaugmentation with stockpiling and anaerobic digestion as the currently used methods for the pre-treatment of faecal material. Our results suggested an overall higher performance of bioaugmentation (DT₅₀ = 32.3 and 66.2 days), compared to stockpiling (DT₅₀ = 95.38 and 113.8 days), and anaerobic digestion (DT₅₀ = 103 and 126.7 days). Interestingly, during anaerobic digestion we observed a hormesis-like low dose induced significant stimulatory effect in biomethanation, which was driven, as

determined by amplicon sequencing analysis, by the increasing abundance of methanogens of the genus *Methanosarcina*.

On the whole, our results highlighted the dispersal potential and negative impact of important for intensive livestock farming antibiotics like SMX, TIA, and TLM, when they end up in agricultural soils through manure application, and, hence, the potential risks for public health. Nevertheless, we have reinforced environmental bioremediation strategies, by demonstrating the power of bioaugmentation for remediating persistent antibiotics in the environment like TIA. This work reinforces the basis set by a, yet, small body of contemporary research, for deeper understanding and problem solving in VA ecotoxicology.

Περίληψη

Τα κτηνιατρικά αντιβιοτικά (ΚΑ) αποτελούν αναπόσπαστο κομμάτι της κτηνοτροφίας. Η παγκόσμια κατανάλωση ΚΑ αποτελεί περίπου το 75% της συνολικής παραγωγής αντιβιοτικών ξεπερνώντας τις ποσότητες που χρησιμοποιούνται για τη θεραπεία ανθρώπινων λοιμώξεων. Μετά τη χορήγηση, τα ΚΑ σε μεγάλο βαθμό δε μεταβολίζονται και απεκκρίνονται από το ζώο μέσω των ούρων και των κοπράνων. Ως εκ τούτου, η οργανική λίπανση του εδάφους με ζωικά περιττώματα, ως κοπριά, που προέρχονται από την κτηνοτροφία οδηγεί σε συστηματική έκθεση των γεωργικών εδαφών σε υπολείμματα ΚΑ και αποτελεί απειλή για το περιβάλλον και τη δημόσια υγεία λόγω πιθανής: (i) τοξικότητας των ΚΑ στις μικροβιακές κοινότητες του εδάφους, που επηρεάζουν σημαντικές λειτουργίες του οικοσυστήματος· (ii) διασποράς της αντοχής στα αντιβιοτικά σε μέλη της μικροβιακής κοινότητας του εδάφους και του άμεσου περιβάλλοντος (π.χ. φυτά). Επιπλέον, η συνεχής έκθεση των γεωργικών εδαφών σε ΚΑ μπορεί να οδηγήσει στην ενίσχυση της βιοαποδόμησης που συνδέεται με τη μικροβιακή ανάπτυξη, παρά την εγγενή αντιμικροβιακή δράση των ΚΑ. Ενώ έγιναν πολυάριθμες προσπάθειες από την ερευνητική κοινότητα, η τρέχουσα γνώση

σχετικά με τον τρόπο που εκτίθενται τα γεωργικά εδάφη στα ΚΑ και τον αντίκτυπο των ΚΑ στις μικροβιακές κοινότητες του εδάφους και του περιβάλλοντος είναι ακόμη περιορισμένη, ενώ η πιθανή εξέλιξη της βιοαποδόμησης που συνδέεται με την ανάπτυξη των ΚΑ σε γεωργικά εδάφη δεν έχει μελετηθεί σε μεγάλο βαθμό και περιορίζεται σε λίγες ομάδες ΚΑ (π.χ. σουλφοναμίδες).

Για την αντιμετώπιση αυτών των κενών στην έρευνα, η παρούσα διατριβή είχε ως στόχο: (i) να διερευνήσει την περιβαλλοντική μοίρα σημαντικών για την κτηνοτροφία ΚΑ όπως η τιαμουλίνη (TIA) και η τιλμικοσίνη (TLM), ξεκινώντας από την κτηνοτροφία και τις ζωικές απεκκρίσεις, μέχρι τα κόπρανα των ζώων και τα γεωργικά εδάφη που εναποτίθενται (ii) να εξετάσει τα πιθανά αποτελέσματα των αλληλεπιδράσεων των ΚΑ και της μικροβιακής κοινότητας του εδάφους (διέγερση βιοαποδόμησης, τοξικότητα και αντιμικροβιακή αντοχή (AMR)), εστιάζοντας στα δύο μη επαρκώς μελετημένα ΚΑ όπως το TIA και το TLM, παράλληλα με τα επαρκώς μελετημένα, όσον αφορά τη συμπεριφορά στο έδαφος, ΚΑ όπως η σουλφαμεθοξαζόλη (SMX). (iii) να ελέγξει την αποτελεσματικότητα της βιοαύξησης χρησιμοποιώντας ένα βακτηριακό στέλεχος που αποδομεί το TIA ως μέθοδο εξυγίανσης μολυσμένων κοπράνων, σε σύγκριση με τις ευρέως χρησιμοποιούμενες μεθόδους της αποθήκευσης σε συνθήκες περιβάλλοντος και αναερόβιας χώνευσης.

Αρχικά, μελετήσαμε την εμμόνη των TIA και TLM κατά τη διάρκεια από τη χορήγηση των χοίρων έως την απέκκριση των κοπράνων και την εφαρμογή στα γεωργικά εδάφη. Χορηγήσαμε TIA και TLM σε χοίρους από το στόμα, μέσω τροφής ή νερού και αξιολογήσαμε: (i) τη συγκέντρωση των υπολειμμάτων των ΚΑ. (ii) την επίδραση των μεθόδων χορήγησης στα επίπεδα απέκκρισης και στο χρονικό μοτίβο απέκκρισης των ΚΑ. (iii) την εμμόνη των υπολειμμάτων των ΚΑ στις κοπριές κατά τη διάρκεια των μεθόδων αποθήκευσης σε συνθήκες περιβάλλοντος και αναερόβιας χώνευσης (iv) την εμμόνη των ΚΑ στα εδάφη όταν εφαρμόζεται απευθείας ή μέσω κοπριών ενισχυμένων με ΚΑ. Τα υπολείμματα των ΚΑ στα ζωικά περιττώματα ποσοτικοποιήθηκαν σε

επίπεδα $0,55-5,99 \text{ mg kg}^{-1}$ για το TIA και $4,27-749,6 \text{ mg kg}^{-1}$ για το TLM. Οι διαφορετικές οδοί χορήγησης (τροφή/νερό) οδήγησαν σε διαφορετικά μοτίβα απέκκρισης και ποσότητες υπολειμμάτων των δύο ΚΑ. Και τα δύο ΚΑ επέδειξαν μεγάλη ανθεκτικότητα κατά τη διάρκεια της αποθήκευσης σε συνθήκες περιβάλλοντος ($DT_{90} = 19,4-119,3$ ημέρες για το TIA και $77,9-165$ ημέρες για το TLM) και την αναερόβια χώνευση ($DT_{90} > 365$ ημέρες). Και τα δύο αντιβιοτικά ήταν πολύ ανθεκτικά όταν εφαρμόστηκαν σε εδάφη με το TLM να είναι το πιο ανθεκτικό. Ο υποκαπνισμός ή η ενσωμάτωση μέσω κοπριάς αύξησε την εμμόνη τους υποδηλώνοντας μικροβιακή αποδόμηση και ενισχυμένη απορρόφηση αντίστοιχα. Συνολικά, η χρήση περιττωμάτων μολυσμένων με TIA και TLM ως κοπριά αναμένεται να οδηγήσει στη διασπορά των ΚΑ με ανεξερεύνητες συνέπειες για το περιβάλλον και την ανθρώπινη υγεία.

Στη συνέχεια, διερευνήσαμε περαιτέρω τις συνέπειες της τακτικής έκθεσης των εδαφών σε ΚΑ δίνοντας ιδιαίτερη έμφαση στις αλληλεπιδράσεις της μικροβιακής κοινότητας του εδάφους με τα υπό μελέτη ΚΑ. Για να πραγματοποιηθεί η μελέτη, κάναμε ένα πείραμα εδαφικών μικροκόσμων όπου εφαρμόσαμε επανειλημμένα τα ΚΑ (TIA, TLM και SMX) σε δύο εδάφη με διαφορετικό pH και ικανότητα αποδόμησης των ΚΑ (γρήγορη έναντι αργής όπως καθορίστηκε στην προηγούμενη εργασία μας), είτε απευθείας είτε μέσω ενισχυμένες κοπριές. Αυτή η προσέγγιση είχε ως αποτέλεσμα την επιταχυνόμενη βιοαποδόμηση του TIA μόνο στο «γρήγορο» έδαφος και μόνο με την άμεση εφαρμογή του αντιβιοτικού. Ενώ η επαναλαμβανόμενη εφαρμογή οδήγησε είτε σε βιοαποδόμηση χωρίς καμία αλλαγή για το SMX είτε σε καθυστέρηση της βιοαποδόμησης για το TLM. Οικοτοξικολογικές μετρήσεις έδειξαν ότι το TIA και το SMX ανέστειλαν τον ρυθμό νιτροποίησης (PNR) και την αφθονία των μικροοργανισμών που οξειδώνουν την αμμωνία (AOM), ενώ η παρουσία του TLM οδήγησε σε συνολική αύξηση. Η παρουσία υπολειμμάτων των ΚΑ στο έδαφος επέβαλλε ισχυρές δομικές επιδράσεις στην κοινότητα των προκαρυωτών και των AOM, σε αντίθεση με τις

κοινότητες των μυκήτων και πρωτίστων που ανταποκρίθηκαν κυρίως στην ενσωμάτωση κοπριάς αντί για τα ΚΑ. Το SMX διεγείρει την ανθεκτικότητα στα σουλφοναμίδια, ενώ η κοπριά διεγείρει τα γονίδια ανθεκτικότητας στα αντιβιοτικά (ARGs) και την οριζόντια μεταφορά γονιδίων (HGT). Τέλος, μπορέσαμε να εντοπίσουμε παθογόνα όπως τα *Clostridia*, *Burkholderia-Caballeronia-Paraburkholderia* και *Nocardioide*s που δρουν ως πιθανές δεξαμενές ARG στο έδαφος. Συνολικά, τα ευρήματά μας παρείχαν πρωτοφανή στοιχεία σχετικά με τις επιδράσεις μη μελετημένων ΚΑ στη μικροχλωρίδα του εδάφους ενώ παράλληλα, τόνισαν τους κινδύνους που ενέχει η εφαρμογή μολυσμένης με ΚΑ κοπριάς σε γεωργικά εδάφη.

Στην τελευταία πειραματική ενότητα της διατριβής, αξιολογήσαμε την απόδοση της βιοαύξησης ως μια μέθοδο εξυγίανσης του ΤΙΑ στα κόπρανα και ως εκ τούτου την παρεμπόδιση της περιβαλλοντικής διασποράς των υπολειμμάτων ΤΙΑ. Δοκιμάσαμε την αποτελεσματικότητα της βιοαύξησης σε ενισχυμένα με ΤΙΑ κόπρανα χοίρου σε δύο επίπεδα συγκέντρωσης (αντιπροσωπευτικά της ποσότητας που μετρήθηκε στο αρχικό πείραμα), 5 και 50 mg kg⁻¹, χρησιμοποιώντας ένα στέλεχος *Sphingomonas* που είχε απομονωθεί προηγουμένως, ικανό να αποδομήσει το ΤΙΑ σε υγρές καλλιέργειες. Για την επίτευξη των στόχων μας, συγκρίναμε τη βιοαύξηση με την αποθήκευση σε συνθήκες περιβάλλοντος και την αναερόβια χώνευση ως τις επί του παρόντος χρησιμοποιούμενες μεθόδους για την προεπεξεργασία των κοπράνων. Τα αποτελέσματά μας έδειξαν μια συνολικά υψηλότερη απόδοση της βιοαύξησης (DT₅₀ = 32,3 και 66,2 ημέρες), σε σύγκριση με την αποθήκευση σε περιβαλλοντικές συνθήκες (DT₅₀ = 95,38 και 113,8 ημέρες) και την αναερόβια χώνευση (DT₅₀ = 103 και 126,7 ημέρες). Άξιο αναφοράς είναι ότι κατά τη διάρκεια της αναερόβιας χώνευσης παρατηρήσαμε μια σημαντική διεγερτική επίδραση στην παραγωγή βιομεθανίου, ορμώμενη από τη χαμηλή δόση ΤΙΑ που εφαρμόσαμε, η οποία ήταν αποτέλεσμα,

όπως προσδιορίστηκε από την ανάλυση αλληλούχισης, της αυξημένης αφθονίας μεθανιογόνων του γένους *Methanosarcina*.

Συνολικά, τα αποτελέσματά μας τόνισαν τη δυνατότητα διασποράς και τον αρνητικό αντίκτυπο των σημαντικών για την κτηνοτροφία αντιβιοτικών όπως τα SMX, TIA και TLM, όταν αυτά καταλήγουν σε γεωργικά εδάφη μέσω της εφαρμογής κοπριάς, και, ως εκ τούτου, τους πιθανούς κινδύνους για τη δημόσια υγεία. Ωστόσο, ενισχύσαμε τις περιβαλλοντικές στρατηγικές βιοαποκατάστασης, επιδεικνύοντας τη δύναμη της βιοαύξησης για την αποκατάσταση έμμενων αντιβιοτικών στο περιβάλλον, όπως το TIA. Αυτή η εργασία ενισχύει τη βάση που τέθηκε από ένα μικρό κομμάτι της σύγχρονης ερευνητικής κοινότητας, για βαθύτερη κατανόηση και επίλυση προβλημάτων στην οικοτοξικολογία των ΚΑ.

Chapter 1

Introduction

1. Veterinary Antibiotic Use In Livestock Production

VAs are chemical compounds with antibacterial properties, used therapeutically and preventively, but also as growth factors (Johnston 1998; Lees et al., 2020). Therapeutic utilization refers to the treatment of bacterial diseases afflicting individual animals or groups of diseased animals. Prophylactic use aims at containing the spread of a disease and preventing illness prior to the occurrence of clinical signs. The final application of VAs pertains to performance enhancement, including improvement of health by boosting the growth rate, feed conversion and yield. This is achieved by providing VAs continuously at subtherapeutic levels as feed additives. The mechanisms linked with growth promotion are poorly understood, nevertheless, associated hypotheses include the overall control of pathogenic microorganisms, the reduction of essential nutrients catabolism by the VA-controlled intestinal microflora, and possible antibiotic direct/indirect effects on the physiology/morphology of the intestinal epithelium (Butaye et al. 2003; Plata et al., 2003).

VAs made their first appearance alongside antibiotics used for human diseases in the 1920s when penicillin was discovered to combat mastitis (Lees et al., 2020). However, the first record of VA administrations was in 1937 when sulfonamides were used to treat bovine mastitis (Lees et al., 2020). Following mastitis, other diseases such as calf pneumonia and poultry coccidiosis were significantly limited due to antibiotic administration (Jones, 1946; Campbell, 2008). Overall, VA presence signified the intensification of livestock farming, allowing low/middle income countries to meet the growing demands for food derived from animal biomass, following the rising average incomes (Van Boeckel et al., 2015). Modeling on VA use place some countries with rising (undergoing intensification of farming) or established economies among the top users of VAs globally, over the total share of 99,502 metric tons of active ingredient (73 % of the total production of antibiotics), with this number predicted to increase by 8 % to 107,472 tons by 2030 (Mulchandani et al., 2023). Specifically, China, Brazil,

India and the United states of America alone account for more than 50 % of the VA global use.

Regardless of antibiotic success, in the early stages of their discovery there were many concerns for human health implications caused by VAs. Next to that, both human and livestock antibiotic misuse/overuse taking place ever since antibiotics were mass produced, have been considered as the drivers of utmost importance for AMR (a set of mechanisms employed by microorganisms to tolerate/resist the effects of antimicrobials/antibiotics) selection/evolution (Holmes et al., 2016). This was proven through the spread of non-naturally occurring resistance to synthetic antibiotics over the last decades. Nevertheless, the development of resistance to antibiotics in patients at hospitals was originally considered to be unrelated to VA utilization in farms (Lees et al., 2020). Up until the 1960s it was believed that AMR selection in livestock farming was a local problem that will vanish after VA withdrawal. Notably, contemporary knowledge suggested vertical as the sole mode of resistance transfer (Lees et al., 2020). In 1958–1960, researchers have concluded that resistance selection occurring on livestock farms could also harm humans through zoonotic infectious diseases, and in 1965, a scientific paper proved that horizontal resistance transfer (the major mode of AMR dispersal) could occur between animal and human associated bacteria (Anderson and Datta, 1965; Smith, 1968; 1969; 1970; 1971). In 1968, a committee was established for examining the banning of the use of VAs as growth promoters and revoked the legislation allowing non-prescribed antibiotic use (Lees et al., 2020).

EU has banned antibiotic use as feed additive in 2006, USA followed in 2017 while China banned the use of VAs as growth promoters in 2019 (legislation effective by 2020) (Prescott, 2017; Kirchhelle, 2018; Lees et al., 2020). In the EU, despite this banning, unrestricted preventive and therapeutic use of antibiotics kept the administration levels high. Preventive use was recently restricted to individual animals (effective from Jan 28, 2022) within the context of the European One Health Action Plan, after the success of banning of preventive antibiotic use in livestock by the Danish

government since 2011 (More, 2020). The results of the regulatory amendments, however, will require some time to be evaluated.

1.1 Benefits and Risks of Veterinary Antibiotic Use

Antibiotic use in farm animals has been beneficial for the animal wellbeing and human prosperity. In the early stages of antibiotic use in livestock production, important infections like mastitis and pneumonia were treated with significant recovery rates. The benefits of VA use were apparent in several occasions, for example during World War II, farm productivity was enhanced due to minimization of the costly caregiving for diseased animals (Lees et al., 2020). In addition, weight gain and overall growth promotion was achieved through low dosage VA administration rather than the implementation of expensive vitamin B12 supplements (Gustafson, 1991; Jones & Ricke, 2003). Claims exist that growth promotion or preventive use associated VA consumption, fulfilled the needs for affordable animal protein for the middle and low-income countries (Hao et al., 2014) leading to safer and cost-effective food production. Nonetheless, there is not enough evidence supporting these claims (Landers et al., 2012).

Excessive VA consumption, on the other hand, can harm the environment and public health. The most prevalent complication of VA misuse is antibiotic resistance dispersal in human associated microbiota/microbial-pathogens either directly (e.g. through consumption of livestock products carrying antibiotic resistant bacteria) or indirectly (e.g. through environmental microbiota of the anthroposphere affected by VAs, like e.g. animal excreta receiving agricultural soils) (Landers et al., 2012). The adverse effects of AMR dispersal have been recorded since a long time ago. For example, in 1968, many food-borne deaths of children were recorded due to ineffective antibiotic therapies (Lees, 2020). More recently, the outcome of antibiotic misuse was compared with the effects of nuclear technology misuse through nuclear weapons

testing (Spagnolo et al., 2021), while WHO reports (World Health Organization, 2014; World Health Organization, 2020) state that “A post-antibiotic era – in which common infections and minor injuries can kill – far from being an apocalyptic fantasy, is instead a very real possibility for the 21st century”. To this end, the European Medicines Agency’s environmental risk assessments in 2016 on use of VAs, eventually led to the enforcement of associated regulations and guidelines by the EU in 2018 (Directive 2001/82/EC of the European Parliament, 2018).

Besides AMR selection, another understudied, albeit important, aspect is that of VA ecotoxicity. Beneficial microorganisms may be affected by antibiotic environmental release, as happens in cases of persistent VA compounds in animal’s body that reach the environment via animal excreta release (Perruchon et al., 2022). It is therefore necessary to include comprehensive risk assessment studies for receiving environments in order to preserve the ecosystem services we currently enjoy. Such risk assessment studies are currently largely missing (Murray et al., 2021). Current norms preprend for environmental risk assessment of veterinary medicines are looking, only at Tier II level, on the effects in aquatic and terrestrial ecosystems including though a restricted set of bioindicators (Felis et al., 2020; Murray et al., 2021). This set does not include thorough testing of the effects of VAs on soil microorganisms, relying on the rather crude and outdated OECD 216 N transformation test (Martin – Laurent et al., 2013) and ignores AMR, despite the acknowledged impact of antibiotics on soil microorganisms (Grenni et al., 2018; Murray et al., 2021). Particularly considering the vast amounts of animal excreta used as organic fertilization in agriculture as described in the following sections.

1.2 Categories of Veterinary Antibiotics

Antibiotics are typically classified based on their (i) chemical structure (sometimes denoting the organism of origin), (ii) the level of their ability to inhibit growth or kill cells, (iii) their origin (natural, modified natural or semi-synthetic, synthetic), (iv) their mechanism of action, (v) their spectrum of activity or target specificity, (vi) their clinical use, (vii) their administration route (Calderon and Sabundayo 2007; Nemeth et al., 2015; Smith et al., 2015; Cheng et al., 2020; Yang et al., 2021; Stojković et al., 2023). Respectively with the aforementioned classifications antibiotics can: (i) be grouped according to their chemical structures like β -lactams (e.g. penicillins, cephalosporins), cephamycins, aminoglycosides, quinolones, sulfonamides, tetracyclines, macrolides, polymyxins, pleuromutilins (Mendes and Antunes, 2013); (ii) be bactericidal (they kill bacteria like aminoglycosides, penicillins, cephalosporins, fluoroquinolones, glycopeptides) or bacteriostatic (they inhibit bacterial growth like lincosamides, macrolides, sulphonamides) (Bernatová et al., 2013); (iii) be of natural origin (e.g. several penicillins, streptomycins, chloramphenicols, and tetracyclines), semi-synthetic (e.g. several cephalosporins), synthetic (e.g. fluoroquinolones, chalcones), (iv) act on cell walls (β -lactams like penicillins, cephalosporins), membranes (polymyxins), protein expression (tetracyclins, macrolides), essential biosynthetic pathways (sulfonamides, trimethoprim act on folate biosynthesis), DNA replication (fluoroquinolones), RNA biosynthesis (actinomycin, rifampin) (O'Rourke et al., 2020); (v) being of narrow (e.g. some penicillins, macrolides, vancomycins) or broad (e.g. sulfonamides, fluoroquinolones, macrolides, tetracyclines) spectrum of activity considering target taxa (Ory and Yow, 1963; Melander et al., 2018); (vi) be of clinical use specific e.g. for mastitis (e.g. β -lactams, tetracyclines, gentamycins), or calf diarrhea (e.g. β -lactams, tetracyclines, sulfonamides, trimethoprim) (Eyler and Shvets, 2019); (vii) be administered orally through feed, via injection, via infusion through secretion glands/natural-openings (thus affecting their metabolism and persistence

through the animal body prior excretion) (Zhang et al., 2013; Kim and De Jesus, 2023). Sales of antibiotic classes recorded by the European Medicines Agency (EMA) considering the mg of active substance per population corrected units (PCU; sales normalized according to the overall EU population) are ranked as follows according to the late EMA report (EMA, 2022): penicillins (β -lactams) 31.2 %, tetracyclines 25.8 %, sulfonamides 9.9 %, macrolides 8.5 %, aminoglycosides 5.9 %, lincosamides 4.7 %, fluoroquinolones 2.8 %, polymyxins 2.6 %, pleuromutilins 2.6 %, trimethoprim 1.6 %. In that same report, Greece resides in the above average zone as far as all active substances are concerned and calculated in mg PCU⁻¹, with 108.8 mg PCU⁻¹ over an EU average of 84.4 mg PCU⁻¹.

The EMA has recently advised the European commission on the revision of the categorization of antibiotic classes for veterinary use in the European Union, within the context of existing regulation (EMA, 2020; Schmerold et al., 2023). Based on the use and line of defense of these antibiotics in human medicine, in order to avoid AMR risks and preserve their efficacy, the Antimicrobial Advice *ad hoc* Expert Group (AMEG) has defined four categories of VAs in that document: category A includes antibiotics that should be avoided in veterinary medicine, in consistency with previous EMA classifications, and can exceptionally be used only in non-food producing animals (e.g. companion animals) under special conditions in compliance with the existing associated EU directive 2001/82/EC and Regulation (2019/6); category B refers to restricted use VAs including highest priority critical importance antibiotics (HPClAs) of the preexisting classification systems except for those classes included in category A; category C includes antibiotics for which alternatives exist in human medicine and can be used with caution; category D encompass lower AMR risk antibiotics that, however, should be prudently used (Schmerold et al., 2023; selection of appropriate antibiotic, use minimization, proper record keeping).

Table 1. Summary of the AMEG categorisation adopted by EMA (European Medicines Agency, 2020).

AMEG Categories	Antibiotic class, subclasses	Example of antibiotic(s)
Category A ("Avoid")	Aminopenicillins	mecillinam, pivmecillinam
	Carbapenems	meropenem, doripenem
	Other cephalosporins§ and penems (ATC code J01DI), including combinations of 3rd-generation cephalosporins with beta-lactamase inhibitors	ceftobiprole, ceftaroline, ceftolozane-tazobactam, faropenem
	Glycopeptides	Vancomycin
	Glycylcyclines	Tigecycline
	Ketolides	Telithromycin
	Lipopeptides	Daptomycin
	Monobactams	Aztreonam
	Oxazolidinones	Linezolid
	Penicillins: carboxypenicillins and ureidopenicillins, including combinations with beta-lactamase inhibitors	piperacillin-tazobactam
	Phosphonic acid derivatives	Fosfomycin
	Pseudomonic acids	Mupirocin
	Rifamycins (except rifaximin)	Rifampicin
	Riminoenazines	Clofazimine
	Streptogramins	pristinamycin, virginiamycin
	Sulfones	Dapsone
	Drugs used solely to treat tuberculosis or other mycobacterial diseases	isoniazid, ethambutol, pyrazinamide, ethionamide
	Substances newly authorised in human medicine following publication of the AMEG categorisation.	To be determined.
Category B ("Restrict")	Cephalosporins: 3rd- and 4th-generation, except combinations with beta-lactamase inhibitors	ceftiofur, cefovecin, cefquinome
	Polymyxins	colistin, polymyxin B
	Quinolones: fluoroquinolones and other quinolones	enrofloxacin, ciprofloxacin, ofloxacin, oxolinic acid
Category C ("Caution")	Aminoglycosides (except spectinomycin)	streptomycin, gentamicin
	Aminopenicillins in combination with beta-lactamase inhibitors	amoxicillin-clavulanic acid
	Amphenicols	florfenicol, thiamphenicol
	Cephalosporins: 1st- and 2nd-generation, and cephamycins	cefalexin, cefapirin
	Macrolides (not including ketolides)	tylosin, tulathromycin
	Lincosamides	clindamycin, lincomycin
	Pleuromutilins	tiamulin, valnemulin
	Rifamycins: rifaximin only	Rifaximin
Category D ("Prudence")	Aminopenicillins, without beta-lactamase inhibitors	amoxicillin, ampicillin
	Cyclic polypeptides	Bacitracin
	Nitrofurans derivatives*	Furazolidone
	Nitroimidazoles*	Metronidazole
	Penicillins: Anti-staphylococcal penicillins (beta-lactamase-resistant penicillins)	Cloxacillin
	Penicillins: Natural, narrow spectrum penicillins (beta-lactamase-sensitive penicillins)	benzylpenicillin, phenoxymethylpenicillin
	Aminoglycosides: spectinomycin only	Spectinomycin
	Steroid antibacterials*	fusidic acid
	Sulfonamides, dihydrofolate reductase inhibitors and combinations	sulfadiazine, trimethoprim
	Tetracyclines	oxytetracycline, doxycycline

§ Other than 1st-, 2nd-, 3rd- and 4th-generation

* Authorised for companion animals only

1.3 Use of Veterinary Antibiotics in Livestock Farming: The Pig Farming Case

Antibiotics are being used in all livestock farming systems, with some of these systems having higher antibiotic sales than others. EMA recorded antibiotic sales in the different productive animal systems like cattle, pig, poultry, sheep/goat, fish, rabbit, and horse (EMA, 2022). Out of these pig farming has the highest average recorded sales of VAs in the EU with $20,269 \cdot 10^3$ tons PCU⁻¹, over $18,623 \cdot 10^3$ tons PCU⁻¹ for cattle, and less than $10,000 \cdot 10^3$ tons PCU⁻¹ for the rest categories. No wonder then, why pig farming has attracted the interest of the research community regarding VA use and associated consequences (Van Boeckel et al., 2015). The most commonly used antibiotics in swine farms are tetracyclines, sulfonamides, macrolides, β -lactams, quinolones and pleuromutilins (Arnold et al., 2004; Lekagul et al., 2019). Most of the antibiotics used in pig farming reside in the AMEG B-D categories, with several of them having little alternatives in human medicine (EMA, 2020). Administration-method-wise, oral, via feed or water, is the most frequently implemented route (Lekagul et al., 2019), particularly for group treatments, whereas injection (according also to the antibiotic stability through the digestive tract) is the preferred method for individual treatments (Sjolund et al., 2015; Trauffler et al., 2014).

Antibiotic administration in pigs, is also tightly connected with the animal age and production stage, and is also associated with the animal vulnerability to infections or the modes of use mentioned previously (therapeutic, prophylactic, or growth associated). According to a study by Callens et al. (2012), the vast majority of VAs are administrated during the first ten weeks of age. Other studies report on antibiotic use by stage of pig production for the main amount of VA administration, such as breeder, sucking piglet, weaner and fatterner/finisher (Lekagul et al., 2019). For example, Jensen et al. (2012) reported higher antibiotic consumption in the weaner phase while,

according to Sjolund et al. in (2016), VAs consumption by suckling piglets was more common than weaners.

As previously mentioned, sulfonamides, pleuromutilins and macrolides account for ~20.75 % of the VA sales in the EU. These classes belong into the AMEG categories most frequently prescribed in pig farming, yet, little is known about the environmental fate, the ecotoxicology, and AMR enhancement potential for most of the antibiotics of these groups (Katsivelou et al., 2023). Representative active compounds of these categories, TIA (pleuromutilin), TLM (macrolide) are frequently prescribed VAs with little or no information about their environmental fate, or are used as yardsticks in environmental fate studies, like the sulfonamide sulfamthoxazole.

1.3.1 Tiamulin

TIA (Figure 1) is a diterpene antibiotic, a semi-synthetic derivative of pleuromutilin (EMA, 2014) naturally produced by *Pleurotus mutilus*, synonym *Clitopilus scyphoides* f. *mutilus* (Schlunzen et al, 2004). It inhibits protein synthesis and is used for treatment and prophylaxis of dysentery pneumonia and mycoplasmosis in pigs and poultry at doses of 100-200 mg kg⁻¹ and 160-320 mg kg⁻¹ of feed respectively for 5-10 days, while it is not used in human medicine (EMA, 1999).

It consists of a tricyclic nucleus composed of a cyclo-pentanone, cyclo-hexyl and cyclo-octane, and a (((2-(diethylamino)ethyl)thio)-acetic acid) side-chain on C14 of the octane ring (Schlunzen et al, 2004). TIA inhibits protein synthesis by binding to the 50S

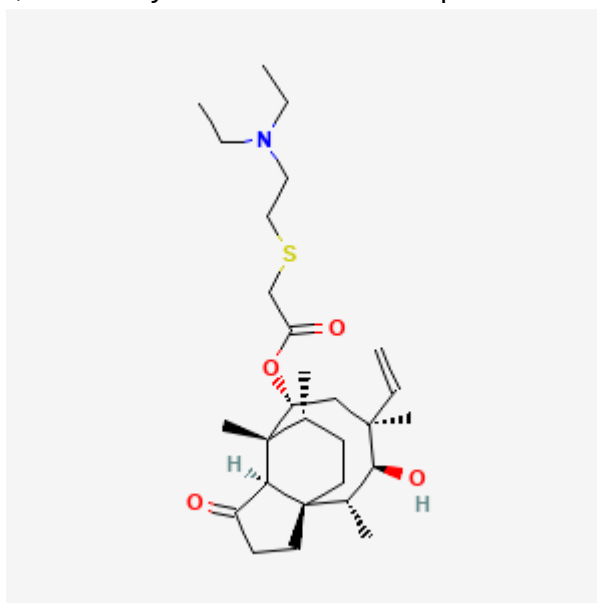


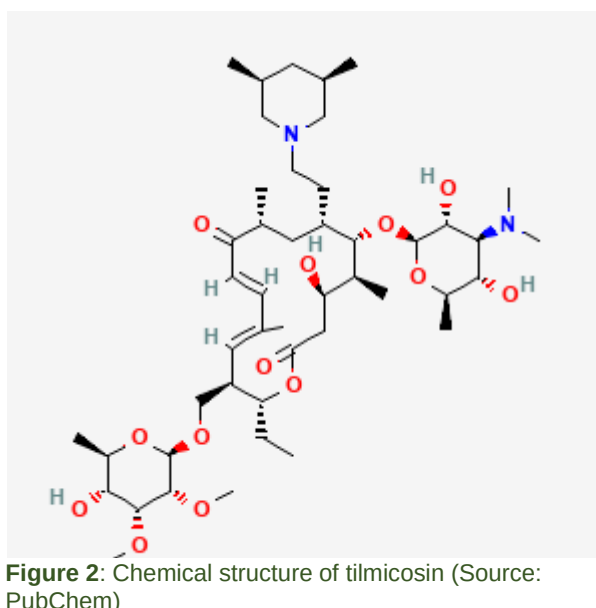
Figure 1: Chemical structure of tiamulin. (Source: PubChem)

ribosomal subunit of bacteria in the peptidyl transferase slot on the ribosome, where it prevents the correct positioning of the CCA ends of tRNA for peptide transferase and subsequent protein production (Poulsen et al., 2001). More specifically, TIA interacts with the domain V of the large rRNA subunit by affecting nucleotides proximation to the peptidyl transferase center. The targeted nucleotides show high phylogenetic conservation in rRNA sequences (Poulsen et al., 2001).

TIA is effective against gram-positive bacteria and *Mycoplasma*. For example, it is used in veterinary medicine to treat and prevent dysentery caused by *Brachyspira hyodysenteriae* and enzootic pneumonia in pigs and mycoplasmal infections in poultry (EMA, 1999; Sun et al., 2016). According to EMA (1999) 35% of the orally administered dose of TIA is excreted through urine and 65% through manure. TIA is metabolized in animals under phase I metabolic routes that includes hydroxylation of the mutilin part, which is the ring part, followed by S-oxidation and N-deethylation on the side chain. Notably, 2 β - and 8 α -hydroxylation and N-deethylation appear to be the main metabolic pathways of TIA in farm animals (Sun et al., 2016).

1.3.2 Tilmicosin

TLM (Figure 2) (20-deoxo-20- [3,5-dimethylpiperidin-1- yl]desmycosin) is a semi-synthetic macrolide antibiotic, a derivative of tylosin which is produced by *Streptomyces fradiae* (Debono et al., 1989; Kaneko et al., 2007). It inhibits protein synthesis and is used for the treatment of respiratory diseases in pigs, cattle and poultry, with doses for pigs ranging between



8-16 mg kg of animal body weight for 15-21 days, while it is toxic to humans in the dose range used in animals with this toxicity manifested by heart attack (Christodouloupoulos et al. 2010; Oakes and Seifert, 2008). It is a 16-member ring molecule with two charged nitrogen atoms (Papich, 2016).

The mode of action of TLM involves inhibition of bacterial growth by binding to the 23S rRNA component of the bacterial 50S ribosomal subunit and preventing peptide bond formation and translocation during protein synthesis (Gerchman et al., 2011). This occurs more frequently in bacteria with a small number of rRNA operons, such as mycoplasmas. TLM has enhanced activity against *Pasteurella multocida*, *P. hemolytica*, *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae*, *Streptococcus suis*, *Actinomyces pyogenes* and others (Ose, 1987; Debono et al., 1989). Its high efficacy against many Gram-positive and some Gram-negative microorganisms, have led to the establishment of its use as a broad spectrum antibiotic in various host animals (Picavet et al., 1991; Moore et al. 1996; Hoar et al. 1998; de la Concha-Bermejillo et al., 1998; Stipkovits et al. 2001; Christodouloupoulos et al. 2010; Mateusen et al. 2010). It is also known that TLM exhibits a dose-dependent (Zhang et al., 2020) negative inotropic and positive chronotropic cardiotoxic effect in animals that can induce cardiac damage and sudden death (Er et al., 2014). In farm animals >90% of the initial oral dose of TLM is excreted in feces and the rest in urine (EMA, 2014). According to Kennington and Readnour (1998) no microbiologically active metabolites of TLM are formed in significant amounts in animal body. In general, TLM is thought not to be metabolized (Montesissa et al., 2003).

1.3.3 Sulfamethoxazole

SMX (Figure 3) (4-amino-N-(5-methylisoxazol-3-yl)-benzene sulfonamide) is a broad-spectrum bacteriostatic sulfonamide. Structure-wise, it is an isoxazole (1,2-oxazole) compound with a methyl group at the 5-position and a 4-

aminobenzenesulfonamido group at the 4-position (Prasannamedha and Kumar 2020). It acts by blocking the important for DNA anabolism folate pathway (Khalil et

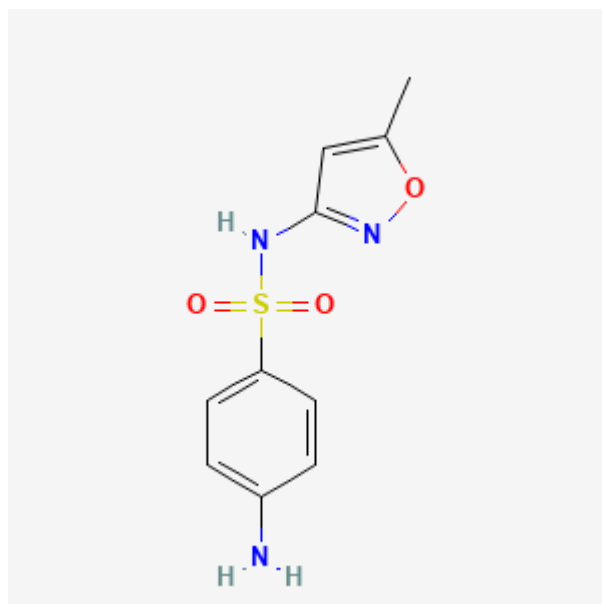


Figure 3: Chemical structure of sulfamethoxazole
(Source: PubChem)

al., 2003; Yun et al., 2012; Kemnic and Coleman, 2023). Most bacteria synthesize folic acid from para-aminobenzoic acid (PABA). SMX is a structural analog of the endogenous substrate PABA that interferes with bacterial growth by suppressing dihydropteroate synthase, the enzyme responsible for bacterial conversion of PABA to dihydrofolic

acid. This leads to blocking of the tetrahydrofolate synthesis and, eventually, the synthesis of bacterial purines and DNA. Further success against purine biosynthesis by bacteria, is provided by the simultaneous administration of trimethoprim that blocks dihydrofolate reductase in the downstream dihydrofolate to tetrahydrofolate transformation (Gobel et al., 2005; Papich 2016). SMX and trimethoprim are used in the prophylaxis and treatment of urinary tract infections soft tissue infections, prostate infections and pneumonia (Gobel et al., 2005; Papich 2016).

SMX is effective against many Gram-negative bacteria such as *Escherichia coli* and *Klebsiella pneumoniae*, it is also reported to have enhanced activity against *Acinetobacter* spp. and *Pseudomonas aeruginosa* (Mittal et al., 2009; Foxman B, 2010; Djordjevic et al., 2013). Up to 80% of SMX and its metabolites are excreted through urine in animals. The main metabolite of SMX is N-acetyl-sulfamethoxazole, measured at levels approaching 80% of the administered parent compound in pigs (Guo et al., 2023). However, when found in the environment, N-acetyl-sulfomethoxazole can undergo a retransformation to the parent compound (Gobel et al., 2005). SMX metabolism includes acetylation at the N4 nitrogen atom by arylamine N-

acetyltransferase (NAT) enzymes, oxidation at the same N4 nitrogen atom, which is catalyzed by CYP2C9.9, glucuronidation of the N1 nitrogen atom and hydroxylation of the C5 methyl group (Zhou et al., 2009; Baran et al., 2011; Guo et al., 2023).

2. Fate of Veterinary Antibiotics and Solutions to Mitigate Contamination

Environmental dispersal of VAs depends on their persistence and the management of animal excretes which are often the carriers of VAs in the environment. An illustration of the route of environmental exposure and associated consequences is provided in Figure 4 and detailed in this section.

2.1. Veterinary Antibiotics: From Administration to Excretes

VAs are administered to farm animals through feed, water, or injection to prevent or treat infections caused by pathogenic microbes (EMA, 2014). The annual consumption of antibiotics by humans is far surpassed by the consumption of VAs (Maron et al., 2013; UBA, 2014). Upon uptake, VAs undergo metabolic transformations (Reemtsma and Jekel, 2006; Botelho et al., 2015). The amount of metabolized VAs within the animal body depends on the antibiotic, the treated animal species, its age and its medical state. However, a major fraction (40-90%) of most VAs is excreted unmodified, in the form of the parent compound (Kümmerer, 2009; Pan and Chu 2017). The quantity of excreted VAs depends on the type of antibiotic, the dosage and the animal under administration (Botelho et al., 2015). For example, 90-95% of tetracyclines and sulfonamides are usually excreted intact by animals (Heuer et al., 2008; Sukul et al. 2009)

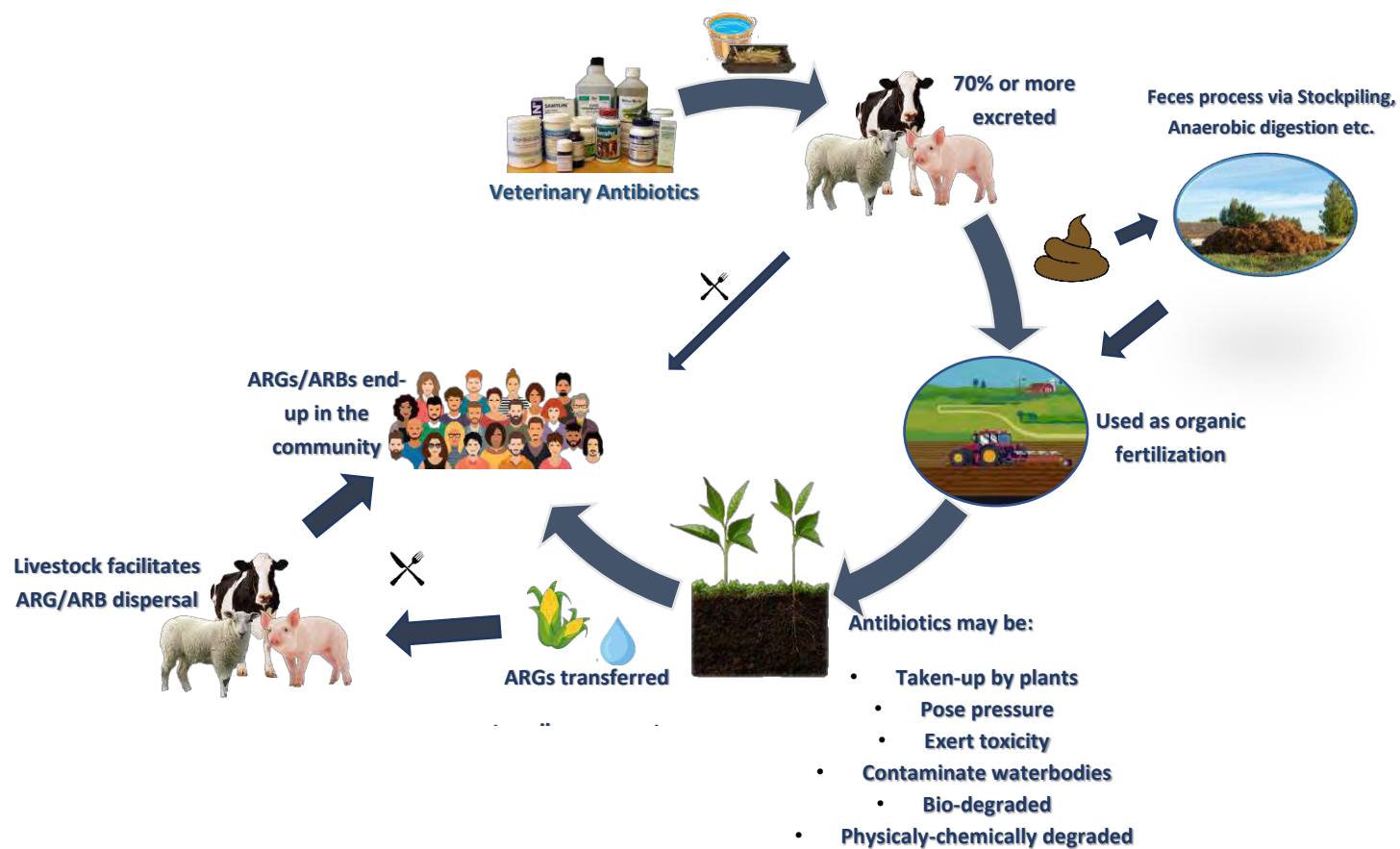


Figure 4: The route of VA dispersal into the environment and its consequences.

2.2 VA-Contaminated Manures: Use and Treatments

Manure derived from animal excreta is used in agriculture as organic fertilization to enrich soil in nitrogen (N), phosphorus (P) and potassium (K) and organic matter (Dou et al., 2001; Szogi et al., 2015). Manures used in plant production are derived to a great extent from heavy-VA-use intensive livestock farm excreta. With several antibiotics being persistent, the application of such contaminated manures result in VA release (up to 75% or more) in agricultural soils (Perruchon et al., 2022; Xu et al., 2022). This way, manure enriched with antibiotics or their transformation products may exert toxic effects to soil microbiota or enhance associated AMR phenomena with possible implications on human health (Chen et al., 2019; Stanton et al., 2020; Heuer et al., 2011). In Europe, approximately 75-85% of animal excreta are stocked in piles or stored in aerobic lagoons before their application in fields aiming also at pathogen and antibiotic residue removal, next to nutrient preservation (Jorgensen and Halling-Sorensen, 2000; Oliver et al., 2020). However, these treatments do not always result in complete dissipation of VAs (Kemper, 2008, Zhao et al., 2010). Large VA amounts used in conventional livestock farming usually lead to heavily contaminated manures ending up in agricultural soils, with many studies reporting antibiotic levels ranging from $\mu\text{g/kg}$ to mg/kg . Ultimately, this practice leads to continuous exposure of agricultural fields to VAs with detrimental effects on the environment and public health through VA accumulation to natural water resources and plants, as well as antibiotic resistance spread and toxicity to the soil microbial community (Heuer & Smalla, 2007; Falkowski et al., 2008; Chung et al., 2017; Charuaud et al., 2019).

Methods of manure treatment prior to their application in agricultural soils include (Liu and Wang, 2020; Dong et al., 2022): (i) stockpiling for storage of the manure and its nutrients next to removal of pathogens/antibiotics prior agricultural prior soil

application; (ii) solid-liquid separation with the solid fraction usually being richer in P and organic N, whereas inorganic N is found in the liquid phase; (iii) composting for the transformation of biodegradable organic matter to stabilized manure with fixed nutrients less likely to be leached after application; (iv) anaerobic digestion with simultaneous biogas production. These treatments result in the reduction of the manure volume, the removal of pathogens and antibiotics, and recycling of nutrients. Stockpiling and anaerobic digestion are the currently preferred approaches for treating manure, whereas bioaugmentation is a relatively novel and ecofriendly alternative.

2.2.1 Stockpiling

Stockpiling is the most common manure management method (Kim et al., 2012; Staley et al., 2021). Animal feces are stored in piles without any pretreatment until their use as organic fertilizers. The piles of animal excreta are stockpiled for periods of 60-180 days. Stockpiling is a cost-effective technique that is characterized by low labor cost and simplicity. During storage, digestion induced temperature rise to levels above 55 °C, for prolonged periods of more than 15 days (Staley et al., 2021), result in the reduction of VA residual levels, reduction of microbial population (including pathogens) and reduction in ARG abundance.

2.2.2 Anaerobic digestion

Anaerobic Digestion (AD) is a relatively well characterized process involving a series of steps where microorganisms break down organic matter in an oxygen-depleted environment (Angelidaki and Sanders 2004). It is characterized by four phases: hydrolysis, acidogenesis, acetogenesis and methanogenesis. In hydrolysis organic polymeric molecules like proteins, fatty acids and hydrocarbons are hydrolyzed into their soluble monomers. These are then converted into organic acids during acidogenesis and then into acetic acid, carbon dioxide and hydrogen during acetogenesis. By the end of AD, biogas - a combination of primarily methane and

carbon dioxide, along with secondary compounds, hydrogen sulfide, hydrogen, and ammonia - is produced by the conversion of acetic acid during methanogenesis (Rodriguez-Chiang, 2017; Angelidaki and Sanders 2004). AD can transpire in many different temperature levels, the most commonly used are the mesophilic (32-35°C) and the thermophilic (50-60°C) digestion (Song et al., 2004; Rodriguez-Chiang, 2017).

2.2.3 Bioaugmentation

Bioaugmentation is a bioremediation strategy whereby microorganisms or plants are used for the removal, reduction, degradation, or immobilization of environmental pollutants from the air, water, soil, flue gasses, industrial effluents etc., (Kamaludeen et al., 2003; Khalid et al., 2017; Yuvraj, 2022). Bioaugmentation is considered an inexpensive and environmentally sustainable process that is steadily gaining ground in the realm of environmental clean-up applications (Ashraf et al., 2019).

During bioaugmentation the addition of indigenous or exogenous microorganisms result in boosting the degradation process of the contaminant in the polluted environment (Nzila et al., 2016). The method is based on the introduction of microorganisms that are able to degrade contaminants and transform them into harmless compounds in the polluted environment (Herrero, 2015). Hence, the introduced microorganisms need to be able to biotransform the pollutant and be able to survive in a range of environmental conditions (in terms of pH, temperature, aeration etc.). Some of the most common applications of bioaugmentation includes soil remediation (Lebeau, 2008; Mrozik, 2010), wastewater treatment (Herrero, 2015; Nzila et al., 2016). Bioaugmentation was implemented for solving environmental problems like those caused by major crude oil spills, like those of the *Exxon Valdez* supertanker in the Alaskan seas in 1989 and the BP *Deepwater Horizon* drilling rig in the Gulf of Mexico in 2010 (Atlas and Hazen, 2011). A relatively understudied field of research is that of bioaugmentation of manures contaminated with VAs and other veterinary drugs (Shi et al., 2021).

3. Effects of the manure introduced VAs on soil and associated habitats

VAs are subjected to an array of changes whilst released in soils. Once in soil, they may remain intact (persist) and mobile in the aquatic phase, or adsorbed on the organic matter or the clay particles depending on the VA properties (Jechalke et al., 2014; Cycoń et al. 2019; Yang et al., 2021). Furthermore, they can be abiotically or biotically transformed into other compounds with or without toxic activity against microorganisms (Jechalke et al., 2014; Long et al., 2020; Yang et al., 2021). Finally, products of the VA metabolism while within the animal or products of abiotic/biotic degradation, once in the manure and/or soil, may re-form to their parent compounds biotically or abiotically, as e.g. identified in the case of certain sulfonamides. Hence, VA interactions with soil microbes result in different phenotypic manifestations. Such manifestations may have negative or positive aspects like (Jechalke et al., 2014; Cycoń et al. 2019; Long et al., 2020; Yang et al., 2021): (i) ecotoxicity where the toxic properties of the applied chemicals described in section 1.2 are exerted against both target and off-target organisms found in natural environments, out of the scope of the antibiotic administration, implying the lack of thorough ecotoxicity associated investigation (negative aspect concerning off-target microorganisms); (ii) antibiotic stress induced HGT of AMR, a major contemporary societal concern described further on (negative aspect); (iii) induction of growth-linked microbial biodegradation or biotransformation of VAs, both useful for bioremediation strategies (positive aspect). Below we will focus further on the different outcomes of the interactions of VAs with the soil microbiota.

3.1 Toxicity effects on the soil microbial communities and affiliated plant microbial habitats

The potential off-target toxicity of antibiotics in soils is an issue that has recently received the attention of the research community (Jechalke et al., 2014; Revellin et al., 2018; Cycoń et al. 2019; Katsivelou et al., 2023). VAs presence in soil might inhibit many soil microorganisms that carry out very important ecosystem functions like that of nutrient cycling (Falkowski et al., 2008; Graham et al., 2016). Revellin et al (2019) first showed that the continuous exposure of soil to a combination of VAs led to remarkable alterations in the composition of the community of nitrogen fixing bacteria in the roots of sorghum plants. Recently, Katsivelou et al (2023) reported significant structural and functional changes imposed in the soil microbial community upon its systematic exposure to TIA, TLM and SMX. Given such examples, Brandt et al. (2015) emphasized the importance of incorporating novel assays into the environmental risk analysis regulatory framework, to evaluate the toxicity of VAs on the soil microbial community.

Although the main focus of this dissertation is on the soil, the potential mobility of VAs from soil to other environmental compartments including plant uptake adds further complication to the risk associated with the presence of VAs in soil. So far several studies have reported a enrichment of plant tissues in VA in agricultural sites receiving application of manures (Bartikova et al., 2016; Chung et al., 2017). Kumar et al. (2005) observed chlortetracycline accumulation in tissues of various plants (such as onions, corn, and cabbage) grown in soils amended with manure containing antibiotics. Manure mediated uptake of sulfamethazine was reported for potatoes, corn and lettuce (Dolliver et al., 2007), while several studies on tetracyclines and sulfonamides showed similar reports for other plants like wheat, willow, maize (Grote et al. 2007; Hu et al. 2010; Michelini et al. 2012). Distribution of VAs in plant tissues

varies among studies (Michelini et al. 2012; Hu et al. 2010), while the plant growth stage and weather conditions also play important role (Tasho and Cho 2016). Reports on negative VA impact on the growth of vegetables were linked with direct effects or indirect effects on plant growth, the latter occurring through VA interactions with endophytes regulating plant growth (Kong et al., 2007; Liu et al., 2009b; Zhang et al., 2023). Among plant observed growth patterns, also hormetic responses were observed suggesting increased plant metabolism at low VA concentrations (Migliore et al. 2010b; Michelini et al. 2014). Both the endophytic and epiphytic microorganisms have been demonstrated to be affected by both manure-mediated and direct VA application (Zalewska et al., 2021).

3.2 Antibiotic Resistance Development and Dispersal in Soil due to VA Contamination

AMR arises from the evolution or enrichment of acquired or naturally occurring mechanisms in microbes that protect them from antimicrobials (Prestinaci et al., 2015). All taxonomic groups of microbes can evolve AMR (fungi, viruses, protozoa and bacteria). Antibiotic resistance specifically pertains to the bacterial resistance against antibiotics. Resistance is commonly measured as a function of minimum inhibitory concentration (MIC), the lowest antibiotic concentration that will achieve inhibition of bacterial growth. Susceptibility is the range of average MICs for an antibiotic among strains of the same bacterial species. If it is in the resistant part of the range, this specific species is considered to have intrinsic resistance to that antibiotic. Although some bacteria can naturally be resistant to antibiotics, or evolve resistance through random mutations and natural selection, others may acquire ARGs horizontally from associated microorganisms.

To put things into perspective, one has to consider that most antibiotics are multi-role (e.g. defense, communication related), naturally produced molecules, existing in the environment since millions to billions of years ago as evidenced by identified primordial amino acid derivatives or evolutionary analysis of biosynthetic pathways (Davies and Davies, 2010; Munita and Arias, 2016). It is difficult to reconstruct the evolutionary history of horizontally transferable genetic material, as very frequently is the case for ARGs, due to differences in estimated evolutionary rates when multiple hosts are involved (Perry et al., 2016). However, *in silico* analysis (of ARGs and antibiotic biosynthetic pathways) and experimental evidence (e.g. permafrost samples) suggest a parallel evolution between antibiotic resistance and production, dating back to millions/billions years ago. Two are the major modes of antibiotic resistance, the intrinsic and the acquired (Munita and Arias, 2016). Intrinsic resistance refers to resistance determinants related to the makeup of an organism that defines its basic physiology/morphology, i.e. under the absence of the selective pressure of an antibiotic. E.g. the absence of cell walls at *Mycoplasma* species make them intrinsically resistant to cell-wall formation affecting antibiotics. Acquired resistance, on the other hand, may result from mutations in chromosomal genes, or the acquisition of genetic material from other resistant microorganisms. Main intercellular genetic material transfer modes are those of conjugation (conjugative/mobilizable plasmid transfer between a donor and a recipient cell; frequently considered as the dominant mode of intercellular genetic material transfer in a bacterial population under selective pressure conditions), transformation, and transduction (Frost et al., 2005). Besides intercellular transfer, also intermolecular ARG mobility can take place with modes exploiting elements like composite transposons (copy/cut and paste mechanisms), integrons (integration of gene cassettes), and genomic islands (alike integrons involving large transferable units found in chromosomes) (Frost et al., 2005; Kosek et al., 2021). AMR mechanisms fall into the following main categories (Figure 5) (Reygaert, 2018): (i) limited uptake of an

antibiotic (e.g. modulation of the number or selectivity of aquaporins frequently involved in the passive antibiotic transport); (ii) modifying a drug target (e.g. mutation of the β -lactam targets transpeptidases); (iii) inactivating a drug (e.g. hydrolysis of β -lactams by β -lactamases); and (iv) active drug efflux pumps (e.g. ATP-binding cassette - ABC - transporters). Intrinsic resistance might use limiting uptake, antibiotic inactivation, and antibiotic efflux while acquired resistance might use modification of antibiotic target, antibiotic inactivation, and antibiotic efflux (Reygaert, 2018). All four mechanisms can only be used by Gram-negative bacteria, while Gram-positive bacteria do not usually use mechanisms involving limited uptake or upregulation of drug efflux mechanisms.

Infections by antibiotic resistant microbes are already widespread across the globe. The most prevalent antibiotic resistant bacteria (ARB) associating to deaths are: *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* (Murray et al. 2022). AMR has been prioritized by WHO as one of the top 10 global public health threats facing humanity (WHO, 2020). This severe problem is characterized as Antibiotic Resistance Crisis. Drug resistant infections were estimated to contribute to 4.95 million deaths globally in 2019. However, it is estimated that global deaths due to AMR might reach 10 million per year by 2050, providing no significant actions would be carried out (O'Neill, 2016).

The systematic exposure of agricultural fields to VAs due to organic fertilization leads to selection of antibiotic resistance (Heuer and Smalla, 2007; Jechalke et al, 2014). Manure serves as a reservoir of ARB, ARGs and pathogens occasionally enriching also indigenous ARGs (Andrews et al., 2004; Heuer and Smalla, 2007;

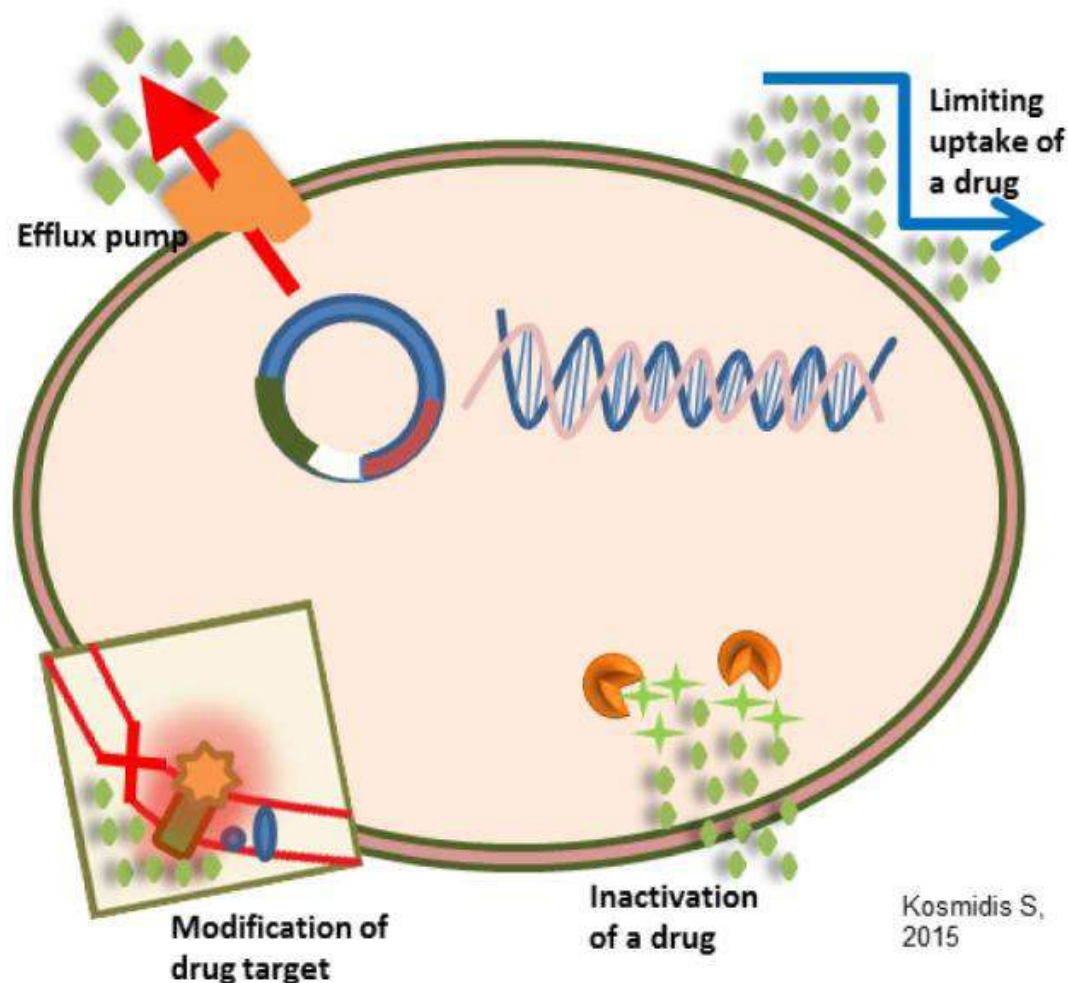


Figure 5: General antimicrobial resistance mechanisms. (Adopted from Kosmidis S. 2015.)

Ferens and Hovde, 2011; Looft et al., 2012; Zhou et al., 2012; Udikovic-Kolic et al, 2014; Chen et al., 2016; Katsivelou et al., 2023). As previously mentioned ARG transfer is tightly linked to HGT with bacterial conjugation being one of the most prevalent mechanisms. To this end, plasmid backbone genes comprise common markers of HGT possibly associated with ARG transfer (Jechalke et al., 2013). Also, the *int1* intermolecular HGT marker has been indicated as an ARG and anthropogenic pressure associated HGT marker whose abundance was positively correlated with manure application in agricultural soils (Jechalke et al., 2014; Gillings et al., 2015; Zhang et al., 2018; Li et al., 2021; Katsivelou et al., 2023). *Int1*, is a class 1 integron, that encodes for a gene cassette integrase, mainly prevalent among Gram-negative

bacteria (Stalder et al., 2012), while the sulfonamide resistance gene cassette residence on the class 1 integron was recently proposed as a marker of clinical relevance (Quintela-Baluja et al., 2021). When ARB are prevalent in the soil microbial community they might pose a threat for human health (Marshall and Levy, 2011). A study by Forsberg et al (2012) revealed the role of the soil microbial community as a reservoir of ARGs that could be transferred to bacterial pathogens. They identified identical genetic cassettes of antibiotic resistance in both soil bacteria and human pathogens.

3.3 Veterinary Antibiotic Growth Linked Biodegradation By Soil Microorganisms

An array of studies over the last decades established that soil microbial communities are able to biodegrade several VAs and use them as nutrient for energy gain. The majority of available studies to date deal with tetracyclines, sulfonamides and macrolides (Topp et al., 2013; Topp et al., 2016; Terzic et al., 2018; Harrabi et al., 2019). Topp et al (2013) showed that continuous exposure of agricultural soils to VAs can enhance the capacity of the microbial communities or select for microbes able to degrade antibiotics for nutrient/energy profit. Such an approach contrasted common perception that the systematic exposure to VAs can only lead to the evolution of VA resistance traits. These studies provided the proof of concept that the continuous exposure of soil microorganisms to VAs can trigger the acclimation of the soil microbial community towards the use of previously unknown catabolic pathways for energy gain through the biodegradation of VAs. In this context, Sentchilo et al (2013) further identified links between mobile genetic elements (MGE) with growth-linked biodegradation genes. Such findings stimulated research involving the isolation of bacteria able to degrade VAs for utilization as carbon/energy source (Leng et al., 2016;

Revellin et al., 2018; Wen et al., 2018). Some studies also posed questions about the need of the co-existence of separate, antibiotic resistance mechanisms, and a growth-linked biodegradation mechanism for microbial proliferation in antibiotic stressed environments. A positive answer to such a question was provided in the case of the actinobacterial *Microbacterium* strain C448, isolated from long term exposed soils to sulfamethazine (Topp et al., 2013). Genomic analysis showed the presence of both sulfonamide resistance genes (*sul1* resided close to *intl* and a relaxase coding gene), and the antibiogenesis *sadABC* locus (Martin-Laurent et al in 2014; Martin-Laurent et al., 2019). Such combination of traits was proven to be useful for bioaugmentation, as described in a recent study where the *Microbacterium* C448 was demonstrated to be conditionally competitive and efficient in the mineralization of a sulfonamide, sulfamethazine (Billet et al., 2021). Overall, antibiogenesis is a very useful tool added in the toolbox of bioremediation as part of a sustainable future aligning with the objectives of the One Health initiative.

4. Aims

The extensive use of VAs in livestock farming poses a threat for ecosystem functioning and public health. VAs are not completely metabolized in the animal body and hence they are released at high amounts in the animal excreta. VA-contaminated manures are subsequently applied in agricultural soils for organic fertilization, usually after being subjected to different treatments like stockpiling or anaerobic digestion, aiming at reducing their VA/pathogenic content. Even after such treatments, high levels of VAs and of potential pathogenic microorganisms persist in manures, and hence end up in arable fields, with an expected impact on the soil and associated plant microorganisms regarding toxicity and resistance to antibiotics.

The main aims of this thesis were (a) to determine the persistence of VAs along their modular route from animals to feces and eventually to agricultural soils and hence identify the true level of exposure of agricultural soils to the studied VAs (Chapter 2) (b) to investigate the multimodal effects of VAs on the soil microbial community appearing as enhanced biodegradation, soil microbial toxicity or stimulation of AMR (Chapter 3); (c) to explore the potential of different methods, including bioaugmentation, as a method for curation of VA-contaminated manures (Chapter 4).

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Chapter 2

Following the route of veterinary antibiotics tiamulin and tilmicosin from livestock farms to agricultural soils

The work presented in Chapter 2 is included in the scientific paper:

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1. Introduction

VAs have a major role in livestock farming where they are administered to animals to treat or prevent microbial infections, increase body weight, or improve feeding efficiency (Song and Guo, 2014; EMA, 2019). VAs are most commonly administered orally to the animals (> 50%), while a small number are injected (ca. 10%). Regardless of the method of administration, most of the VA dosed (40–90%) is excreted through urine and feces either intact (parent compound) or in the form of transformation products, which are often as bioactive as the parent compound (Song and Guo, 2014; Pan and Chu, 2017).

In Europe, animal excretions are temporarily stocked in piles before used as manures in agricultural soils (Jorgensen and Halling-Sorensen, 2000; Van Epps and Blaney, 2016). Alternatively, animal feces are used as substrates in anaerobic digestion for biogas production (Karim et al., 2005; Bauer et al., 2014). The persistence of VAs in feces during stockpiling or anaerobic digestion will determine the level of exposure of agricultural soils to VAs upon manuring. Previous monitoring studies have suggested that the levels of VAs in manures ready to be applied in agricultural soils could vary from $\mu\text{g/kg}$ to mg/kg (Zhao et al., 2010). Once in soil, VAs are subject to a range of biotic and abiotic process including sorption on soil colloids, degradation by soil microorganisms, plant uptake, and transportation to surface water and groundwater systems (Cycon et al., 2019; Song and Guo, 2014). The systematic exposure of agricultural soils to VAs could lead to a range of undesirable effects on the environment and on public health like: (a) toxic effects on the soil biota driving key ecosystem services (Graham et al., 2016); (b) contamination of natural water resources (Grenni et al., 2017); (c) dispersal of ARGs among members of the soil microbial community (Heuer and Smalla, 2007; Jechalke et al., 2014) that can be further transmitted to the rural and the urban population (Marshall and Levy, 2011; Forsberg et al., 2012); (d)

transport to edible plant parts facilitating the dispersal of ARGs (ARGs) to human pathogens residing on plant surfaces with reciprocal undesirable effects for consumers health (Solomon et al., 2010; Pan and Chu, 2017). To determine the risk associated with the exposure of agricultural soils to VAs we need to have a thorough idea of the level and the duration of the exposure of soils to VAs. Good knowledge of the persistence of VAs along their route from animals to feces and via intermediate treatment to agricultural soils is required.

In this study, we focused on two VAs, TIA and TLM, which are both commonly used in livestock farming in Europe and for which little is known about their persistence and environmental fate. TIA is a semi-synthetic derivative of pleuromutilin and it is used for the treatment of enteric and respiratory infections of pigs and poultry (EMA, 2014). In pigs approximately 35% and 65% of the dose is excreted in urine and feces respectively (EMA, 1999). Little is known regarding the persistence of TIA in feces and soil. TIA was particularly persistent in animal feces and manure with $DT_{50} > 200$ days (Schlüsener et al., 2006; Berendsen et al., 2018), whereas it showed limited persistence in the only soil study available ($DT_{50} = 16$ days) (Schlüsener and Bester, 2006). TLM is a macrolide synthesized from tylosin mostly used to treat respiratory infections in calves and pigs (EMA, 2014; Geijlswijk et al., 2018). Six days after administration more than 90% of the TLM dose was excreted (EMA, 2014). Today we know little about the environmental persistence of TLM with its DT_{90} values in feces ranging from 38 to 346 days, depending on the animal origin of feces (Berendsen et al., 2018), while in soil its DT_{90} was higher than 365 days (Berendsen et al., 2021).

The main aim of the current study was to determine the level of exposure of agricultural soils to TIA and TLM. In this quest we determined the persistence of the two VAs along their modular route from animals to feces and eventually to agricultural soils. We initially posed the question how different administration modes affect the excretion

kinetics of TIA and TLM in pigs. We subsequently tested the hypothesis that different treatment of feces like anaerobic digestion or stockpiling at ambient conditions will affect differently the persistence of TIA and TLM. Beyond purely measuring the persistence of VAs, we also monitored the potential effect of VAs on the anaerobic digestion process itself through methane and volatile fatty acid (VFA) production measurements. Finally, we determined the persistence and dissipation of TIA and TLM in a range of agricultural soils under different application regimes (directly in soil or via feces amendment) and explored the role of soil microorganisms (fumigated vs non fumigated soils) and different application regimes (application in soil vs application through treated feces) in the dissipation of VAs.

2. Materials and methods

2.1. Chemicals

For TIA and TLM application in the pig farm the following commercial formulations were used: (a) Vetmulin® 100 g kg⁻¹ Premix and Vetmulin® 450 mg g⁻¹ for TIA administration through feed and water respectively, (b) Tilmove® 200 g kg⁻¹ Premix (administration through feed) and Tilmove® 250 mg ml⁻¹ Oral solution (through water) in the case of TLM. For residue analysis analytical standards of TIA fumarate (Vetranal®Sigma-Aldrich, purity > 98%) and TLM (HPC Standards, purity 92.8%) were used, while in all laboratory experiments the technical standards of TIA fumarate (Biosynth®Carbosynth, purity > 97%) and TLM (Biosynth®Carbosynth, purity > 85%) were utilized for the preparation of the solutions applied on soils and feces.

2.2. Residue analysis of VAs

2.2.1. Extraction of VAs from feces and soil

TIA and TLM were extracted from feces following the method described by Wang et al. (2020), with slight modifications. Briefly, 1 g of feces was weighed in a 50 ml Teflon tube (Nalge Nunc International, Rochester, NY, USA) and 10 ml of extraction solution (water: methanol: acetone 50:25:25 v/v/v with 25 mM EDTA, 0.6 M NaCl and 2% ammonia) were added. The mixture was vortexed for 1 min and then sonicated in an ultrasound bath for 15 min. Subsequently, the sample was centrifuged at 10,000 $\times g$ for 5 min at 4 °C and the supernatant was collected. Feces were extracted once more with a fresh aliquot (10 ml) of the extraction solution. The combined supernatant was passed through a Poly-Sery HLB cartridge (CNW Technologies, Duesseldorf, Germany) preconditioned with 5 ml of methanol followed by 6 ml of water. Afterwards, the cartridge was washed with 5 ml of water, vacuum-dried for 3 min, and the antibiotic eluted twice with 7 ml of 9:1 methanol: acetonitrile (v/v), containing 0.2% or 0.4% formic acid for TIA and TLM respectively. Samples were vacuum-dried at 35 °C, resuspended in 0.5 ml (TIA) or 1 ml (TLM) of a water: acetonitrile (50:50 v/v) solution, filtered through a 0.22 μm filter (PTFE Hydrophobic Syringe Filter, Zhejiang ALWSCI Technologies, Hangzhou, China), and analyzed by HPLC as described below. A method described by Lee et al. (2017) was used for the extraction of TIA and TLM from soil. Briefly, 5 g of soil were weighed in a 50 ml Teflon tube and 2.5 ml of water were added. The mixture was vortexed for 1 min and left for 10 min to equilibrate. Subsequently, 5 ml of acetonitrile, 0.5 g of NaCl and 0.05 ml of acetic acid (0.025 ml for the acidic soils) were added and the mixture was vortexed for 5 min prior to centrifugation at 8,000 g for 5 min at 25 °C. An aliquot (3 ml) of the supernatant was collected and vortexed for 1 min with 0.075 g of PSA. After centrifugation the clear supernatant was filtered through a 0.45 μm syringe before analyzed by HPLC.

2.2.2. HPLC analysis

The concentration of VAs in the extracts was determined in a Shimadzu HPLC-PDA system equipped with a CNH Athena RP C18–150 mm column (CNW Technologies, Duesseldorf, Germany). TIA was eluted using a mobile phase of 60:40 acetonitrile: water (v/v) + 1.25% ammonia (flow rate of 1 ml min⁻¹) and it was detected at 208 nm. TLM residues were determined using a gradient elution of acetonitrile (solvent A) and water + 0.1% H₃PO₄ (solvent B). The initial mobile phase composition was maintained at 90% solvent B for 3 min, then changed linearly to 65% (3–4 min) and held 11 min (4–15 min), then followed by a return to the initial conditions within 1 min (15–16 min) and kept 5 min (16–21 min) for the chromatograph column equilibration. Detection was achieved at 287 nm at a flow rate of 0.7 ml min⁻¹ and a column temperature set at 60 °C. The injection volume was in all cases 20 µl. Method validation tests involving fortification of feces with VAs at three concentration levels (0.1, 1 and 10 mg kg⁻¹) showed recoveries of 97.0 ± 0.5%, 81.6 ± 0.7% and 80.0 ± 8.9% for TIA and 100.0 ± 3.5%, 82 ± 2.8% and 100 ± 7.9% for TLM. Similar recovery tests for TIA and TLM at the same fortification levels in soil showed recoveries of 93.0 ± 9.2%, 93 ± 8.8%, 101.5 ± 9.6% for the former and 82 ± 5.7%, 90.4 ± 1.6%, 85.7 ± 7.5% for the latter.

2.3. Excretion kinetics of TIA and TLM under different administration regimes

This study was carried out in a commercial pig farm (HOIROPAL, Palamas, Greece) and VAs admission has been performed according to common practices of the farm. All animals enrolled in the study were weaned piglets in the pre-fattening phase (30–38 days old) which were divided into 5 groups of 100 animals each. Each group was located in a separate room and it was further separated into four subgroups of 25

piglets. Each group received a different treatment as follows: (a) TIA administered through feed, (b) TIA administered through water, (c) TLM administered through feed, (d) TLM administered through water and, (e) untreated controls. In all cases VAs were administered to animals at the recommended dose rate, as described in the products label: (a) 10 and 12 mg of TIA and TLM per day and kg of body weight for 7 consecutive days when administered through feed; (b) 8.8 and 20 mg of TIA and TLM respectively for 5 consecutive days in the case of administration through water. No visible changes in the feeding and watering behavior of the treated and untreated piglets was observed during the administration period. Four samples from each treatment (one per subgroup) were collected at 2, 4, 6, 9, 11, 13, and 23 days after the first administration and stored at -20°C until extracted for HPLC analysis.

2.4. Persistence of VAs in the faecal material during storage at ambient conditions

Fresh feces of young pigs non-treated with any antibiotic were collected from the same farm as above to be used in the laboratory. For each VA, feces were divided into three 150-g portions, each treated with different amounts of stock solutions of TIA (in methanol) and TLM (in acetonitrile) prepared at three different concentrations (100, 1000 and $10,000\text{ }\mu\text{g ml}^{-1}$) to achieve final VA concentrations of 0.5, 5 and $50\text{ }\mu\text{g g}^{-1}$ respectively. The range of nominal application rates for the two VAs was selected based on the residual levels of the two compounds measured in pig feces at the end of the excretion kinetic study (see Section 2.3.). Before VA application, feces were amended with ddH₂O to adjust moisture content to 70% of their water holding capacity (determined as described by Papadopoulou et al., 2018) and mixed by hand to ensure uniform distribution. Subsequently samples were placed in aerated plastic bags and stored at ambient temperature and sunlight conditions for 60 days (July-August 2020).

Triplicate samples were collected from each treatment at 0, 15, 30, 45, and 60 days and analyzed by HPLC for VA residues. During storage the moisture content of feces was maintained constant with regular additions of ddH₂O when needed.

2.5. Persistence of VAs in faecal material during anaerobic digestion and their effects on bimethanation

Anaerobic digestion experiments were performed in collaboration with the Laboratory of Agricultural Structures and Equipment, Aristotle University of Thessaloniki, Greece.

2.5.1. Experimental setup

Feces were collected, handled and treated with TIA and TLM at three concentration levels (0.5, 5 and 50 µg g⁻¹) as described in the previous section. The inoculum used for the anaerobic digestion was obtained from Biogas Lagada S.A. (Lagada, Thessaloniki). It was pre-incubated at 37 ± 0.5 °C until no significant methane production was detected to deplete any eventual residual biodegradable organic material (Angelidaki et al., 2009). For each VA-concentration level combination triplicate batch anaerobic digesters were prepared along with triplicate controls containing VA-untreated feces (blanks) and inoculum controls (no feces). Methane produced from the inoculum controls was subtracted from the methane production in all feces-containing samples. Batch anaerobic digestion tests were conducted in glass bottles (batch reactors) of 0.3 L. The ratio of total solids (TS) to volatile solids (VS) of the feces and the inoculum was determined as described by APHA (2005). The feces loading factor was 1.5 g VS and the feed to inoculum ratio (F/I) was 1 in all batch reactors. The F/I ratio was based on the initial VS of the feces and the inoculum. Upon addition of the designated amounts of inoculum and feces in each batch reactor, their operating volume was adjusted to 0.16 L with addition of ddH₂O. The headspace of each batch reactor was purged with nitrogen gas (99.99% purity) for approximately 2

min to ensure anaerobic conditions. Subsequently, all batch reactors were sealed and placed in a heat chamber at 37 ± 0.5 °C and they were daily manually mixed. Headspace samples were taken for biogas composition analysis almost daily for 37 days. In addition, five parallel sacrificial batch reactors were set for each treatment and collected at 0, 7, 17, 27, and 37 days to determine eventual changes in the VFA profile and VA dissipation along the anaerobic digestion process. Regarding VA residue analysis, samples were centrifuged for 15 min at 10,000 rpm. The solid phase was extracted and analyzed in HPLC, while the liquid phase was diluted 1:2 with water, filtered to remove suspended solids and directly analyzed in HPLC.

2.5.2. Methane measurements

Samples (0.15 ml) were daily collected with a gas-tight syringe and analyzed in a gas chromatograph (GC-2010plusAT, Shimadzu, Japan) equipped with a thermal conductivity detector (GC-TCD) and two columns: (a) GC-Column, FS, Valco-PLOT HayeSep D and (b) VP- Molesieve Column, FS, ValcoBond, 5AFused Silica. Helium gas was used as a carrier gas and the GC was calibrated by using a certified gas containing 60% methane- 40% carbon dioxide (Kalamaras et al., 2020). CH₄ concentration was used to calculate the accumulative production. The measured methane volumes were adjusted to the volumes at standard temperature (0 °C) and pressure (1 atm). The experiment was terminated when methane production during three consecutive days was < 1% of the accumulated volume of methane, as established by Holliger et al. (2016).

2.5.3. VFA measurements

VFA measurements were performed as described by Christou et al. (2021). Briefly, 5 ml samples from each batch reactor were extracted with 0.1 ml of H₃PO₄ and the pH was measured. Samples were centrifuged twice for 10 min at 12,000 rpm and then analyzed in a gas chromatograph (GC-2010plusAT, Shimadzu) equipped with a flame ionization detector (GC-FID) and a ZB-FFAP column (Zebron-Phenomenex). Column

oven temperature was set at 60 °C for the first 3.5 min, then increased at 25 °C/min rate to 130 °C and then at 10 °C /min to 235 °C, with a final hold of 5 min. The injection sample volume was 1 µl and helium was used as carrier gas.

2.6. Soil dissipation of VAs

Four soils with different physicochemical characteristics in terms of pH, organic carbon content and soil texture (Annex I: Table S1) were collected in the region of Thessaly, Greece, from different field sites named Livadi, Zappeio, Rodia, and University. Fresh soil samples were sieved and stored at 4°C until use, with the storage period not exceeding 2 weeks. Fresh feces of untreated pigs were collected from the same commercial pig farm as described above and they were stored at ambient temperature for a period of 30 days simulating a short storage period of feces before manuring. Each soil was divided into three portions of 400 g. One of these samples was fumigated with chloroform as described by Lagos et al. (2021). Briefly, soils were fumigated under vacuum with chloroform for 7 days at 30°C in a vacuum desiccator. Samples were the incubated moist at room temperature (23–25°C) for a week and the soil was subjected to a second fumigation for 7 days to ensure the eradication of any microbial propagules survived during the first fumigation event. Upon completion of the second fumigation period traces of chloroform were removed from by repeated vacuum application and the fumigated soils were left overnight before further processed. The two remaining soils were maintained without fumigation (non-fumigated). The moisture content in all soils was adjusted to 40% of their water holding capacity. The fumigated (FuS) and one of the non-fumigated samples (S) per soil were treated directly with methanol or acetonitrile solutions of the two VAs aiming to a final concentration of 2.5 µg g⁻¹. The second non-fumigated soil sample was amended with feces (2% on a weight basis), which had been treated with either of the two VAs aiming to a final soil concentration of 2.5 µg g⁻¹. This nominal concentration was selected based on the residual amounts of VAs found in animal feces after ambient storage or anaerobic digestion assuming a

manure application rate of 2% (Xu et al., 2008). Only for the Zappeio soil, we employed two extra treatments to get insights into the role of feces in TIA dissipation in soil. Hence one non-fumigated sample of the Zappeio soil was treated with TIA-amended fumigated feces (treatment S-FuFe) and one fumigated soil sample was treated with TIA-amended feces (treatment FuS-Fe). In these treatments the final TIA dose in soil was again $2.5 \mu\text{g g}^{-1}$. Subsequently, for all treatments samples were divided into subsamples of 20 g which were placed in aerated plastic bags and incubated in the dark at 25°C. Immediately after treatment (0 days) and 5, 10, 25, and 50 days after, triplicate samples per soil and treatment were analyzed for TIA and TLM residues by HPLC. In summary our experimental setup resulted in the following treatments for each of the soils: (i) non-fumigated soil directly amended with VAs (S) (ii) fumigated soil directly amended with VAs (FuS) (iii) non-fumigated soil treated with VA-amended feces (S-Fe).

2.7. Data analysis

The single first order (SFO) and three biphasic kinetic models [Hockey Stick (HS), first order multi-compartment (FOMC) and double first order in parallel (DFOP)] were used to calculate the dissipation kinetics for TIA and TLM in feces and soil (FOCUS, 2006). The χ^2 test as well as visual inspection were used to assess the agreement between calculated and observed data. Data analysis was performed with the mkin v1.0.3 package (Ranke, 2020) of the R software v4.0.2 (R Core Team, 2021). Cumulative methane production was modeled using the complex substrate adapted versions (Zwietering et al., 1990) of frequently used microbial growth models, also used to model cumulative biogas production (Ware and Power, 2017), like: (a) the Logistic model, (b) the Gompertz model, (c) the Richards and Stannard model (the latter two resolve to the same formula after the Zwietering modification). The Logistic model provided the best consensus Akaike Information Criterion (AIC), Bayesian Information Criterion and log likelihood values (Annex I: Table S2) and was therefore used in

following analysis. Modeling was performed with the nlme v3.1–151 package (Pinheiro et al., 2020) of the R software according to the command set provided in the supporting information. VFA production patterns were also modeled for obtaining their 50% disappearance time (DT₅₀) using the drc v3.0–1 package (Ritz et al., 2016) of the R software. For doing so we have tested all available 3-, 4- and 5-parameter models and chose the consensus per treatment (4 parameter Brain-Cousens for TIA and 4 parameter Weibull for TLM and the control; Annex I: Table S3). Multiple Student's t-tests were performed between treatments for pairwise comparisons of the maximum CH₄ yield, the maximum CH₄ production rate, the CH₄ production lag time model parameters, and the 50% VFA dissipation time (DT₅₀). The obtained P-values were adjusted for multiple hypothesis testing with the False Discovery Rate (FDR), or Benjamini-Hochberg algorithm (Benjamini and Hochberg, 1995).

2.8. Ethical statement

The study was carried out in compliance with the national animal welfare regulations and following the context of relevant EU legislation for animal experimentations (Directive 86/609/EC). There was absolutely no handling of the animals and samples were collected from the pen floor. Treatment was performed by registered veterinarians, according to the farm routine procedures, and caused no suffering. Samples were collected only after the farmers' consent had been obtained.

3. Results and discussion

3.1. Excretion kinetics of TIA and TLM in pig faecal material under different administration regimes

We first explored the excretion temporal dynamics of TIA and TLM under the hypothesis that different administration regimes will result in different residual amounts and different excretion temporal patterns of the VAs in pig excreta. The mode of

administration did not affect the concentration levels of TIA which ranged from 1.21 to 5.99 $\mu\text{g g}^{-1}$ in the excreta of pigs receiving TIA via feed compared to 0.55–4.86 $\mu\text{g g}^{-1}$ in the excreta of pigs receiving TIA via water (Fig. 1a and b). On the contrary the mode of administration strongly affected the temporal residual patterns of TIA in pig feces. When administrated through food, TIA was detected at higher levels during the 7-day period of administration and dropped to significantly lower levels right after the first days of the withdrawal period (Fig. 1a). Whereas, when administered through water TIA was detected at low levels in feces during the 5-day administration period and peaked during the withdrawal period from day 9 onwards (Fig. 1b). The different temporal excretion patterns of TIA in the different administration regimes might be associated with differences in the distribution of the VA in the animal tissues. Previous studies have noted that administration of VAs through feed or subcutaneously could result in differences in the concentrations of the VAs in the animal organs (Halling-Sørensen et al., 2001; Qiu et al., 2016).

In contrast to TIA, the mode of administration had a strong influence on the residual concentrations of TLM in feces. Administration through feed led to significantly higher residues of TLM in pig feces (11.5–749.6 $\mu\text{g g}^{-1}$) (Fig. 1c), compared to its levels in feces upon water administration (4.3–92.6 $\mu\text{g g}^{-1}$) (Fig. 1d). This could be associated with the high water solubility of TLM (566,000 mg L^{-1}) which might have facilitated its higher excretion through urine, not considered and analyzed in our study. In support of this, Qiu et al. (2016) showed that the distribution of four different sulfonamide VAs in pig feces and urine depended on the water solubility of the tested compounds with the more water-soluble compounds (sulfachloropyridazine, sulfadimoxine and sulfaquinoxaline) being detected mostly in urine compared to the more lipophilic sulfamerazine which partitioned in feces. TLM showed similar temporal residual patterns in pig feces regardless of the mode of administration, with high residual

amounts observed during the 7- and 5-day administration period and decreasing gradually during the following withdrawal period.

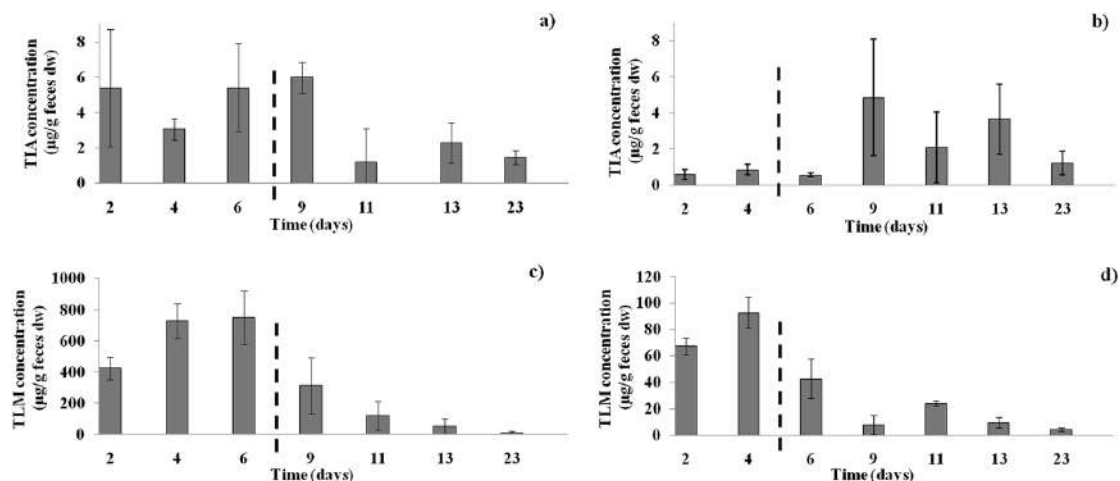


Figure 1: The temporal kinetic patterns of TIA (a, b) and TLM (c, d) in pig feces after their administration through feed (a, c) or water (b, d). Each value is the mean of three replicates $\pm$ standard deviation. The vertical dotted line indicates the ending of the administration period of VAs which was day 7 for feed administration and day 5 for water administration.

Between the two studied VAs and regardless of the mode of administration, TIA was detected in feces at significantly lower concentrations throughout the study ($0.55\text{--}5.99\text{ }\mu\text{g g}^{-1}$) compared to TLM ($4.3\text{--}749.6\text{ }\mu\text{g g}^{-1}$) (Fig. 1). Although TLM was applied at higher amounts ($1008\text{--}1200\text{ mg/piglet}$ during the administration period) compared to TIA ($528\text{--}600\text{ mg/piglet}$) the differences observed in feces could not be explained just by the difference in the dose rates. We speculate that the higher water solubility of TLM compared to the lipophilic TIA (0.524 mg L^{-1}) might have resulted in lower absorption of the former by the internal animal tissues facilitating its higher excretion in feces.

To date a few studies have followed the dynamics of the residues of VAs in animal excreta with most of them focusing on tetracyclines (oxytetracycline, doxycycline), fluroquinolones (ciprofloxacin, enrofloxacin) (Peng et al., 2016; Serrano et al., 2010), suflonamides (sulfamerazine, sulfachloropyridazine, sulfadimoxine, sulfaquinoxaline)

(Qiu et al., 2016) and penicillins (amoxicillin) (Peng et al., 2016), but not on macrolides like TLM and pleuromutulins like TIA. In all these studies, VAs showed a rapid dissipation after the end of the administration period with their residues reaching to levels below the limit of detection within 4–5 days (Peng et al., 2016; Qiu et al., 2016). This was not the case in our study where TIA and TLM dissipated at a slower rate with residual amounts in feces, exceeding 1.46 and 11.52 $\mu\text{g g}^{-1}$ respectively, at the end of a 16- to 18-day withdrawal period. Our results demonstrate that TIA and TLM could persist at considerable levels in animal feces for at least 23 days when administered to animals according to the label and suggest that longer withdrawal periods should be implemented for TIA and TLM in pig farming units to minimize the risk for carryover of VA residues in agricultural soil.

3.2. Persistence of TIA and TLM in faecal material

Following up on the persistence of VA residues in pig feces at the end of the withdrawal period, we further explored the dissipation of TIA and TLM during: (a) storage in stockpiles under ambient conditions and (b) anaerobic digestion. These are two of the most frequently methods applied for the treatment of pig feces before their application in agricultural soils (Van Epps and Blaney, 2016).

3.2.1. Persistence of TIA and TLM in faecal material during storage at ambient conditions

The dissipation of TIA and TLM in feces during storage at ambient conditions varied with the former showing a slightly lower persistence, with DT_{50} values of 5.9–35.9 days, compared to the latter (23.5–49.8 days) (Fig. 2, Table 1). The dissipation of TIA followed a dose-dependent pattern which became more evident when looking at the DT_{90} values, that varied from 19.3 days at the lowest dose rate (0.5 $\mu\text{g g}^{-1}$) to 119.3 days at the highest dose rate (50 $\mu\text{g g}^{-1}$). On the contrary, TLM dissipation showed no dose-dependent pattern (Fig. 2, Table 1). Berendsen et al. (2018) reported DT_{50} values

for TIA and TLM, applied at $0.5 \mu\text{g g}^{-1}$, of 101 and 47 days respectively, compared to 5.9 and 39.5 days observed in our study at the same dose rate. The persistence of TIA could be further prolonged under anaerobic storage conditions of liquid pig manure as denoted by Schlüsener et al. (2006) who reported no appreciable degradation of $2 \mu\text{g g}^{-1}$ of TIA after 180 days. It should be noted that apart from the complete dissipation of TIA in faecal material treated with the lowest dose rate ($0.5 \mu\text{g g}^{-1}$), in all other cases both VAs persisted at the end of the 60-day storage period at levels ranging from 20% to 40% of their starting levels. The DT_{90} values of TIA and TLM in our study suggest that stockpiling of feces containing these VAs at levels which are expected to be found at the onset of the storage period will not lead to complete dissipation of their residues and further handling or extension of the storage period beyond 4–6 months might be necessary to ensure complete removal.

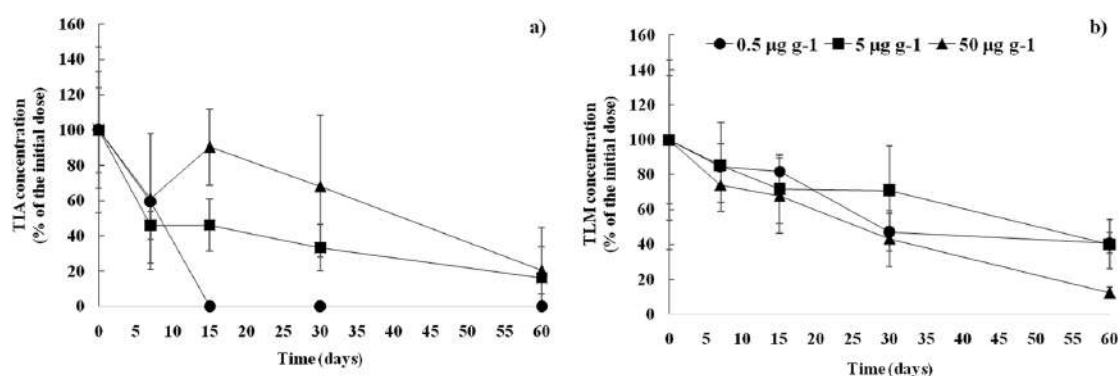


Figure 2: The dissipation of TIA (a) and TLM (b) in pig faecal material during storage at ambient conditions. Each value is the mean of three replicates $\pm$ standard deviation.

Table 1: The dissipation kinetic parameters of TIA and TLM applied in faecal material at three different dose rates (0.5, 5 and 50 $\mu\text{g g}^{-1}$) during storage at ambient conditions. The kinetic models used to fit the data and calculate the dissipation parameters are indicated as follows: SFO; HS.

Antibiotic	Dose rate ($\mu\text{g g}^{-1}$)	Model	DT ₅₀ (days)	DT ₉₀ (days)	k _{deg} (days ⁻¹)	k ₁ (days ⁻¹)	k ₂ (days ⁻¹)	t _b (days)	χ^2 (%)
TIA	0.5	SFO	5.85	19.4	0.119				Inf
	5	HS	5.95	83.6		0.116	0.021	5.98	12.63
	50	SFO	35.9	119.3	0.019				17.86
TLM	0.5	SFO	39.5	131.2	0.018				7.39
	5	SFO	49.8	165.5	0.014				5.21
	50	SFO	23.5	77.9	0.030				5.54

Table 2: The dissipation kinetic parameters of TIA and TLM applied in faecal material at three different dose rates (0.5, 5 and 50 $\mu\text{g g}^{-1}$) during anaerobic digestion. The kinetic models used to fit the data and calculate the dissipation parameters are indicated as follows: SFO; HS.

Antibiotic	Dose rate ($\mu\text{g g}^{-1}$)	Model	DT ₅₀ (days)	DT ₉₀ (days)	k _{deg} (days ⁻¹)	k ₁ (days ⁻¹)	k ₂ (days ⁻¹)	t _b (days)	χ^2 (%)
TIA	0.5	HS	11	276.3		0.116	0.006	5.71	4.61
	5	SFO	>365	>365	<0.0001				4.94
	50	SFO	>365	>365	<0.0001				11.67
TLM	0.5	HS	25.6	49.5		<0.0001	0.067	1.53	11.14
	5	HS	82.6	361.1		0.067	0.006	3.51	7.13
	50	SFO	157	>365	0.004				12.17

3.2.2. Persistence of TIA and TLM during anaerobic digestion and effects on bimethanation

Beyond stockpiling, animal feces are often used as feed for anaerobic digestion and biogas production (Ward et al., 2008). Most of the studies available have looked into the effects of VAs on bimethanation (Sanzet al., 1996; Lallai et al., 2002; Beneragama et al., 2013), while less is known about the dissipation of VAs during anaerobic digestion (Mohring et al., 2009; Mitchell et al., 2013; Panseri et al., 2013). Both VAs tested were persistent during anaerobic digestion (Fig. 3). TIA was more persistent than TLM with their DT_{50} values ranging from 11 to > 365 days (extrapolated) and from 25.6 to 157 days (extrapolated), respectively (Table 3). The dissipation of both VAs seemed to follow a dose-dependent pattern with a significant retardation in their dissipation at high dose rates. This was more prominent for TIA which showed negligible dissipation when applied at the 5 and the 50 $\mu\text{g g}^{-1}$ dose rate ($DT_{50} > 365$ days). Regarding TLM, its dissipation showed a clear dose-dependent pattern with its DT_{50} values increasing gradually from 25.6 at the 0.5 $\mu\text{g g}^{-1}$ application rate to 82.6 and 157 days at the 5 and 50 $\mu\text{g g}^{-1}$ rates respectively (Fig. 3b, Table 2). Little is known about the removal rates of TLM and TIA during anaerobic digestion. Yin et al. (2019) showed that TLM was the sole VA of the ones tested (florfenicol, tylosin the others) that did not completely dissipate during the anaerobic digestion process, although its dissipation percentage was still above 98% at starting concentrations higher than those used in the current study (100, 200, 300 and 400 mg kg^{-1}). In a similar study Sui et al. (2018) reported only a partial dissipation of TLM during anaerobic digestion of pig manure. Our findings indicate a limited dissipation of TIA and TLM during anaerobic digestion suggesting a potential toxicity of these VAs on the active microbiota in feces (Wang et al., 2006).

Beyond the dissipation of VAs, we followed the dynamics of the anaerobic digestion process in the VA-treated and untreated feces. The presence of both VAs had a

significant inhibitory effect on methane production (Fig. 4a and b; Annex I: Fig. S1; Table S4).

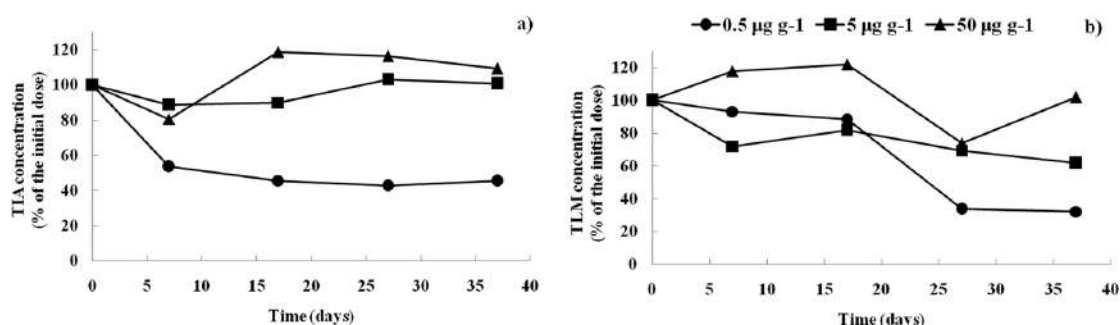


Figure 3: The dissipation patterns of TIA (a) and TLM (b) in animal faecal material during anaerobic digestion.

When the total measured methane production in the different treatments was considered, all TLM treated samples and the samples treated with the two higher dose rates of TIA (5 and 50 µg g⁻¹) showed significantly lower methane production compared to the untreated control (Annex I: Fig. S1). In contrast to our findings, Yin et al. (2019) did not identify any strong inhibitory effects of TLM on the biomethanation process. VAs, often, have a negative effect on methanogenesis and the discrepancies in the results between studies may be due to different experimental conditions (Huang et al., 2014; Aydin et al., 2015). Modeling of the cumulative production patterns of methane in the different treatments allowed us to calculate the maximum estimated methane production (y_{max}) and the maximum methane production rate (k_{max}). Both TLM and TIA significantly inhibited the maximum estimated methane production at all dose rates in a dose-dependent manner (Annex I: Table S4; $FDR \leq 0.05$). TLM significantly reduced methane production rates only at the highest dose rate (50 µg g⁻¹), compared to TIA which significantly reduced methane production rate at the two higher dose rates (5 and 50 µg g⁻¹) with the inhibitory patterns showing a clear dose-dependent pattern (Annex I: Table S4).

We further measured and modeled the production of VFAs during the anaerobic digestion process (Fig. 4c and d). Total VFAs were depleted in all treatments during anaerobic digestion starting from concentrations $> 1250 \text{ mg L}^{-1}$ at day 1 and decreasing to $< 200 \text{ mg L}^{-1}$ at the end of the study. In total 10 VFAs were detected in all treatments with acetic acid, propionic acid, butyric acid, and lactic acid being the most abundant at the onset of anaerobic digestion. Acetic acid remained the most abundant VFA at the end of the study (Annex I: Fig. S2). Total VFAs measured at the end of the study were significantly reduced only in the feces treated with the two higher concentration levels of TIA, whereas no significant effects of TLM were observed (Annex I: Fig. S2). When VFA production temporal measurements were fitted to the Brain-Cousens model, which showed the best fit to the data, we noted a trend of increasing production of VFAs in the TIA-treated feces compared to the blank at day 8 (Fig. 4c), whereas no such differences were evident for TLM (Fig. 4d). When the DT_{50} values for total VFAs in the different treatments were calculated and compared (Annex I: Table S5), significant differences were evident only between the highest TIA concentration and the untreated control (became nonsignificant when the Benjamini-Hochberg approach to FDRs was implemented). Overall, our findings suggest that anaerobic digestion of feces containing even low levels of TLM and TIA will not enhance their removal, instead the presence of these VAs is expected to negatively affect bimethanation performance.

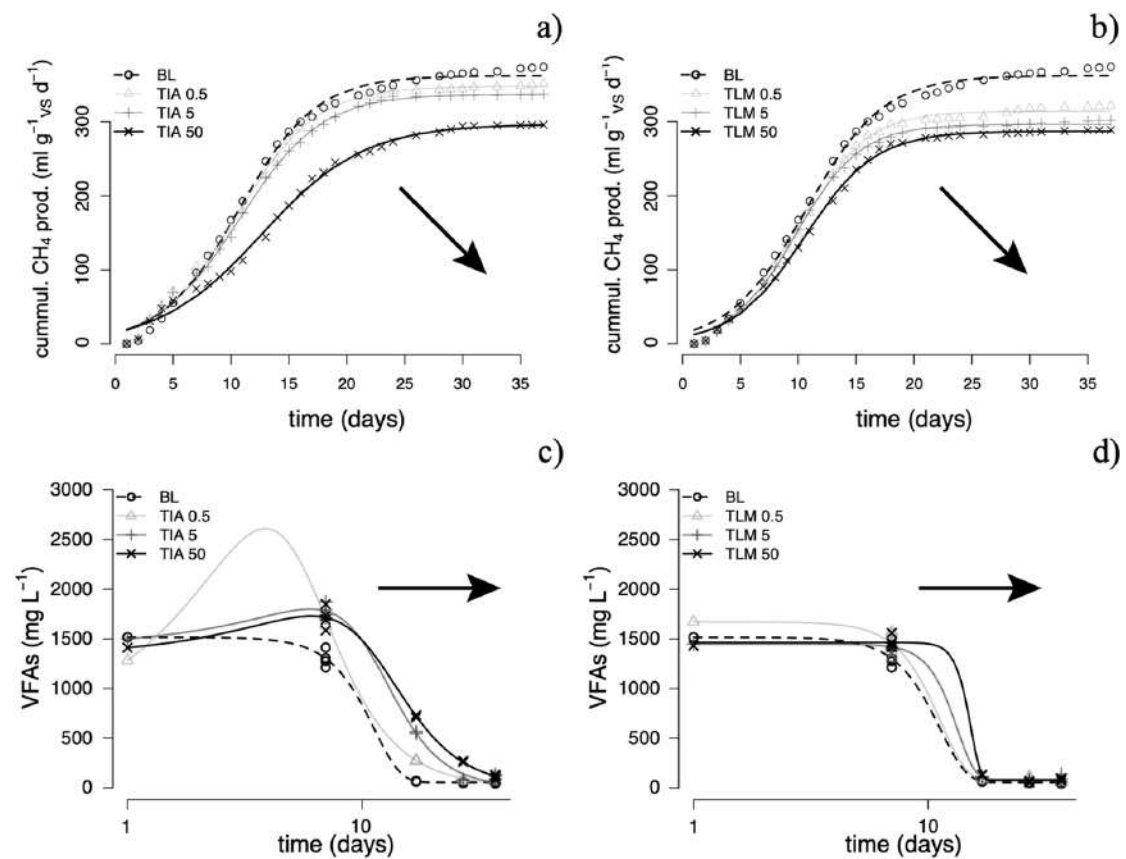


Figure 4: Modeling of the cumulative production of methane (a, b) and VFAs (c, d) during anaerobic digestion of animal feces treated with 0.5, 5 and 50 $\mu\text{g g}^{-1}$ of TIA (a, c) and TLM (b, d). Each value is the mean of three replicates $\pm$ standard deviation

Table 3: The dissipation kinetic parameters of TIA and TLM in four different soils where the VAs were applied directly in non-fumigated (S) or fumigated soil samples (FuS) or through VA-treated feces (S-Fe). Only in Zappeio soil the dissipation of TIA was determined either in soil amended with TIA treated fumigated feces (S-FuFe) or in fumigated soil treated with TIA-treated feces (FuS-Fe). The kinetic models used to fit the dissipation data are indicated as follow: SFO; HS.

Soil	Treatment	Model	DT ₅₀ (days)	DT ₉₀ (days)	k _{deg} (days ⁻¹)	k ₁ (days ⁻¹)	k ₂ (days ⁻¹)	t _b (days)	χ ² (%)
Tiamulin									
Livadi	S	SFO	80.0	265.7	0.009				1.163
	FuS	HS	360.9	>365		0.053	0.002	1.6	1.254
	S-Fe	SFO	>365	>365	<0.0001				1.503
University	S	SFO	31.7	105.3	0.022				5.822
	FuS	SFO	42.8	142.3	0.016				9.065
	S-Fe	SFO	60.0	199.4	0.012				3.85
Rodia	S	SFO	20.1	66.9	0.034				5.944
	FuS	SFO	33.7	112.1	0.021				6.319
	S-Fe	HS	10.9	174.1		0.064	0.007	19.8	5.572
Zappeio	S	HS	94.5	>365		0.046	0.005	4.6	4.793
	FuS	SFO	30.9	102.6	0.022				10.83
	S-Fe	SFO	57.1	189.6	0.012				7.885
	S-FuFe	HS	162.0	>365		0.114	0.001	4.2	1.601
	FuS-Fe	HS	9.8	49.4		0.071	0.040	1.1	3.742
Tilmicosin									
Livadi	S	SFO	141.1	>365	0.005				7.16
	FuS	SFO	>365	>365	<0.0001				4.257
	S-Fe	SFO	170.2	>365	0.004				6.244
University	S	HS	158.4	>365		0.052	0.003	3.2	3.116
	FuS	SFO	>365	>365	0.001				2.118
	S-Fe	SFO	>365	>365	0.001				3.875
Rodia	S	SFO	121.1	>365	0.006				4.105
	FuS	SFO	>365	>365	0.001				5.631
	S-Fe	HS	>365	>365		0.099	0.001	28.6	1.896
Zappeio	S	SFO	128.7	>365	0.005				3.864
	FuS	SFO	348.6	>365	0.002				2.921
	S-Fe	HS	>365	>365		123.5	<0.0001	4.0	5.48

3.3. The dissipation of TIA and TLM in agricultural soils

Our measurements of the persistence of VAs in the earlier stages of their lifecycle suggested the presence of residual amounts of TIA and TLM in feces before application to agricultural soils. Therefore, we evaluated the dissipation of TIA and TLM in a range of different soils under two application regimes aiming to determine the inherent soil persistence of these VA compounds (direct soil application) and their soil persistence under realistic practical conditions (reaching soil through application of VA-containing feces). Furthermore, we explored the nature of the dissipation observed, biotic or abiotic, by including soil fumigated controls.

The dissipation of the two VAs varied in the different soils and treatments (Figs. 5 and 6). TIA was generally less persistent, with DT_{50} values in the non-fumigated soils ranging from 20.1 to 94.5 days, compared to TLM with corresponding DT_{50} values of 121.1–158.4 days (Table 3). The few soil studies available for TIA and TLM have reported DT_{50} values which are in the same range with our values. For example, Schlüsener and Bester (2006) reported a DT_{50} value of TIA of 16 days, although the compound did not completely degrade in soil after 120 days. More recently, Berendsen et al. (2021) reported a TLM DT_{50} of 108 days in a sandy soil from the Netherlands. TIA was more persistent in the soils Livadi and Zappeio characterized by higher organic carbon content (over 2%) in line with its higher lipophilicity and hence higher adsorption affinity onto soil organic matter compared to the less lipophilic TLM, which did not show a clear soil-dependent behavior. Overall, our findings suggest that once released in soil TIA and particularly TLM are expected to persist with unknown effects to the soil microbiota, the evolution and dispersal of novel ARGs and translocation to other environmental compartments.

We further explored the contribution of soil microbes in the dissipation of TIA and TLM. Despite their biocidal nature, several previous studies have verified the major

contribution of soil microbiota in the soil dissipation of VAs (Accinelli et al., 2007, 2017). Soil fumigation resulted in a significant reduction in the dissipation rate of TIA in all soils except Zappeio ($DT_{50\text{fum}} = 30.9$ days vs $DT_{50\text{s}} = 90.1$ days) (Table 3, Fig. 5). In contrast, the inhibitory effect of fumigation on the dissipation of TLM was uniform in all soils tested ($DT_{50\text{fum}} > 345$ days vs $DT_{50\text{s}} = 121.1\text{--}158.4$ days). These findings suggest a key role of the soil microbiota in the degradation of these pharmaceuticals. In line with our findings, Berendsen et al. (2021) showed that the degradation of TLM in a single soil was strongly hampered by soil sterilization.

To further explore the mechanism behind the differential dissipation patterns of TIA in the Zappeio soil, we tested its dissipation in fumigated soil samples amended with TIA-treated feces (FuS-Fe) and also in nonfumigated soil samples amended with TIA-treated fumigated feces (SFuFe). In the former, the dissipation of TIA was significantly accelerated with $DT_{50} = 9.8$ days, conversely in the latter the dissipation of TIA was strongly retarded ($DT_{50} = 162$ days) (Table 3). These results suggest that in the specific soil, characterized by high organic carbon and clay content, feces act more as a reservoir of microbes that could degrade TIA, especially in the absence of competition by the soil microbiota, rather than a surface for the adsorption of the VA. Previous studies have suggested that acclimated swine manure from livestock farms using TIA could be a source for the isolation of bacterial able to effectively degrade TIA (Nguyen et al., 2019).

Eventually, we explored the dissipation of TIA and TLM under realistic soil exposure conditions, whereby VAs reach soil through the application of VA-contaminated manures. The dissipation of TIA was significantly retarded in all soils with the sole exception again of the Zappeio soil where a faster dissipation was evident when TIA was applied in soil through feces ($DT_{50\text{s}} = 90.1$ days vs $DT_{50\text{s+Fe}} = 57.1$ days). Regarding TLM, we observed a significant increase in its persistence when applied to

soil through feces, with extrapolated DT₅₀ values ranging from 170.2 to over 365 days (Table 3).

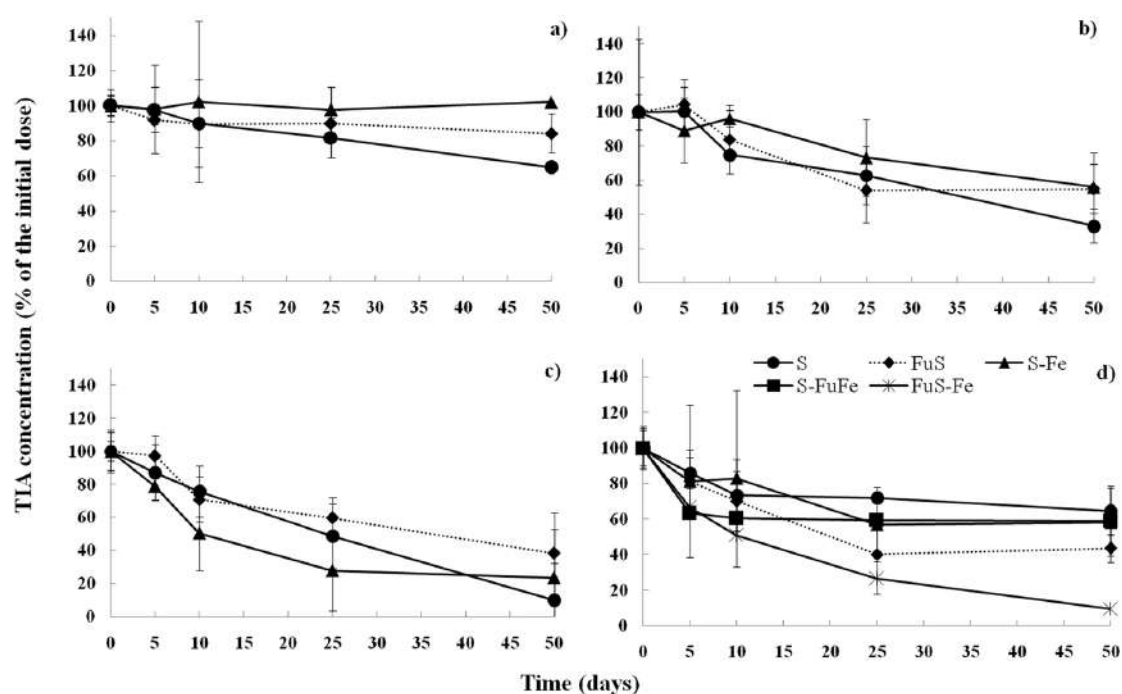


Figure 5: The dissipation patterns of TIA in four different soils named (a) Livadi, (b) University, (c) Rodia and (d) Zappeio, using different modes of application as follows: (S) direct application in non-fumigated soil; (FuS) direct application in fumigated soil; (S-Fe) application in non-fumigated soil through TIA-treated feces. Only in the Zappeio soil the dissipation of TIA was studied in further two treatments: (S-FuFe) application in nonfumigated soil through TIA-treated fumigated manure; (FuS-Fe) application in fumigated soil through TIA-treated feces. Each point is the mean of three replicates with error bars denoting the standard deviation.

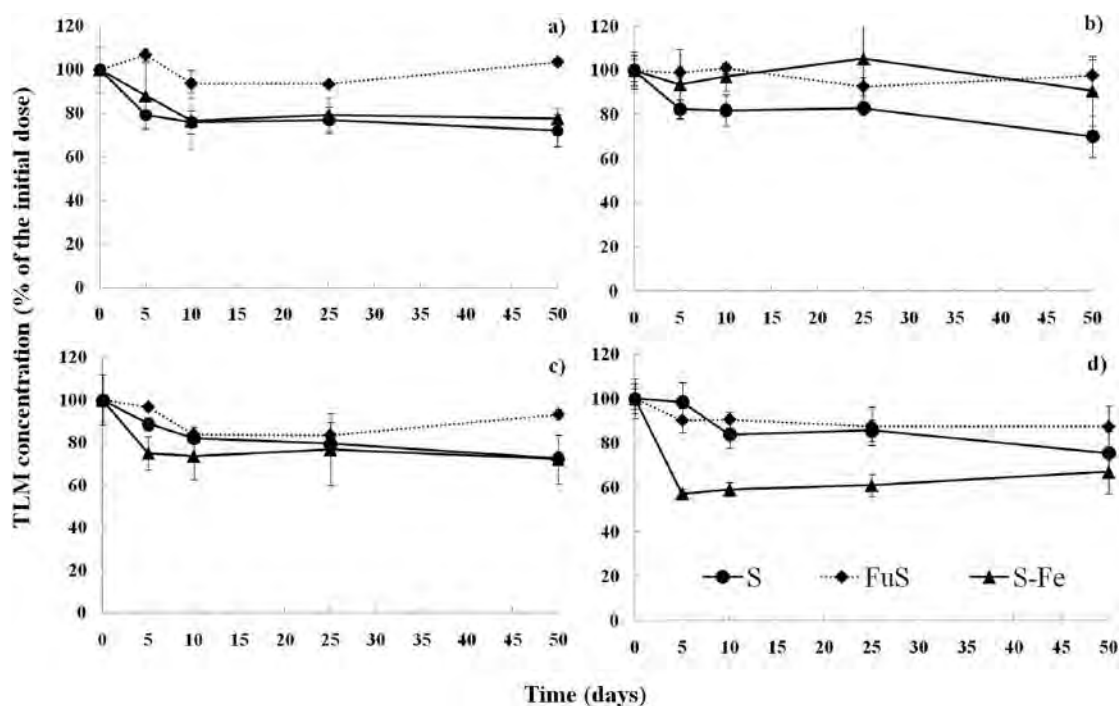


Figure 6: The dissipation patterns of TLM in four different soils named (a) Livadi, (b) University, (c) Rodia and (d) Zappeio, using different modes of application as follows: (S) direct application in non-fumigated soil; (FuS) direct application in fumigated soil; (S-Fe) application in non-fumigated soil through TLM treated feces.

4. Conclusions

Our study provides first evidence for the persistence of TIA and TLM along the chain from animal administration to agricultural soils. Overall TLM appears to be excreted at higher amounts in animal feces and it was more persistent in feces during ambient storage and in soil, compared to TIA. Beyond their persistence, both compounds negatively affected bimethanation suggesting that the use of animal feces originated from swine farms using TIA and TLM for biogas production should be done with caution since the presence of these VAs even at low levels could compromise the efficiency of the process. Overall, our data suggest that the administration of TIA and TLM in pig farms and the subsequent use of feces for manuring of agricultural soils are expected to lead to dispersal of residues of these VAs in the soil environment with yet unexplored consequences for environmental contamination and human or animal health.

Following studies will focus on the effects of these VAs on the soil microbial community and on their contribution in the dispersal of ARGs along the soil-plant continuum.

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Annex I – Chapter 2 Supplementary Data

Table S1: The physicochemical properties of the four different soils used in Chapter 2

Soil	pH	Organic carbon content (%)	Sand (%)	Silt (%)	Clay (%)
Livadi	5.9	2.31	40.82	47.82	11.36
University	8.8	0.60	37.64	26.0	36.36
Rodia	7.9	1.37	45.82	36.0	18.18
Zappeio	6.5	2.30	36	20	44

Table S2: CH₄ model selection associated statistics (Akaike information criterion - AIC; Bayesian information criterion - BIC; log likelihood) for all tested models. Best values per model are provided in red.

	MODIFIED LOGISTIC			MODIFIED GOMPERTZ			MODIFIED RICHARDS STANNARD		
	AIC	BIC	loglik	AIC	BIC	loglik	AIC	BIC	loglik
BL	223,4826	229,0874	-107,741	178,4632	184,068	-85,2316	180,4644	187,4704	-85,2322
TIA.0.5	211,3842	216,989	-101,692	225,8764	231,4812	-108,938	212,6916	219,6976	-101,346
TIA.5	205,238	210,8428	-98,619	220,1115	225,7163	-106,056	205,3467	212,3527	-97,6734
TIA.50	211,0947	216,6995	-101,547	222,9492	228,554	-107,475	212,8983	219,9043	-101,449
TLM.0.5	195,4876	201,0924	-93,7438	200,5513	206,1561	-96,2757	187,7854	194,7914	-88,8927
TLM.5	184,5709	190,1757	-88,2855	176,6112	182,216	-84,3056	157,8364	164,8424	-73,9182
TLM.50	188,045	193,6498	-90,0225	211,5485	217,1533	-101,774	189,4856	196,4916	-89,7428

Table S3: VFA models and model selection associated statistics (W1.4: four-parameter Weibull 1; W2.3: three-parameter Weibull 2; W2.4: four-parameter Weibull 2; LL.4: four-parameter log logistic; LL2.3: three-parameter log logistic 2; BC.4: four-parameter Brain-Cousens). Best model Akaike information criterion (AIC) values are provided in red.

BL							
	treatment	mod	AIC	Estimate	Std. Error	Lower	Upper
BL_W1.4_DT10	BL	W1.4	143,21	6,286	0,463	5,238	7,335
BL_W1.4_DT50	BL	W1.4	143,21	10,253	0,916	8,18	12,326
BL_W1.4_DT90	BL	W1.4	143,21	14,003	2,065	9,332	18,675
BL_LL.4_DT10	BL	LL.4	143,211	6,597	0,32	5,874	7,321
BL_LL.4_DT50	BL	LL.4	143,211	8,864	1,16	6,241	11,488
BL_LL.4_DT90	BL	LL.4	143,211	11,91	3,421	4,17	19,65
TIA 0.5							
	treatment	mod	AIC	Estimate	Std. Error	Lower	Upper
TIA 0.5_BC.4_DT10	TIA 0.5	BC.4	121,609	13,549	10,064	-9,659	36,756
TIA 0.5_BC.4_DT50	TIA 0.5	BC.4	121,609	17,509	13,02	-12,515	47,533
TIA 0.5_BC.4_DT90	TIA 0.5	BC.4	121,609	34,737	27,998	-29,828	99,302
TIA 0.5_W2.3_DT10	TIA 0.5	W2.3	155,834	9,225	1,48	5,877	12,574
TIA 0.5_W2.3_DT50	TIA 0.5	W2.3	155,834	12,436	1,225	9,665	15,207
TIA 0.5_W2.3_DT90	TIA 0.5	W2.3	155,834	19,869	2,209	14,873	24,866
TIA 0.5_LL2.3_DT10	TIA 0.5	LL2.3	156,85	2,477	0,516	1,31	3,645
TIA 0.5_LL2.3_DT50	TIA 0.5	LL2.3	156,85	2,684	0,217	2,193	3,175
TIA 0.5_LL2.3_DT90	TIA 0.5	LL2.3	156,85	2,89	0,097	2,671	3,11
TIA 5							
	treatment	mod	AIC	Estimate	Std. Error	Lower	Upper

TIA 5_BC.4_DT10	TIA 5	BC.4	139,621	11,354	0,869	9,351	13,357
TIA 5_BC.4_DT50	TIA 5	BC.4	139,621	15,384	0,642	13,903	16,865
TIA 5_BC.4_DT90	TIA 5	BC.4	139,621	26,901	2,704	20,666	33,136
TIA 5_W1.4_DT10	TIA 5	W1.4	149,641	14,148	12,724	-15,194	43,49
TIA 5_W1.4_DT50	TIA 5	W1.4	149,641	16,259	3,55	8,073	24,446
TIA 5_W1.4_DT90	TIA 5	W1.4	149,641	17,767	3,842	8,907	26,627
TIA 5_LL.4_DT10	TIA 5	LL.4	149,642	14,421	12,913	-15,357	44,198
TIA 5_LL.4_DT50	TIA 5	LL.4	149,642	16,184	4,334	6,191	26,178
TIA 5_LL.4_DT90	TIA 5	LL.4	149,642	18,163	6,55	3,059	33,268
TIA 50							
	treatment	mod	AIC	Estimate	Std. Error	Lower	Upper
TIA 50_BC.4_DT10	TIA 50	BC.4	150,662	12,559	0,783	10,789	14,33
TIA 50_BC.4_DT50	TIA 50	BC.4	150,662	17,822	0,712	16,211	19,432
TIA 50_BC.4_DT90	TIA 50	BC.4	150,662	35,479	3,143	28,368	42,589
TIA 50_W2.3_DT10	TIA 50	W2.3	162,817	10,258	1,079	7,853	12,662
TIA 50_W2.3_DT50	TIA 50	W2.3	162,817	15,938	0,851	14,041	17,835
TIA 50_W2.3_DT90	TIA 50	W2.3	162,817	31,823	3,071	24,98	38,666
TIA 50_W2.4_DT10	TIA 50	W2.4	164,761	10,459	1,674	6,671	14,247
TIA 50_W2.4_DT50	TIA 50	W2.4	164,761	15,811	1,051	13,434	18,188
TIA 50_W2.4_DT90	TIA 50	W2.4	164,761	30,242	8,45	11,126	49,358
TLM 0.5							
	treatment	mod	AIC	Estimate	Std. Error	Lower	Upper
TLM 0.5_W2.4_DT10	TLM 0.5	W2.4	144,697	6,828	0,265	6,217	7,438
TLM 0.5_W2.4_DT50	TLM 0.5	W2.4	144,697	8,32	1,417	5,052	11,587

TLM 0.5_W2.4_DT90	TLM 0.5	W2.4	144,697	11,345	5,319	-0,921	23,611
TLM 0.5_LL.4_DT10	TLM 0.5	LL.4	144,704	6,665	0,478	5,563	7,767
TLM 0.5_LL.4_DT50	TLM 0.5	LL.4	144,704	8,937	1,896	4,566	13,308
TLM 0.5_LL.4_DT90	TLM 0.5	LL.4	144,704	11,984	5,463	-0,613	24,581
TLM 5							
	treatment	mod	AIC	Estimate	Std. Error	Lower	Upper
TLM 5_W1.4_DT10	TLM 5	W1.4	120,308	9,109	1,187	6,372	11,846
TLM 5_W1.4_DT50	TLM 5	W1.4	120,308	12,549	0,883	10,512	14,586
TLM 5_W1.4_DT90	TLM 5	W1.4	120,308	15,392	0,71	13,754	17,03
TLM 5_LL.4_DT10	TLM 5	LL.4	120,338	8,371	0,861	6,387	10,356
TLM 5_LL.4_DT50	TLM 5	LL.4	120,338	10,708	0,971	8,468	12,947
TLM 5_LL.4_DT90	TLM 5	LL.4	120,338	13,696	1,416	10,432	16,961
TLM 50							
	treatment	mod	AIC	Estimate	Std. Error	Lower	Upper
TLM 50_LL.4_DT10	TLM 50	LL.4	137,244	12,445	15,215	-21,975	46,864
TLM 50_LL.4_DT50	TLM 50	LL.4	137,244	14,142	10,21	-8,954	37,238
TLM 50_LL.4_DT90	TLM 50	LL.4	137,244	16,07	3,579	7,973	24,167
TLM 50_LL2.4_DT10	TLM 50	LL2.4	137,245	2,505	0,921	0,422	4,589
TLM 50_LL2.4_DT50	TLM 50	LL2.4	137,245	2,64	0,544	1,409	3,87
TLM 50_LL2.4_DT90	TLM 50	LL2.4	137,245	2,774	0,169	2,392	3,156

Table S4: Pairwise statistics for the CH₄ model maximum estimated production, maximum estimated production rates and lag time. Significant P and FDR values are provided in red.

treat.1	treat.2	max total CH ₄ (ymax)					p-value	FDR
		mean.1	mean.2	std.1	std.2	t		
BL	TIA.0.5	362,43785	348,16045	15,374419	12,646649	3,9281842	0,000465	0,0023249
BL	TIA.5	362,43785	337,42384	15,374419	11,649658	7,1026666	6,72E-08	4,70E-07
BL	TIA.50	362,43785	296,93293	15,374419	15,715518	16,319375	1,83E-16	2,92E-15
BL	TLM.0.5	362,43785	314,18899	15,374419	8,9848255	14,840537	2,33E-15	3,26E-14
BL	TLM.5	362,43785	296,59495	15,374419	7,3517355	21,161952	1,38E-19	2,63E-18
BL	TLM.50	362,43785	287,18839	15,374419	8,2210604	23,640507	5,98E-21	1,26E-19
TIA.0.5	TIA.5	348,16045	337,42384	12,646649	11,649658	3,420087	0,0018245	0,0072979
TIA.0.5	TIA.50	348,16045	296,93293	12,646649	15,715518	13,909514	1,28E-14	1,66E-13
TIA.0.5	TLM.0.5	348,16045	314,18899	12,646649	8,9848255	11,994124	5,65E-13	6,21E-12
TIA.0.5	TLM.5	348,16045	296,59495	12,646649	7,3517355	19,307563	1,80E-18	3,25E-17
TIA.0.5	TLM.50	348,16045	287,18839	12,646649	8,2210604	22,140036	3,86E-20	7,72E-19
TIA.5	TIA.50	337,42384	296,93293	11,649658	15,715518	11,336877	2,28E-12	2,28E-11
TIA.5	TLM.0.5	337,42384	314,18899	11,649658	8,9848255	8,6502795	1,20E-09	1,08E-08
TIA.5	TLM.5	337,42384	296,59495	11,649658	7,3517355	16,233904	2,11E-16	3,16E-15
TIA.5	TLM.50	337,42384	287,18839	11,649658	8,2210604	19,297537	1,83E-18	3,25E-17
TIA.50	TLM.0.5	296,93293	314,18899	15,715518	8,9848255	-5,2210867	1,9999875	1
TIA.50	TLM.5	296,93293	296,59495	15,715518	7,3517355	0,1066981	0,9157388	1
TIA.50	TLM.50	296,93293	287,18839	15,715518	8,2210604	3,0093186	0,005265	0,015795
TLM.0.5	TLM.5	314,18899	296,59495	8,9848255	7,3517355	8,3008277	2,89E-09	2,31E-08
TLM.0.5	TLM.50	314,18899	287,18839	8,9848255	8,2210604	12,14352	4,14E-13	4,97E-12
TLM.5	TLM.50	296,59495	287,18839	7,3517355	8,2210604	4,671585	5,88E-05	0,0003528

max CH4 production rate (k)								
treat.1	treat.2	mean.1	mean.2	std.1	std.2	T	p-value	FDR
BL	TIA.0.5	27,338617	25,538138	5,2267264	4,1324213	1,4800584	0,1492837	1
BL	TIA.5	27,338617	24,011904	5,2267264	3,5755972	2,8772956	0,0073203	0,1098047
BL	TIA.50	27,338617	16,805694	5,2267264	2,944844	9,6164317	1,13E-10	2,37E-09
BL	TLM.0.5	27,338617	27,580168	5,2267264	4,0701394	-0,1997163	1,1569499	1
BL	TLM.5	27,338617	26,589939	5,2267264	3,5271857	0,6503307	0,5204283	1
BL	TLM.50	27,338617	23,423389	5,2267264	3,223317	3,4921941	0,0015077	0,0241236
TIA.0.5	TIA.5	25,538138	24,011904	4,1324213	3,5755972	1,5297616	0,136553	1
TIA.0.5	TIA.50	25,538138	16,805694	4,1324213	2,944844	9,4257553	1,78E-10	3,56E-09
TIA.0.5	TLM.0.5	25,538138	27,580168	4,1324213	4,0701394	-1,9283056	1,9366715	1
TIA.0.5	TLM.5	25,538138	26,589939	4,1324213	3,5271857	-1,0603544	1,702554	1
TIA.0.5	TLM.50	25,538138	23,423389	4,1324213	3,223317	2,2101224	0,0348678	0,4881486
TIA.5	TIA.50	24,011904	16,805694	3,5755972	2,944844	8,5208524	1,65E-09	3,14E-08
TIA.5	TLM.0.5	24,011904	27,580168	3,5755972	4,0701394	-3,6075031	1,9988913	1
TIA.5	TLM.5	24,011904	26,589939	3,5755972	3,5271857	-2,8114202	1,9913909	1
TIA.5	TLM.50	24,011904	23,423389	3,5755972	3,223317	0,6695935	0,5082381	1
TIA.50	TLM.0.5	16,805694	27,580168	2,944844	4,0701394	-11,747029	2	1
TIA.50	TLM.5	16,805694	26,589939	2,944844	3,5271857	-11,663032	2	1
TIA.50	TLM.50	16,805694	23,423389	2,944844	3,223317	-8,3020349	2	1
TLM.0.5	TLM.5	27,580168	26,589939	4,0701394	3,5271857	1,0070351	0,3219718	1
TLM.0.5	TLM.50	27,580168	23,423389	4,0701394	3,223317	4,3852202	0,0001312	0,0023623
TLM.5	TLM.50	26,589939	23,423389	3,5271857	3,223317	3,6298296	0,0010443	0,0177524

Table S5: Pairwise comparison statistics for the VFA DT₅₀ values of the performed treatments. Significant P and FDR values are provided in red.

treat.1	treat.2	DT50.1	DT50.2	std.1	std.2	t_diff	p_H0	FDR
BL	TIA 0.5	11,276671	771,23887	4,300285	3269,0161	0,8381973	0,41707	1,00000
BL	TIA 5	11,276671	86,134883	4,300285	134,64976	2,0034761	0,06643	1,00000
BL	TIA 50	11,276671	98,510113	4,300285	99,423022	3,1605442	0,00752	0,15787
BL	TLM 0.5	11,276671	11,329116	4,300285	7,2703052	0,022386	0,98248	1,00000
BL	TLM 5	11,276671	13,356294	4,300285	2,9263802	1,4415287	0,17309	1,00000
BL	TLM 50	11,276671	15,28667	4,300285	7,8505757	1,615231	0,13026	1,00000
TIA 0.5	TIA 5	771,23887	86,134883	3269,0161	134,64976	0,7549932	0,46371	1,00000
TIA 0.5	TIA 50	771,23887	98,510113	3269,0161	99,423022	0,7416412	0,47149	1,00000
TIA 0.5	TLM 0.5	771,23887	11,329116	3269,0161	7,2703052	0,8381381	0,41710	1,00000
TIA 0.5	TLM 5	771,23887	13,356294	3269,0161	2,9263802	0,8359039	0,41831	1,00000
TIA 0.5	TLM 50	771,23887	15,28667	3269,0161	7,8505757	0,8337728	0,41947	1,00000
TIA 5	TIA 50	86,134883	98,510113	134,64976	99,423022	0,2665789	0,79398	1,00000
TIA 5	TLM 0.5	86,134883	11,329116	134,64976	7,2703052	2,0001797	0,06682	1,00000
TIA 5	TLM 5	86,134883	13,356294	134,64976	2,9263802	1,9483509	0,07329	1,00000
TIA 5	TLM 50	86,134883	15,28667	134,64976	7,8505757	1,8939045	0,08070	1,00000
TIA 50	TLM 0.5	98,510113	11,329116	99,423022	7,2703052	3,1531781	0,00762	0,15787
TIA 50	TLM 5	98,510113	13,356294	99,423022	2,9263802	3,0867454	0,00867	0,16464
TIA 50	TLM 50	98,510113	15,28667	99,423022	7,8505757	3,0087126	0,01007	0,18124
TLM 0.5	TLM 5	11,329116	13,356294	7,2703052	2,9263802	0,9326207	0,36801	1,00000
TLM 0.5	TLM 50	11,329116	15,28667	7,2703052	7,8505757	1,3335733	0,20524	1,00000
TLM 5	TLM 50	13,356294	15,28667	2,9263802	7,8505757	0,8307296	0,42112	1,00000

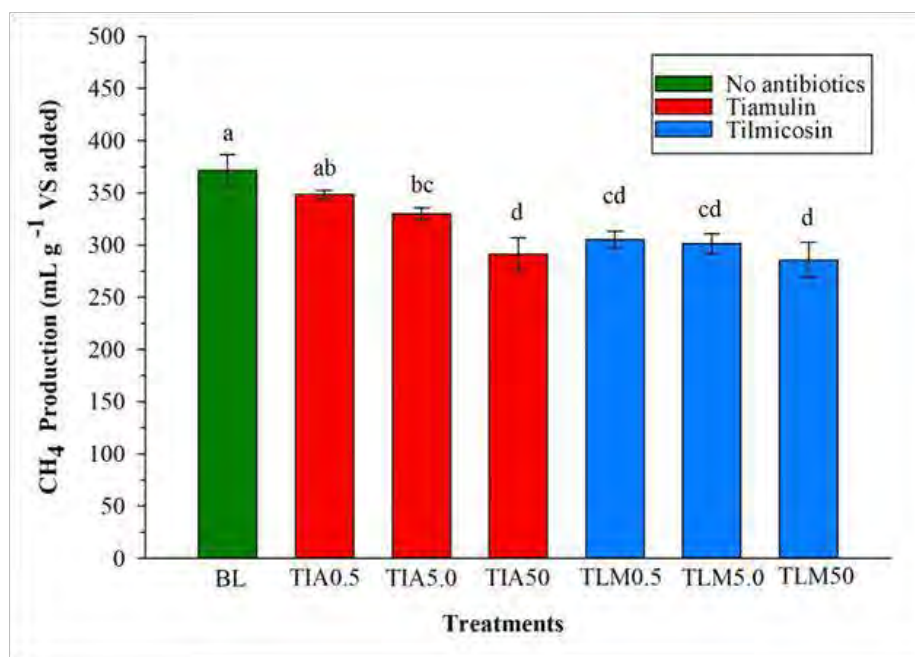


Figure S1: The total methane production during anaerobic digestion of animal feces treated with different dose rates (0.5, 5 and 50 $\mu\text{g g}^{-1}$) of the VAs TIA and TLM. Each value is the mean of three replicates $\pm$ the standard deviation. Within each VA bars designated with the same letter are not significantly different at 0.05 level. BL: blank treatment not treated with VAs.

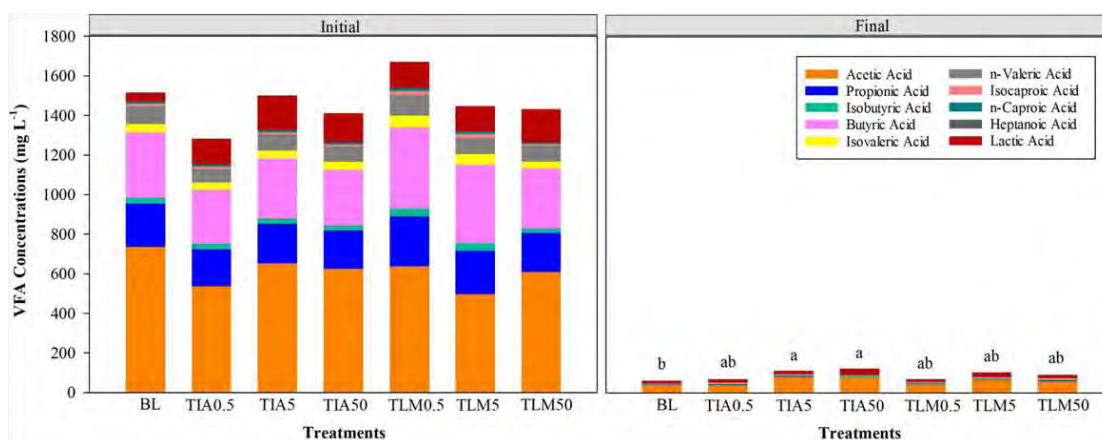


Figure S2: The concentration of VFAs detected at the start and the end of the anaerobic digestion of animal feces treated with different dose rates (0.5, 5 and 50 $\mu\text{g g}^{-1}$) of the VAs TIA and TLM. Each value is the mean of three replicates $\pm$ the standard deviation. BL: blank treatment not treated with VAs.

Chapter 3

Accelerated dissipation, soil microbial toxicity and dispersal of antimicrobial resistance in soils repeatedly exposed to tiamulin, tilmicosin and sulfamethoxazole

The work presented in Chapter 3 is included in the scientific paper:

Katsivelou E, Perruchon C, Karas P A, Sarantidou A, Pappa E, Katsoula A, Ligda P, Sotiraki S, Martin-Laurent F, Vasileiadis S, Karpouzas D G (2023). Accelerated dissipation, soil microbial toxicity and dispersal of antimicrobial resistance in soils repeatedly exposed to tiamulin, tilmicosin and sulfamethoxazole. *Sci Total Environ* 893, 164817, <https://doi.org/10.1016/j.scitotenv.2023.164817>.

1. Introduction

Dispersal of AMR may become a severe burden for the healthcare system, threatening the achievements of modern medicine. This is highlighted in a recent WHO report (World Health Organization, 2014; World Health Organization, 2020) where it is stated that “a post-antibiotic era – in which common infections and minor injuries can kill – far from being an apocalyptic fantasy, is instead a very real possibility for the 21st century”. This risk is further intensified by certain agricultural practices like manuring, which facilitates the dispersal of AMR (Jechalke et al., 2014). Manures are used in agriculture to enrich arable soils with organic matter and nutrients. They are usually derived from conventional livestock farms that rely on the use of VAs to combat infections of productive animals. Although the use of VAs for animal biomass increase has been prohibited in the EU since 2006 (Reg.1831/2003/EC), the administration of VAs as preventive and therapeutic measures is still allowed, contributing to the high levels of VA administration in livestock farming. Preventive use was recently restricted to individual animals (Reg. 2019/6 and repealing Dir. 2001/82/EC, effective since Jan 28, 2022), within the context of the European One Health Action Plan (COM/2017/0339), following the successful banning of the mass preventive VA use in livestock in Denmark in 2011 (More, 2020). However, the potential impact of these regulatory amendments at EU level will still require some time to become apparent.

With several VAs being persistent and not metabolized by animals, they can reach up to 75 % or more of their administered dose in the animal excretes (faeces, urine) (Perruchon et al., 2022; Xu et al., 2022). The subsequent use and application of the animal excreta as organic fertilizers in agricultural soils can exert (i) a selection pressure towards the emergence of AMR, and (ii) ecotoxicological effects on soil ecosystems (Chen et al., 2019; DeVries and Zhang, 2016; Semedo et al., 2018; Stanton et al., 2020; Zheng et al., 2022). Next to the diffusion of VAs from manure to

soil, the potential further flow of manure-derived ARGs and microorganisms to agricultural soils, and from there to humans or animals, pose a major health risk (Jechalke et al., 2014). Considering the extent of ecosystem services provided by soil microorganisms, such as nutrient cycling, nutritional support to plant growth, biocontrol of pathogens, climate regulation, biodegradation of xenobiotics, production of therapeutic bioactive compounds and others, the protection and preservation of soil microbial diversity and functioning should be prioritized (Bender et al., 2016). Hence, there is an urgent need for inclusion of more endpoints concerning soil microbial communities in the environmental risk assessment of antibiotics (Brandt et al., 2015).

Despite the recent interest on the behaviour of antimicrobials in manure-amended soils, and on the ecotoxicological effects of VAs on soil microorganisms (Cleary et al., 2016; Jechalke et al., 2014), we still have major knowledge gaps for several commonly used VAs such as the synthetic pleuromutilin TIA and the macrolide TLM. These two VAs are among the most heavily used broad-range spectrum VAs (Cycoń et al., 2019; European Medicines Agency, 2016). Moreover, existing studies focus on the effects of VAs on the abundance and diversity of total bacteria, the soil enzyme activities, and ARG enrichment, with only a handful of studies considering effects on taxonomic groups like fungi, protists and ammonia-oxidizing microorganisms (AOM), which are essential for key ecosystem functions (Cycoń et al., 2019; Nguyen et al., 2022). Consequently, the mechanisms underpinning the potential ecotoxicity of VAs on soil microbial communities remain poorly understood. Furthermore, little is known about the effects of the flow of microorganisms and AMR from manures during organic fertilization of agricultural soils to the receiving soils and further up the food chain.

This study aims to assess the effects of repeatedly applied VAs, either directly or through fortified manures, on the soil microbiota. To reach this objective, a microcosm study was employed using specific soils and VAs based on our previous findings (Perruchon et al., 2022). Three VAs, (a) a well-studied sulfonamide like SMX; (b) a

much less studied pleuromutilin, TIA; and (c) a yet unstudied in such a context macrolide, TLM, were repeatedly applied in two soils at environmentally relevant dose rates, either directly or through fortified swine manure. We (i) followed the dissipation of VAs in soil and particularly the potential of acceleration of their dissipation under a repeated application setup; (ii) assessed the effects of VAs (and of manures) on the diversity of broad taxonomic groups of the soil microbial food-web like prokaryotes, fungi, and protists and on sensitive functional microbial groups like AOM; (iii) monitored the evolution of ARGs and of HGT marker genes; (iv) identified microbial groups that could be profiled as AMR reservoirs in soil.

2. Materials and methods

2.1. Soils, veterinary antibiotics and faecal material

Technical standards of TIA fumarate (Biosynth® Carbosynth, Staad, Switzerland – purity >97 %), TLM (Biosynth® Carbosynth, purity >85 %) and SMX (ChemCruz®, Dallas, TX, USA – purity of 99.9 %) were utilized for the preparation of the solutions applied on soils and faecal material.

Two soils differing in their biodegradation capacity and pH, as described in our previous work (Perruchon et al., 2022), were used in our microcosm study. The Rodia soil with the highest pH value (7.9) showed a higher biodegradation capacity for TIA and TLM. On the other hand, the Livadi soil with the lowest pH (5.9) showed a lower biodegradation ability for the same two VAs (see Annex II: Table S1 for properties).

The faecal material used for soil application was free of the studied VAs (SMX, TIA, TLM), as verified by HPLC analysis. It was collected from a commercial pig farm (HOIROPAL, Palamas, Thessaly, Greece) from 30 to 45-day old piglets that did not receive any of the VAs used in the present study. In addition, any possible previous

treatment with other antibiotics was curative and, it was terminated before the start of the study. Alternatively, in cases where the administration of VAs was necessary to be continued, the treated animals were removed from the test group, ~10 days before the collection of the manure. The collected manure was stored at 4 °C in the dark for maximum 3 weeks until downstream use.

2.2. Experimental setup

Fresh soil samples were sieved through a 2 mm mesh and stored at 4 °C until downstream use. In a laboratory microcosm study, the soils were subjected to three repeated VA applications, either directly, or through fortified swine manure. The two soils were amended with manure at a final rate of 2 % (w/w). This application rate corresponds to 25 t of manure ha⁻¹, and it is considered as the maximum amount of swine manure that could be amended in soil without entailing any risk for soil health (considering the potential manure high heavy metal content) (Königer et al., 2021). In these calculations we assumed that the manure was incorporated in the top 10 cm of soil, while our soil bulk density was 1.5 g cm⁻³. It should be noted that the manure used was collected from weaning (<3 weeks post-lactation) piglets supplemented, through their feed, with 72 mg kg⁻¹ of zinc as growth promoter and diarrhoea preventive (Castillo et al., 2008). Two different modes of delivery of the VAs to soil were selected: (i) direct application to determine the inherent capacity of VA molecules to enhance their degradation or imposed toxicity onto soil microorganisms, or (ii) application through fortified manures to explore their behaviour (enhanced degradation or toxicity) mimicking more realistic exposure conditions. In both cases we aimed for a nominal concentration of VAs in soil of 2.5 mg kg⁻¹ soil (dry weight). These levels are consistent with our previous findings regarding the detection of residues of TIA and TLM in the excreta of animals treated with the specific VAs under normal veterinary practices

(Perruchon et al., 2022). For all VAs, corresponding control treatments including soils treated with manure without VAs and with just water without VAs, were also included. Upon treatment of soils, their moisture content was adjusted to 40 % of their water holding capacity and it was maintained throughout the study with regular water additions. Treated samples were placed in aerated plastic bags and incubated in the dark at 25 °C. Throughout the experiment and at each application cycle triplicate sub-samples were collected at days 0, 7, 15, 25 and 50 for TIA and TLM, and at days 0, 2, 4, 8, 15 and 25 for SMX, and were used for downstream analysis. TIA and TLM were applied on 50-day intervals based on our previous dissipation data in the studied soils (Perruchon et al., 2022), unlike SMX where a shorter time interval of 25 days was selected based on data derived from a preliminary dissipation study, and the overall low persistence of SMX in soil (Martin-Laurent et al., 2019).

2.3. Residue analysis of VAs

TIA, TLM and SMX were extracted from soil as described in Perruchon et al. (2022). Briefly, 5 g of soil were mixed with 2.5 ml of water in a 50 ml Teflon tube. They were then vortexed for 1 min and were left for 10 min to equilibrate. Five ml of acetonitrile, 0.5 g of NaCl and 0.05 ml of acetic acid (0.025 ml for the acidic soils) were added to the mixture which was homogenized by vortexing for 5 min, before being centrifuged at 8000g for 5 min at 25 °C. Three ml of the supernatant were collected and vortexed for 1 min together with 0.075 g of Primary/Secondary Amine EnvirocleanR PSA (BGB Analytik AG, Böckten, Switzerland). The mixture was centrifuged at 8000g for 5 min at 25 °C, the supernatant was filtered through a 0.45 µm filter, and the filtrates were analysed via HPLC as described below. The concentrations of VAs in the extracts were determined in a Prominence Modular HPLC-PDA (Shimadzu, Kyoto, Japan) system equipped with a CNH Athena RP C18–150 mm column (CNW Technologies,

Düsseldorf, Germany). TIA and TLM elution and detection conditions were as described by Perruchon et al. (2022). SMX was eluted using a mobile phase of 40:60 methanol: water (v/v) (flow rate of 1 ml min⁻¹) and it was detected at 268 nm. The injection volume was in all cases 20 µl. Method validation tests involving spiking of faeces and soil samples with the tested VAs at three concentration levels (0.05, 0.5 and 5 mg kg⁻¹) showed mean recoveries of >81 % and relative standard deviations lower than 11 %.

2.4. Determination of potential nitrification rate

PNR were determined as described previously (Kandeler, 1995). Briefly, 5 g of soil were mixed with 20 ml of (NH₄)₂SO₄ 1 mM and 0.1 ml of NaClO₃ 1.5 M. The mixture was incubated for 5 h at 25 °C. Controls were also incubated in parallel for 5 h at -20 °C to inhibit nitrification. After incubation, samples and controls were left to adjust to room temperature and 5 ml of KCl 2 M solution were added followed by light shaking and filtration. Subsequently, 5 ml of the samples were mixed with 3 ml of NH₄Cl 0.19 M buffer and 2 ml of colour reagent. The samples were homogenized and left for 15 min at room temperature for colour development. Standard curves were prepared from standard NaNO₂ solution at concentrations of 0, 0.2, 0.4, 0.8 and 1.0 µmol NO₂⁻¹-N ml⁻¹ and the sample contents were measured at 520 nm.

2.5. DNA extraction

Two hundred and fifty mg of soil were used for DNA extraction using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany). DNA integrity was checked by electrophoresis and its quantity was measured using Qubit dsDNA BR Assay Kit fluorometer (Invitrogen, Waltham, MA, USA). The extracted DNA was stored at -20 °C

for further analysis. DNA was extracted from soil samples collected at days 0 and 40 after each application of TIA and TLM and at days 0 and 25 after each application of SMX.

2.6. qPCR analysis of the abundance of the 16s rRNA gene, selected ARGs, the *intl1*, and the *amoA* gene

The abundance of 10 marker genes was monitored in a QuantStudio® 5 Real-Time PCR Instrument (384-well Block, Applied Biosystems by Thermo Fisher Scientific). Standard curves and associated statistics for all tested genes are provided in File S1 (see Annex II). The 16S rRNA gene was used to determine total bacterial abundance (Annex II: Table S2). The gene coding for the integrase of class-1 integrons (*intl1*) was used as a marker for HGT (Stalder et al., 2012) and anthropogenic pressure (Gillings et al., 2015). The abundance of several ARGs, related or not related to the applied VAs, such as the dihydropteroate synthase variant-group 1 (*sul1*), the dihydrofolate reductase variant 1 (*dfrA1*), the tetracycline resistance coding *tetM*, and the fluoroquinolone resistance gene *qnrD* was monitored to determine the evolution and dispersal of ARGs upon VA and/or manure application. Genes involved in nitrogen cycling, such as the α subunit of ammonia monooxygenase (*amoA*) and the β subunit of nitrite oxidoreductase (*nxrB*), were used to determine the abundance of ammonium-oxidizing archaea (AOA) and bacteria (AOB), and of *Nitrobacter* and *Nitrospira* nitrite-oxidizing bacteria (NOB), respectively. The primer combinations and thermal cycling conditions used are listed in Table S2 (Annex II). Absolute quantification of target genes was performed with the KAPA SYBR FAST kit (KAPA Biosystems, Wilmington, MA, USA) in 20 μ l reactions with 20 μ M final concentration for each primer and 0.4 μ g ml⁻¹ of bovine serum albumin (BSA; New England Biolabs®, Ipswich, MA, USA). Standard curves were constructed using serial dilutions of PCR generated and Sanger

sequencing verified clone inserts following the procedure described previously (Rousidou et al., 2013).

2.7. Amplicon sequencing

To determine the effects of VAs on soil microbial diversity, several phylogenetic marker genes were selected (Annex II: Table S3): the V4 hypervariable region of the 16S rRNA gene for total prokaryotes; the ITS genomic region for fungi; the 18S rRNA gene for protists; and the *amoA* gene for AOB and AOA. Sample-wise multiplex indexing and pooling for sequencing was performed according to our in-house protocol (Vasileiadis et al., 2022; Vasileiadis et al., 2018b). To amplify the selected targets, PCR was performed in 10- μ l reactions using the following reagents: Q5 High-Fidelity 1 $\times$ Master Mix (New England Biolabs®, Ipswich, MA, USA), BSA (New England Biolabs®, Ipswich, MA, USA) 0.4 mg μ l⁻¹, primers (each) 0.5 μ M, DNA 0.1 or 10 ng per reaction depending on the target gene (Annex II: Table S3). The combination of primers and thermal cycling conditions used are listed in Table S4 (see Annex II). Subsequently, a second, sample-wise tagging PCR with indexed primers was performed. Reactions were performed in 20- μ l volumes with the following reagents: Q5 High-Fidelity 1 $\times$ Master Mix, primers (each, with one being indexed) 0.5 μ M, 2 μ l of the first reaction as template. Final products were pooled at equal masses. The PCR products were purified with the NucleoMag® NGS Clean-up and Size Select Kit (Macherey-Nagel, Duren, Germany). The pooled PCR products were sequenced at an Illumina HiSeq 2X250bp at Admera Health (New Jersey, USA). The sole exception were the AOB *amoA* amplicons which were sequenced, due to their longer size, at an Illumina MiSeq with the 2 $\times$ 300bp module using the v3 reagents at the Genome Center of the Biomedical Research Foundation Academy of Athens (BRFAA, Athens, Greece). The sequencing data were submitted to the Sequence Read Archive (SRA) of the National

Center for Biotechnology Information (NCBI) and are publicly available under the BioProject accession number PRJNA922348.

2.8. Statistical analysis and bioinformatics

2.8.1. Antibiotic dissipation kinetics

The SFO and three biphasic kinetic models (HS, FOMC and DFOP) were used to calculate the dissipation kinetics for TIA, TLM and SMX as described previously (FOCUS, 2006). The χ^2 test as well as visual inspection were used to assess the agreement between calculated and observed data. Data analysis was performed with the mkin v1.0.3 package (Ranke, 2020) of the R software v4.1.3 (R Core Team, 2023), while the R software was also used for all analytical tasks described below.

2.8.2. q-PCR marker data hypothesis testing

The abundance data from the various marker genes were expressed as ratios to the 16S rRNA gene and they were subjected to analysis of variance (ANOVA) and the Tukey's test in case of significant ANOVA test, or to the non-parametric equivalents of Kruskal-Wallis and the Wilcoxon ranksum test.

2.8.3. Amplicon sequencing data bioinformatic analysis

The retrieved sequences were demultiplexed according to our pipeline (Vasileiadis et al., 2022), and they were further processed with the dada2 v1.24.0 (Callahan et al., 2016) package modules of the R software. Sequence reads were quality trimmed at the first instance of ≤ 2 Phred Q values at the 3' to 5' direction. Sequences with unknown bases, max error rates of 0.7 per 100 bp of reads, or shorter than 150 bp post trimming (or 100 bp for the short AOA amplicons) were removed from the dataset. Remaining sequences were dereplicated and error corrected with the dada2 machine learning and rare event removal algorithm. Passing read-pairs were assembled to amplicon

sequence variants (ASVs) with the dada2 read-pair assembler. Generated contigs were controlled for chimeric amplicons using the native de novo approach (Callahan et al., 2016). Finally, sequences were classified with the naïve Bayesian classifier (Wang et al., 2007) using: (i) the Silva v138 database (Yilmaz et al., 2014) and an 80 % bootstrap cutoff for total prokaryotes; (ii) the general v10.05.2021 internal transcribed spacer (ITS) UNITE database (Nilsson et al., 2019) for the ITS2 amplicons with a 50 % bootstrap cutoff; (iii) the Abell et al. (2012) reference database for AOB and the Alves et al. (2018) reference database for AOA with a 50 % bootstrap support cutoff after database pre-processing for compatibility with the dada2 annotation module. Regarding protist annotation, the DECIPHER v2.22.0 (Wright, 2016) idTaxa algorithm was implemented using the v4_13_March2021 PR2 (Vaulot et al., 2022) database. After annotation, relational database objects were formed with the Phyloseq v1.38.0 (McMurdie and Holmes, 2013) R package and off-target sequences were removed from the generated object.

2.8.4. α - and β -diversity analysis

The obtained final phyloseq objects were subjected to a series of statistical tests at α - and β -diversity levels. Diversity indices like the Shannon, the inverse Simpson's, the Fisher's α , the observed richness and the abundance based coverage estimator (ACE) were calculated with the Vegan v2.6-4 (Dixon, 2003) and the Entropart v1.6-11 (Marcon and Hérault, 2015) R packages. Hypothesis testing was performed (ANOVA followed by Tukey's as post hoc test or their non-parametric counterparts Kruskal-Wallis and Wilcoxon rank-sum) for depicting possible index differences between the various groupings using the Agricolae v1.3-5 (de Mendiburu, 2021) package. The Vegan R package was also used for performing permutational multivariate analysis of variance (PERMANOVA) was applied to assess the effects associated with the VA treatment, the application method (directly or through fortified manure), and the application cycle on the microbial community structures. Next to that, the average

linkage hierarchical clustering of samples according to their Bray-Curtis dissimilarities was also used for assessing sample clustering (Bray and Curtis, 1957).

2.8.5. ASV winnowing and differential abundance testing

Considering either both soils, or each soil individually, the obtained ASV matrices were “winnowed” (aka certain ASVs were retained for downstream analysis) according to ASV-prevalence, random forest importance scores, and ASV-keystoneness scoring. First, a minimum participation of ASVs in all three replicates of at least two samples was necessary for considering them for downstream analysis. Then, the pime v0.1.0 (Roesch et al., 2020) algorithm was used for selecting the random forest out of bag (OOB) error rate based minimum prevalence cutoffs among samples of the same antibiotic treatment, in order to retain ASVs in the analysis. Following, network analysis was performed with the SpiecEasi v1.1.2 algorithm (Kurtz et al., 2015), while the igraph v1.3.5 package was used for the keystone index calculations (degree, indirect degree, betweenness centrality, closeness centrality, transitivity) and the network graph simplification (self-looping removal and minimum spanning tree algorithm implementation). Then, an overarching keystone index was calculated using a heuristic scoring system where: (i) all keystone indices were scaled between 0 and 1; (ii) the betweenness centrality, closeness centrality, and transitivity indices were reduced in importance by multiplying their scaled values by a factor of 0.2, whereas the indices of degree and indirect degree were retained to the scaled values, deeming them this way more important; (iii) the final keystone index equalled to the mean of the index values obtained by ii. ASVs with scores at the upper quartile according to this ranking system were retained for the final downstream network analysis. Following the described “winnowing” process, network analysis based on the SpiecEasi algorithm was performed for a composite dataset derived from: (i) the winnowed datasets; (ii) the relative to 16S rRNA gene copies of the quantified ARGs and the nitrogen cycle associated genes; and (iii) the measured PNR values. The winnowed

datasets were further subjected to the Kruskal-Wallis and the post hoc pairwise Wilcoxon rank-sum tests approach for differential abundance assessment using the Benjamini-Hochberg (Benjamini and Hochberg, 1995) p-value correction method for multiple hypothesis testing.

2.9. Ethical considerations

The study was carried out in compliance with all national animal welfare regulations and following the context of relevant EU legislation for animal experimentations (Directive 86/609/EC). There was no handling of the animals and samples were collected from the pen floor. Samples were collected with the farmer's consent.

3. Results

3.1. Dissipation of VAs in soil

The SFO kinetic model or the best fitting biphasic model, depending on the obtained χ^2 statistic (FOCUS, 2006), were used for the calculation of the DT₅₀ values for the studied VAs. SMX was the least persistent VA with DT₅₀ values of 1.1–9.9 days and 0.6–5.7 days in the soils where the VA was applied directly or through fortified manure, respectively. Regardless of the application method, SMX showed a clear deceleration in its dissipation along repeated application cycles, as suggested by the gradual increase in its DT₅₀ values at both tested soils (Fig. 1, Annex II: Table S5). TLM was the most persistent VA with DT₅₀ values of 97.7 to >365 days (extrapolated) and 115.5 to >365 days in the soils which were treated with the VA either directly or through fortified manure, respectively (Fig. 1, Annex II: Table S5). Repeated application of TLM led to a reduction in its persistence in all cases, with the sole exception of the high-pH

soil which was treated directly with TLM. Nevertheless, the long persistence of TLM led to a gradual accumulation of its residues along the successive application cycles. The dissipation of TIA varied in the two tested soils, with a cumulative pattern in the case of the low-pH soil and an accelerated dissipation in the high-pH soil (Fig. 1, Annex II: Table S5). In both soils, TIA had a pronounced reduction of its DT_{50} values in the direct application mode and over cycle progression (DT_{50} values of 13.9 and 7.4 days in the high-pH soil; >365 and 70.8 days in the low-pH soil in the first and third cycle respectively). This pattern was not replicated in the feces-amended soils.

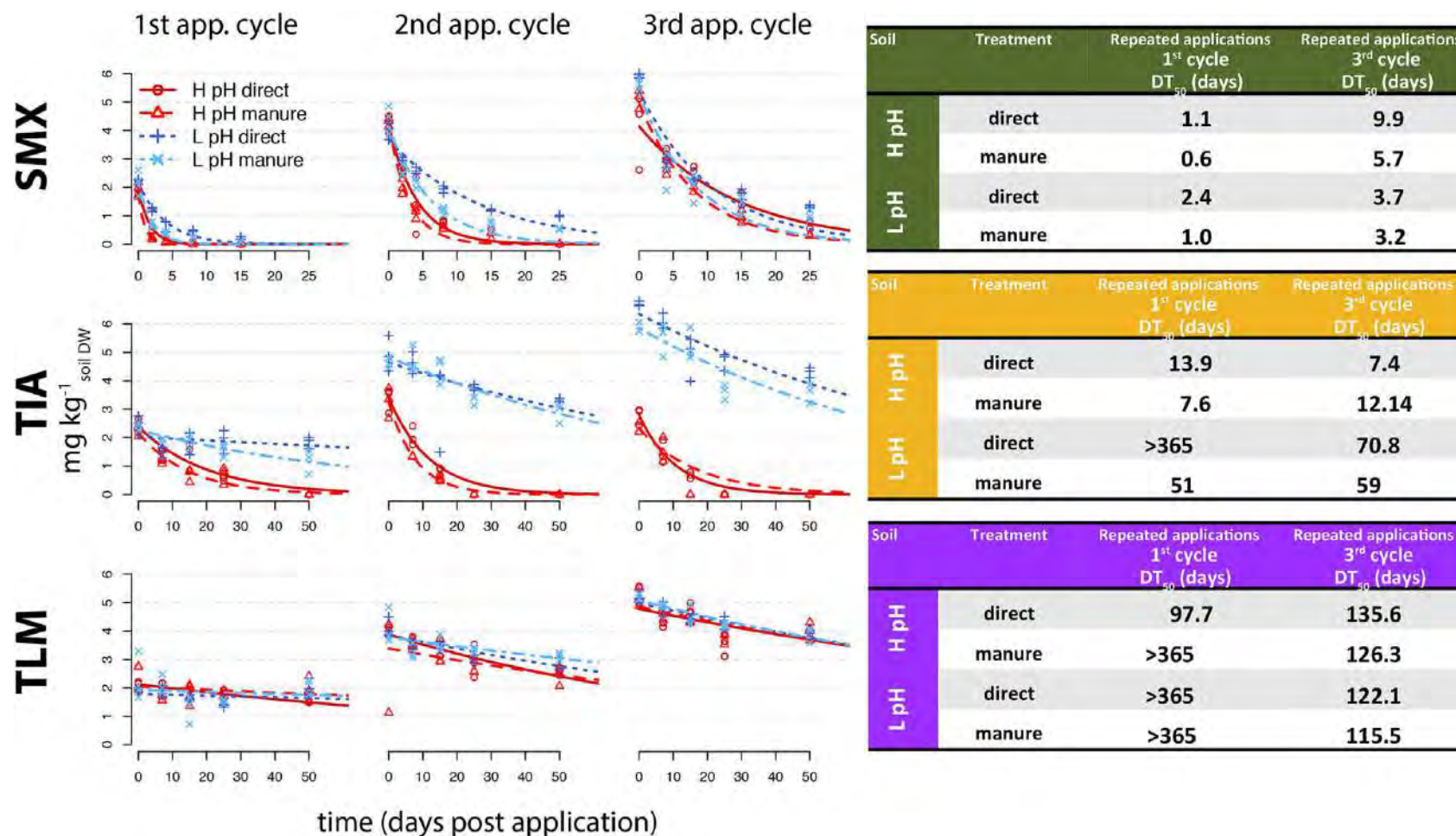


Figure 1: The degradation patterns of the three tested VAs (SMX; TIA; TLM) upon three successive application cycles, when applied directly or through fortified manure at the low-pH (L pH) or the high-pH (H pH) soil. The corresponding DT₅₀ values of the tested VAs calculated by the most appropriate model according to FOCUS (2006) for the first and the third application are presented in the table on the right. All the DT₅₀ values per VA and application mode are given in Table S5 (see Annex II).

3.2. Effects of VAs on soil nitrifier activity and abundance

The effects of VAs and their mode of application (directly or via fortified manure) on N cycling were assessed through measurements of PNR and of the abundance of AOB, AOA, and NOB of the genus *Nitrobacter* and *Nitrospira*. PNR showed high sensitivity to all treatments. SMX exerted the strongest effects on PNR regardless of its application mode and the tested soil (Fig. 2, upper panels per each soil type). During the first application cycle, SMX imposed a significant increase on PNR, whereas this was inversed in the following application cycles leading to strong and persistent inhibition of the PNR. Similarly, the first direct application of TIA led to a significant increase ($p_{adj} \leq 0.05$; Fig. 2 middle panels per soil type) in PNR followed by a significant decrease in the following two cycles. In contrast, when TIA applied in the two soils through fortified manures induced an initial mild negative effect on PNR, which became significant only at the third application cycle. TLM, when applied directly in soil, induced a significant ($p_{adj} \leq 0.05$) increase in PNR in both soils (Fig. 2 lower panels per soil type). This pattern was replicated in the samples of the low-pH soil treated with TLM through fortified manure but not in the corresponding high-pH soil, where a significant decrease ($p_{adj} \leq 0.05$) in PNR was observed at the end of the last two application cycles.

SMX reduced the abundance of the *amoA* gene of AOA and AOB in both soils, with associated significant effects appearing either early (e.g. 25 days after the first application) or late (e.g. after the third application) and being more prominent for AOB than AOA (Annex II: Figs. S1 & S2). The effects of TIA and TLM on the abundance of AOA were similar. Both VAs induced a significant reduction in the abundance of AOA in the low-pH soil and a significant increase the abundance of AOA in the high-pH soil (Annex II: Fig. S1). Conversely, TIA and TLM led to a significant increase in the abundance of the *amoA* gene of AOB in both soils and application modes (Annex II:

Fig. S2). In general, we noted a significant positive effect ($p \leq 0.05$) of all VAs on the abundance of *Nitrobacter* (Annex II: Fig.

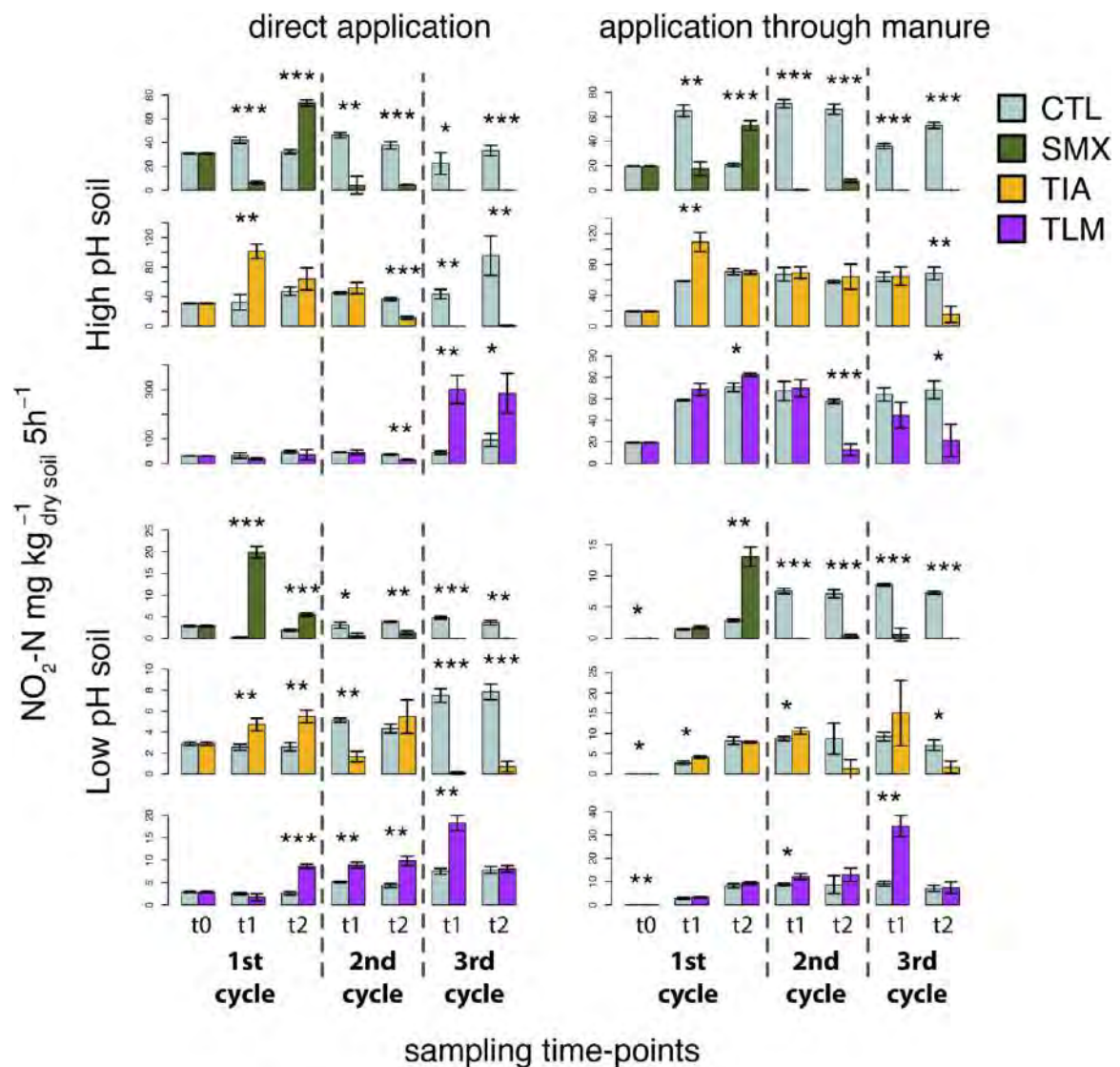


Figure 2: The effects of repeated applications of VAs on PNR in the high (Rodia) and low (Livadi) pH soils receiving VAs directly or via spiked manures. Within each time point significant differences between treated and control samples, as determined by student's t-test, are denoted with asterisks for the Benjamini-Hochberg adjusted p-values for multiple hypothesis testing (sig. codes: '***' 0.001; '**' 0.01; '*' 0.05). CTL: control; SMX; TIA; TLM; t0: time 0 days post application (dpa) within each application cycle; t1: 8 dpa for SMX and 15 dpa for TIA/TLM; t2: 25 dpa for SMX and 50 dpa for TIA/TLM.

S3) and *Nitrospira* (Annex II: Fig. S4): this trend was marked for TIA and TLM, in the low-pH soil. In contrast, in the high-pH soil we noted a different pattern, where all VAs induced a significant increase ($p \leq 0.05$) in the abundance of *Nitrobacter* (Annex II: Fig. S3) and a significant decrease ($p \leq 0.05$) in the abundance of *Nitrospira* starting from the second application and onwards (Annex II: Fig. S4).

3.3. Effects of VAs on microbial diversity

The effects of VAs and their mode of application on the diversity of total prokaryotes, fungi, protists, and AOB/AOA was determined on samples collected at 25 days (for the non-persistent SMX) and at 40 days (for the more persistent TIA and TLM) after each application cycle, via high throughput amplicon sequencing. The studied soil factor, constituted the most important parameter affecting the structure of all analysed microbial groups (Annex II: Fig. S5). Hence, microbial diversity analyses were performed separately for each studied soil.

3.3.1. Effects of VAs and application method on microbial α -diversity

Five α -diversity indices were analysed covering different dominance levels: (i) the observed richness and the ACE predicted richness which capture the diversity of the members of the microbial community of low dominance; (ii) the Shannon index which captures the diversity of the members with low to intermediate dominance; and (iii) the Inverse Simpson and Fisher's α indices which capture the diversity of the more dominant and highly dominant members of the community. In the high-pH soil, we noted a clear negative effect of all tested VAs on the α -diversity of fungi from the second application and onwards, and at both application modes, while negative effects on the α -diversity indices of protists by either SMX or TIA were evident after the second and third application (Fig. 3). In the low-pH soil, regardless of their mode of application all VAs induced a significant reduction in at least one α -diversity index for the community of fungi and protists, either after the first or the second application respectively. In addition, SMX and TIA negatively affected the α -diversity of prokaryotes with their effects varying by the mode of application. In contrast, VAs had a positive effect on the α -diversity of AOA which was limited to certain treatments and it lasted for the first two cycles (Fig. 3).

3.3.2. Effects of VAs and their mode of application on the structure of the soil microbial community

A hypothesis testing approach (PERMANOVA) was adopted to assess the effects of VAs, their mode of application, and their application cycle on the structure of the analysed microbial groups. Effect size (variable explained variance: R^2) was selected as an effect index of each tested variable. In the case of the high-pH soil, the employed VA treatment was a very important factor affecting the structure of prokaryotic, AOB and AOA communities for all three VAs (Fig. 4, Annex II: Fig. S6). Protists and fungi, on the other hand, were most affected by the mode of application of the VAs (direct vs through fortified manure). In the case of the low-pH soil, a less clear trend was observed, with the mode of application being an impact factor for protists and fungi, but the VA treatment and the application cycle showing varying importance for all tested microbial groups. In the high-pH soil, TLM showed the highest percentage of the VA-treatment explained variance, as determined by the R^2 value (and hence the highest possible effect on the community structure), for prokaryotes, fungi and protists compared to the other two VAs (Figs. 4 and Annex II: S6). In the same soil, TIA and TLM had nearly double R^2 values compared to SMX for AOB and AOA. In the low-pH soil, SMX had the highest R^2 values for prokaryotes, TLM showed the highest R^2 values for fungi, AOB, AOA, and TIA for protists (Figs. 4 and Annex II: S6).

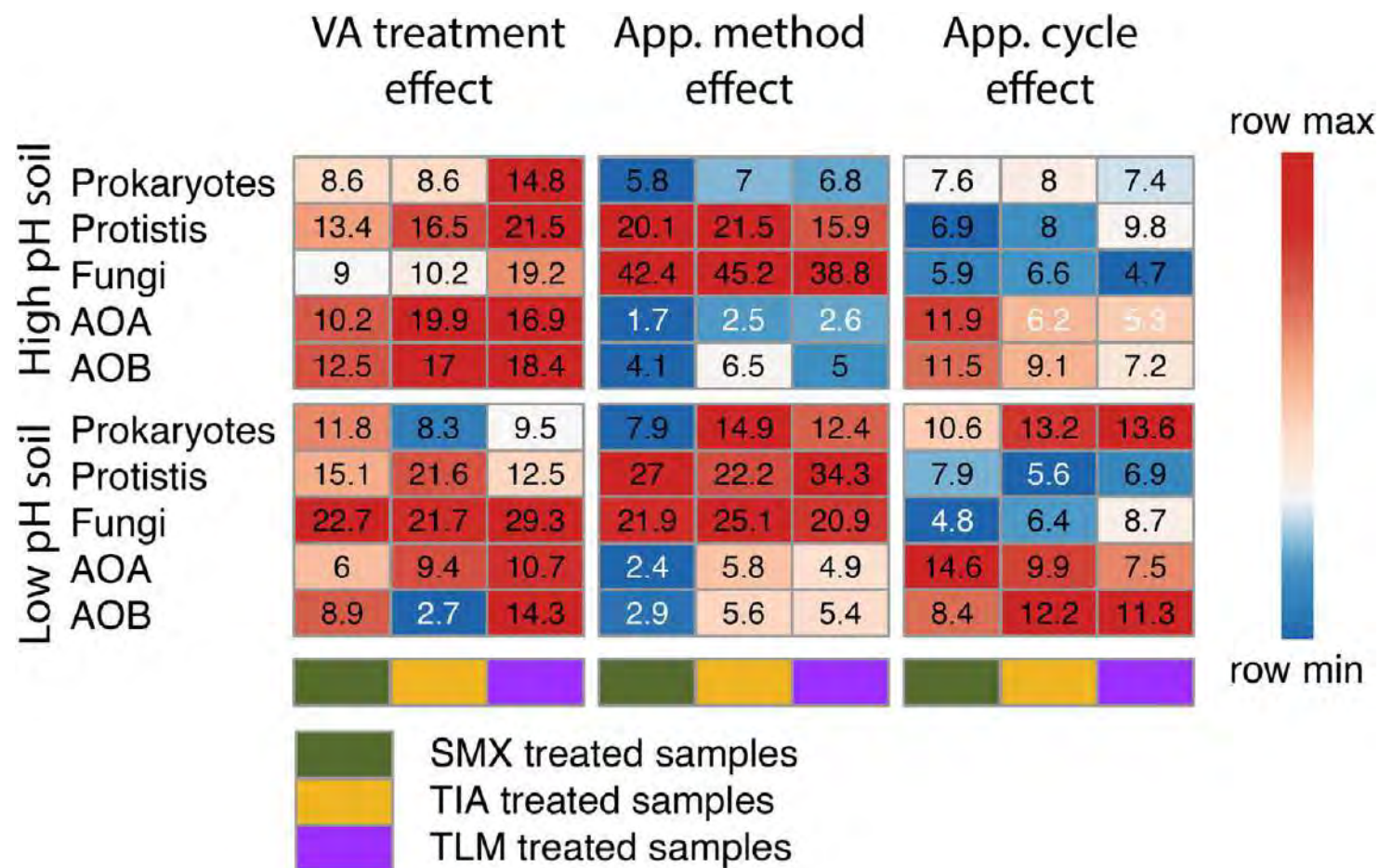


Figure 4: Heatmap of the effect of each factor (VA treatment, application method, application cycle), quantified by the percentage of explained variance (R²), on the tested microbial communities (prokaryotes, protists, fungi, AOA and AOB functional communities) according to the performed PERMANOVA (community structure examined as a function of the VA treatment, the application method and the application cycle). Colours are scaled between the minimum and maximum values of each row. White numbers show insignificant model components ($p > 0.05$). SMX; TIA; TLM. Detailed hierarchical clustering graphs are provided in Fig. S6 (Annex II).

3.3.3. Differentially abundant ASVs associated with VA treatments

A two-step filtering process was adopted for identifying the differentially abundant ASVs among VA treatments. The pime algorithm (prevalence/random-forest based) was used for an initial selection of ASVs which was followed by the performance of a Kruskal-Wallis differential abundance test. Overall, ASVs mostly residing in Actinobacteria and Proteobacteria had clear VA-related patterns (Fig. 5). However, we did not observe any common ASV responders to the VA application in the two soils. We identified ASVs belonging to *Rhodococcus*, *Micrococcaceae* and *Streptomycetaceae* at the high-Ph soil, and *Burkholderia*-*Caballeronia*-*Paraburkholderia*, *Methylobacterium*-*Methylorubrum* at the low-Ph soil that were favoured in the SMX-treated samples. ASVs belonging to *Methylophilaceae*, *Blastococcus* at the high-Ph soil, and *Rhodococcus*, *Sphingomonas* at the low-Ph soil were positively associated with TLM. Finally, ASVs affiliated to different taxa of Actinobacteriota like *Kribbella*, *Rubrobacter*, *Solirubrobacter*, *Gaiella* or Proteobacteria like *Sphingomonas* and CCD24 at the high-Ph soil, and *Flexivirga*, *Conexibacter*, *Rhodococcus*, *Sphingomonas* at the low-Ph soil were favoured by TIA.

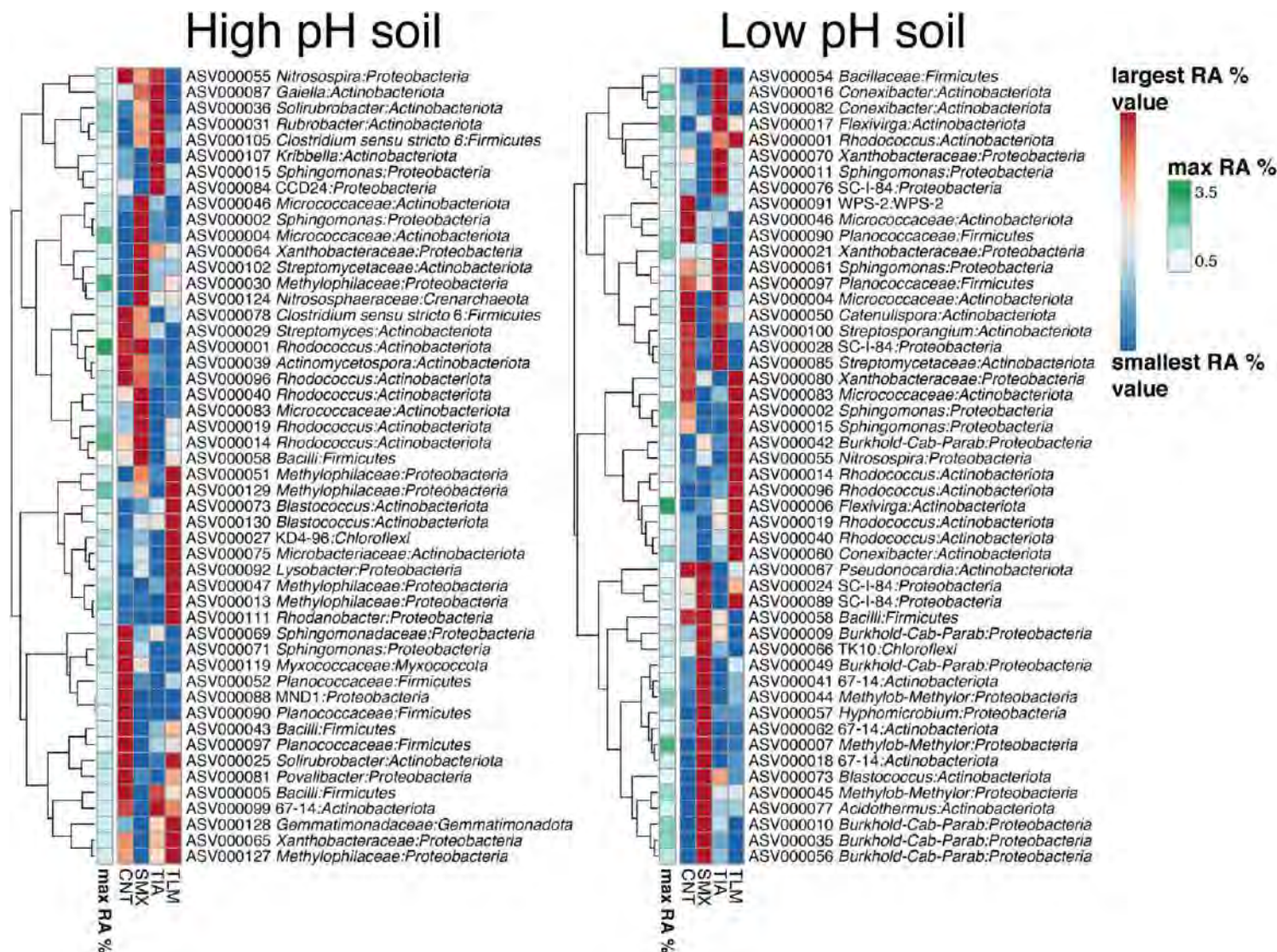


Figure 5: Heatmaps showing the differentially abundant ASVs in the two soils according to the VA treatment employed. Blue and red cell colours depict relative abundance (RA) values scaled between the lowest and highest observed values for each ASV between treatments (i.e. the red and blue cell of ASV000055 in the CTL and TLM respectively for the high-pH soil, show that these treatments had the highest and lowest relative abundances respectively for this specific ASV). The maximum mean relative abundance value among each VA treatment samples for each taxon is indicated by the white to green leftmost column of the heatmaps.

3.3.4. The fate of manure–derived ASVs in soil

The incorporation of manure in the studied soils resulted in a significant increase, higher than an order of magnitude, of the 16S Rrna gene counts ($p < 0.05$) in the case of the low-pH soil, while in the high-pH soil the observed increase was about half of that observed in the low-pH soil and was not statistically significant (Annex II: Fig. S7). The effect of the manure was reflected in a significant increase in the Shannon diversity index in the low-pH soil, but no difference in the high-pH soil was observed. Analysis of the dynamics of the manure-derived taxa (that collectively reached 28–42 % in the manure-treated soils and ~0 % in the unamended soils; Annex II: Fig. S7), showed a quick reduction of their relative abundance at both soils (their relative abundance dropped to zero, 25 days after manure incorporation in the studied soils).

3.4. Abundance of *intl1* and ARGs, and their correlations with prokaryotes

We also monitored the abundance of *intl1* and selected ARGs *sul1* (SMX), *dfrA1* (trimethoprim), *qnrD* (quinolone), and *tetM* (tetracycline)) as markers for assessing the impact of VAs and/or manure application on the HGT frequency and the overall antibiotic resistance potential of the soil microbial communities, respectively. In the case of TIA and TLM, where resistance relies on mutations of the target genes, no resistance specific markers were available to be screened. The relative abundance of the marker genes was assessed for correlations with bacterial taxa aiming to identify potential AMR reservoirs in the analysed soils. The selection of these taxa was based on their prevalence and association with the experimental treatments (pime approach), and their keystone performance (according to network analysis) in the microbial communities. Apart from *tetM*, all other marker genes showed significantly higher relative abundance in the manure-treated samples. This effect of manure was restricted to the low-pH soil for *intl1* and *sul1*. TIA and TLM showed a milder impact on

the relative abundance of all marker genes, whereas SMX induced a significant increase in the relative abundance of *sul1* (Fig. 6A). The relative abundance of *dfrA1* and *qnrD* drastically increased in the samples treated with SMX-fortified manure.

Several “keystone” ASVs annotated as *Myxococcaceae*, *Rhizobiaceae*, *Methylophilaceae* and *Burkholderiales* SC-I-84 showed significant positive correlations with *int1* (Fig. 6B). *Nocardioides* and *Xanthobacteraceae* ASVs showed significant positive correlations with *sul1*. The normalized *dfrA1* counts were positively correlated with ASVs of *Streptosporangium*, *Nocardiaceae* and *Proteobacteria*. The relative abundance of the *tetM* gene exhibited strong positive correlations with several ASVs, primarily from the *Burkholderia-Caballeronia-Paraburkholderia* group (BCP), as well as *Nocardioides*, *Hyphomicrobium*, and *Bacilli*. *Clostridium sensu stricto* 6 showed the highest number of positive correlations with *int1*, *dfrA1* and *qnrD*. Some of these ASVs showed significantly higher abundance in the manure-amended samples (e.g. clostridia and *Streptosporangium*), while others were more abundant in the manure free samples (e.g. BCP and *Nocardioides*) (Annex II: Fig. S8A).

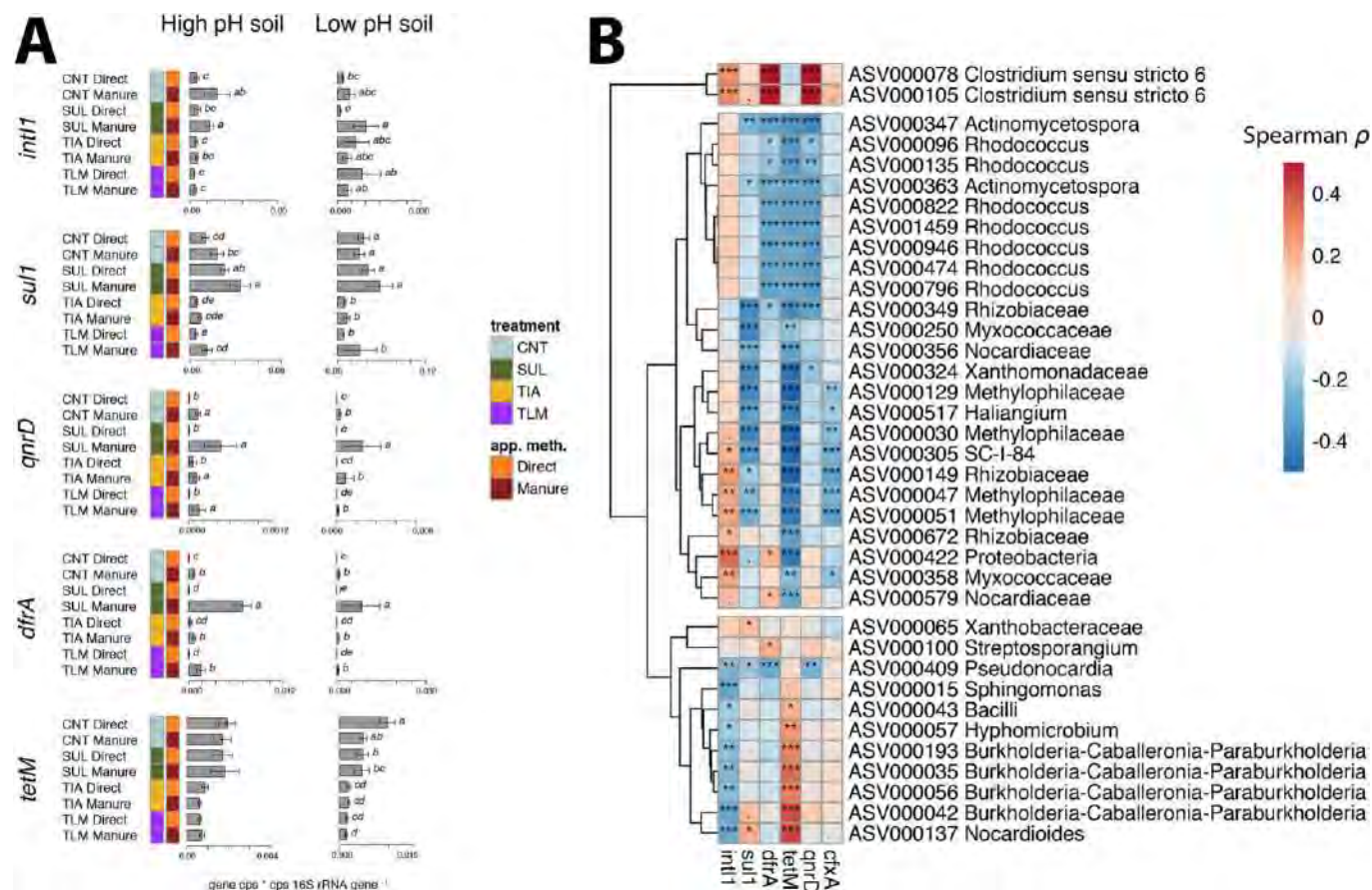


Figure 6: (A) The relative abundance (normalized to the 16S rRNA gene counts) of *int1* and selected ARGs in the different soils (high-pH and lowpH) treated with SMX, TIA or TLM either directly or through fortified manure. For each marker gene in each soil, bars designated by the same letter are not significantly different (α of 0.05); (B) Significant positive or negative (according to Benjamini-Hochberg adjusted p-values) Spearman correlations of winnowed ASVs with the relative abundance of *int1* and selected ARGs. The nature of the correlation (positive or negative) is indicated by the colour of the cell, while the significance and its level is indicated by the asterisks (** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$).

4. Discussion

We explored the outcome of the interaction of VAs with the soil microbiota. We aimed to answer the following questions: (i) Does repeated exposure of soils to VAs lead to accelerated degradation or accumulation of their residues in the soil? (ii) What is the impact of VAs on nitrification and on the abundance and diversity of the key microbial players in this critical N cycling process? (iii) What is the impact of VAs on the microbial diversity across the soil food-web? (iv) What is the effect of the posed antibiotic pressure and the manure incorporation on both related and not-directly linked, yet, clinically relevant ARGs? (v) Are there microorganisms that could fit the profile of resistance reservoirs in soil?

To answer the first question, we examined the degradation of VAs following their repeated application into the two studied soils. The selected VAs were: TIA, a pleuromutilin with no known information about its vulnerability to enhanced biodegradation; TLM, a macrolide with some members of this group being prone to enhanced biodegradation (Topp et al., 2016); and SMX, a sulfonamide prone to enhanced biodegradation in soil (Topp et al., 2013). Repeated applications of TIA led to its accelerated degradation in the soil. This was evident only upon its direct soil application, unlike with the fortified manure application, where the presence of manure seemed to prevent the establishment of this phenomenon (Fig. 2). This is consistent with previous studies that have reported enhanced biodegradation of other VAs in response to long term repeated direct applications of antibiotics, rather than through spiked manures, in soil (Topp et al., 2013; Topp et al., 2016). We speculate that in the presence of the, rich in organic matter content, manure, TIA would adsorb on it, limiting its bioavailability (Wegst-Uhrich et al., 2014) and diminishing the selective pressure for microorganisms, possibly involved in its accelerated dissipation. In line with this hypothesis, Goulas et al. (2018) showed that the application of VA fortified manures

resulted in a reduced bioavailability of sulfamethazine and erythromycin which led to a delayed observation of their enhanced biodegradation for the two studied VAs. Perruchon et al. (2022) recently demonstrated the key role of soil microbes in the degradation of TIA in soil. However, the present report is the first demonstrating the accelerated dissipation of TIA in soil, most probably due to the phenomenon of enhanced biodegradation. TLM was the most persistent tested VA, in line with previous reports (Berendsen et al., 2021; Lagos et al., 2022). Repeated applications of TLM led to its accumulation in both soils, and this, despite the observed acceleration of its dissipation along the repeated application setup. Although accelerated biodegradation of SMX has been observed in soils and wetland sediments in the past (Chen et al., 2022; Martin-Laurent et al., 2019), in our study, repeated applications of SMX did not lead to its accelerated dissipation. This was somewhat surprising, as SMX-treated soils were enriched with ASVs related to *Micrococcaceae* and *Rhodococcus*, known to encompass SMX degraders (Chen et al., 2022; Martin-Laurent et al., 2019).

Many VAs, like those tested here, may be active against a broad spectrum of prokaryotes (Chan et al., 2020; Martin-Laurent et al., 2019; Yu et al., 2020), thus affecting key soil processes like nitrification, performed by AOA/AOB and NOB. With respect to that effect, we attempted to answer our second question: “What is the impact of VAs on nitrification and on the abundance and diversity of the microorganisms involved in this process?”. VAs significantly affected the studied nitrification markers with the effects varying for the different VAs and treatment cycles. SMX was the most inhibitory and TLM was the least inhibitory VA. TIA showed variable effects which spanned from negative to PNR and AOA abundance, to positive on AOB abundance. The higher expected bioavailability of SMX and TIA in soils compared to TLM (less water soluble) could provide an explanation for their higher inhibitory effects onto nitrifiers. No studies are yet available regarding the effects of TIA and TLM on soil nitrifiers, compared to SMX, and other sulfonamides, that have been studied for their

ecotoxicity to nitrifiers (Liao et al., 2019). Previous studies showed that sulfadiazine, another sulfonamide, reduced the abundance of AOB but did not affect AOA (Ollivier et al., 2010; Ollivier et al., 2013), as partially observed in our study. On the other hand, Liao et al. (2019) observed no significant effect of sulfadiazine on PNR and the diversity of AOM at a concentration double of that used in our study. The effects of TIA and SMX on PNR and on the abundance of AOA and AOB showed a clear temporal pattern, with an initial stimulatory effect after the first application and a gradual inversion of this effect (leading to clear inhibition) at the third application cycle. The initial stimulatory effect of VAs on nitrifiers could be ascribed to a hormetic-like effect previously observed for PNR and other enzyme activities after exposure to antibiotics (DeVries and Zhang, 2016) or metals (Han et al., 2019; Langdon et al., 2014; Vasileiadis et al., 2018a). In fact, antibiotic doses below or close to the minimum inhibitory concentration (MICs), stimulated PNR for a period of time ranging from days to weeks, while higher doses were identified as inhibitory (DeVries and Zhang, 2016). Such hormetic-like phenomena are usually associated with structural community shifts, existing functional redundancy and increased metabolic rates (DeVries and Zhang, 2016; Han et al., 2019). Here, we have used VA levels which approximated MICs and might have provided hormetic-like responses in the abundance and diversity of AOA/AOB, which are further reflected in PNR measurements. These results are consistent with potentially increased metabolic rates at the initial treatment cycle (s) followed by inhibition at the later treatment cycle(s) (after prolonged exposure), as expected in hormetic phenomena.

We expanded our research on broader microbial groups and asked the question, “What is the impact of VAs on the microbial diversity across the soil food-web?”. The amplicon sequencing data for prokaryotes, fungi and protists, indicated that both the application of VAs and the mode of their delivery to the soil (direct application or through fortified manure) had strong structural effects. The effects of all VAs on the α -

diversity of the different microbial groups were more prominent on the low-pH soil, most probably because of the longer persistence of VAs in this soil. Prokaryotes showed significant β -diversity responses to the VA treatments. Next to that, we observed an application cycle associated effect of the VAs in the low-pH soil, which was possibly driven by the longer persistence of the VAs in this specific soil (Fig. 4). This was somewhat expected considering that all tested VAs are broad spectrum antibiotics that could affect the structure of the soil prokaryotic communities in various ways spanning from direct toxicity to growth-linked biodegradation (Cycoń et al., 2019). Besides prokaryotes, the α -diversity of protists and fungi was negatively affected by all three VAs. Out of the studied VAs, reports concerning their potential effects on the soil microbial diversity are available only for SMX. These studies explored the impact of SMX, applied through digested manure at higher concentrations than the one used in our study, on the diversity of bacteria and fungi. Garbini et al. (2022) showed that SMX (20 mg kg^{-1}) induced initial but not long-lasting changes in the composition of the soil bacterial community, unlike the fungal community which was not affected. Cheng et al. (2020) showed that the application of SMX (100 to 300 mg kg^{-1}) induced strong and persistent changes in the β -diversity of bacteria and fungi. Our study, although performed at lower concentrations, representative of realistic levels of exposure of soils to SMX, reinforces previous findings regarding its effects on the diversity of bacteria and fungi and gives new information on its ecotoxicity to protists. The mechanisms involved in the ecotoxicity of VAs on the communities of fungi and protists remain unclear. Although, direct toxicity of VAs to fungi or protists cannot be excluded, it is most likely that their effect is indirect. E.g. direct toxicity of VAs to prokaryotes relieves fungi from competition for resources and space (Cheng et al., 2020), or a reduction in the diversity and abundance of the protist prey (e.g. bacteria), may exert a further bottom-up control on the predator diversity per se (Saleem et al., 2013). Recent studies have suggested that the effects of VAs on the diversity of soil protists were mostly indirect, stemming from the restriction of prey abundance and diversity (Nguyen et al.,

2020), that eventually caused a decline of certain groups of soil protists (e.g. bacterivorous consumers *Endomyxa* and *Discosea-Flabellinia*).

Besides VAs themselves, also their mode of delivery in soil, and particularly their application via fortified manure imposed strong effects on the community of fungi and protists. Previous studies have stressed the strong effects of manure on the soil fungal communities (Chen et al., 2016; Du et al., 2020) and on the functional diversity of soil protists. These effects become more intense in the presence of VAs (Nguyen et al., 2020). Comparison of the 16S rRNA gene counts between soil samples directly treated with VAs and the corresponding manure-treated soil samples, showed that manure application introduces a large mass of prokaryotes in soil (Annex II: Fig. S7). However, this effect was transient in both soils and the strictly enteric microorganisms become nearly undetectable 15 days after manure application. Our findings are consistent with previous reports, where incorporation of fresh, composted or stockpiled manure introduced intestinal microorganisms in the soil, and led to a temporary increase in microbial numbers. This was followed by a decrease in the abundance of most enteric taxa to below detection limits shortly after (Lopatto et al., 2019; Peng et al., 2021).

In our following endeavour we sought to answer the question “What is the effect of the posed antibiotic pressure and manure incorporation on both related and not-directly linked, yet, clinically relevant ARGs?” Out of the tested ARGs, the abundance of *sul1* was stimulated by SMX treatment in the high-pH soil and similar trends, but with no significant differences, were observed in the low-pH soil, in line with previous studies (Cheng et al., 2020). The abundance of the *dfrA1* (trimethoprim resistance conferring) gene was also boosted in the soil samples treated with SMX-spiked manure (Fig. 6A). Sulfonamide resistance is widespread, with associated ARG isoforms being detected in areas under low/no anthropogenic impact (Han et al., 2022; Willms et al., 2019). On the other hand, trimethoprim is usually prescribed in combination with sulfonamides in livestock production and its resistance is rarely encountered in naturally occurring soil

microorganisms (Walsh and Duffy, 2013). It is well known that the immediate environment is largely responsible for formation of the neonatal piglet intestinal microbiome during their lactating period (Thompson et al., 2008). To this end, resistance incidence in piglets has been associated with the sow antibiotic treatments (Burow et al., 2019), while in the case of sulfonamide treatments, trimethoprim is very frequently combined with these sulfonamides (Lekagul et al., 2019). Hence, it is not surprising to identify an SMX-mediated boost of *dfrA1* abundance in the manure-treated soils but not in the corresponding soils not amended with manure. This is consistent with the previously reported combined selection of *sul1/dfrA1* in livestock and other human impacted environments which are affected by the prescriptions of such antibiotic combinations (Carmo et al., 2017; Haulisah et al., 2021; Mahbub et al., 2020; Patil et al., 2020). Besides VAs, manure application clearly stimulated the abundance of all tested ARGs (except of *tetM*) and also the abundance of the HGT marker gene *int1*. This was supported by the fact that we observed such increase of these genes also in the samples treated with the non-spiked manures. It should be noted that the applied manure did not have any background contamination with other VAs, not included in our study, and any curative administration of VAs to the piglets had been halted at least 10 days prior to faecal collection. Hence we hypothesize that the broad stimulatory effect of manure on the ARG soil pool could be attributed either to a selection or co-selection of these ARGs due to the supplementation of piglets feedstuff with zinc (used as a growth promoter in the post antibiotic-growth-promoting banning period), or to inherited/transferred intestinal ARG-containing microbiomes during birth/weaning, as suggested by recent studies (Li et al., 2022; Nowland et al., 2021).

As a final goal in our analysis, we set to identify bacteria acting as potential reservoirs of ARGs in soil through the discovery of positive correlations between the abundance of soil bacteria and ARGs. A ubiquitous ARG in soil like *tetM* was positively correlated

with a relatively broad range of opportunistic pathogenic taxa and common soil dwellers like *Burkholderia-Caballeronia-Paraburkholderia*, *Nocardioides*, *Hyphomicrobium* and *Bacilli* (Fig. 6B). *Clostridium sensu stricto* 6 demonstrated strong correlations with two of the tested ARGs (*dfrA1* and *qnrD*) and *int1* (Fig. 6B). *Clostridia* are common and persistent residents of manures and agricultural soils, and potent toxin-producing opportunistic pathogens (Palmer et al., 2019; Usui et al., 2017). Their ability to form spores facilitates their long-term survival even at high temperatures occurring during manure composting (Usui et al., 2017). Manure incorporation enhanced their presence in soil and, along with their ARG carrying capacity, they may create an explosive mixture of antibiotic resistant toxin producers that pose a serious threat for public health.

5. Concluding remarks

We explored the interactions of VAs, applied in soils directly or through fortified-manures, with the soil microbiota. Our results suggest that the outcome of these interactions will depend on the VA, the soil and the mode of VA delivery in soil. From the beneficial side of the story, repeated application of VAs resulted in an accelerated degradation of TIA. Despite this positive note, the establishment of accelerated degradation only upon direct soil application of TIA questions its relevance in realistic exposure scenarios where VAs reach soil through diffusion from contaminated manures. On the other hand, the repeated application of VAs: (i) negatively affected nitrification and its prokaryotic components (especially SMX), reinforcing the potential of AOM as an ecotoxicological surrogate for assessing toxicity of pollutants on the soil microbiota (van Gestel, 2012); (ii) imposed strong effects on the diversity of the various strata of the soil food web with unknown reciprocal effects on microbial community coherence and functioning (prokaryotes, fungi, protists, AOB, AOA); (iii) boosted

related (*sul1*) or unrelated to their application (*dfrA1*, *qnrD*) ARGs. Besides VAs, manure itself introduced in soil a high microbial load that did not persist, still triggered an increase in the abundance and dispersal of ARGs, as suggested by the increase in *int1* abundance. Strikingly, several opportunistic pathogens, including strains of pronounced clinical importance (*Clostridium sensu stricto* 6, *Burkholderia-Caballeronia-Paraburkholderia*, *Nocardioides*, *Hyphomicrobium* and *Bacilli*), identified as soil or manure dwellers, may act as potent AMR reservoirs in soils exposed to VA-contaminated manures. Collectively, our study: (i) extends current knowledge on the soil behavior and the impact of heavily used in livestock practice, still understudied, VAs (e.g. TIA, TLM) on the soil microbiota, (ii) reinforces the need for consideration of more microbial endpoints in the environmental risk assessment of VAs, and (iii) highlights the multiple risks that the release of VA-contaminated manures impose on human and animal health. Our study elucidates interactions of VAs with the soil microbiota and provides precious insights on underlying ecological mechanisms under soil lab microcosm conditions. Hence, a follow up field testing will further assist in validating these findings under more realistic exposure regimes and manure application scenarios.

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Annex II – Chapter 3 Supplementary Data

Table S1. The physicochemical properties of the two different soils (low (Livadi) of high (Rodia) soil pH) used in the present study

Soil	pH	Organic carbon content (%)	Sand (%)	Silt (%)	Clay (%)
Livadi	5.9	2.31	40.82	47.82	11.36
Rodia	7.9	1.37	45.82	36.0	18.18

Table S2. Primers and thermocycling conditions used for qPCR analysis of target genes.

Primers	Thermocycling conditions	Sequence (5' – 3')	Target	Reference
338f 518r	95°C for 15 s, 62°C for 20 s, 72°C for 30 s (35 cycles) 95°C for 15 s, 60°C for 1 min	ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	Bacterial V4 region of the 16S rRNA gene	(Muyzer et al., 1993)
HS463aF HS464R	95°C for 30 s, 62°C for 30 s, 72°C for 60 s (40 cycles) 95°C for 15 s, 60°C for 60 s	CTGGATTTCGATCACGGCACG ACATGCGTGTAATCATCGTCG	<i>Int1</i>	(Hardwick et al., 2008)
nxB-1F nxB-1R	95°C for 5 s, 57°C for 30 s (40 cycles) 95°C for 15 s, 60°C for 60 s	ACGTGGAGACCAAGCCGGG CCGTGCTGTTGAYCTCGTTGA	<i>nxB</i> gene of Nitrobacter	(Vanparys et al., 2007)
nxB169f nxB638r	95°C for 15 s, 56.2°C for 30 s (40 cycles) 95°C for 15 s, 60°C for 60 s	TACATGTGGTGGAACA CGGTTCTGGTCRATCA	<i>nxB</i> gene of nitrospira	(Pester et al., 2014)
Arch-amoAF Arch-amoAR	95°C for 15 s, 53°C for 30 s, 72°C for 45 s (45 cycles) 95°C for 15 s, 60°C for 60 s	STAATGGTCTGGCTTAGACG GCGGCCATCCATCTGTATGT	<i>amoA</i> gene of AOA	(Francis et al., 2005)
amoA-1F amoA-2R	95°C for 5 s, 57°C for 10 s, 72°C for 30 s (45 cycles) 95°C for 15 s, 60°C for 60 s	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	<i>amoA</i> gene of AOB	(Rotthauwe et al., 1997)

Primers	Thermocycling conditions	Sequence (5' – 3')	Antibiotic resistance genes	Reference
tetM_1	95°C for 5 s, 49°C for 30 s (45 cycles) 95°C for 15 s, 60°C for 60 s	ACAGAAAGCTTATTATATAAC TGGCGTGTCTATGATGTTTAC	<i>tetM</i> gene of tetracyclines	(Koike et al., 2007)
qnrD_ (4)-447	95°C for 15 s, 60°C for 20 s (40 cycles) 95°C for 15 s, 60°C for 60 s	ACGACAGGAATAGCTTGGAAGG TCAGCCAAAGACCAATCAAACG	<i>qnrD</i> gene of fluoroquinolones	(Tan et al., 2018)
dfrA1_1	95°C for 15 s, 60°C for 20 s (40 cycles) 95°C for 15 s, 60°C for 60 s	GGAATGGCCCTGATATTCCA AGTCTTGCGTCCAACCAACAG	<i>dfrA1</i> gene of trimethoprim	(Wang et al., 2014)
sul1_3	95°C for 15 s, 60°C for 20 s (40 cycles) 95°C for 15 s, 60°C for 60 s	GACTGCAGGCTGGTGGTTAT GAAGAACCGCACAAATCTCGT	<i>sul1</i> gene of sulfonamides	(Ross and Topp, 2015)

Table S3. Summary table of primers and DNA concentration used in amplicon sequencing analyses.

Target	Amplified gene fragment	Primers	Concentration	µl used as templates in the 10 µl PCR reactions	Reference
Total Prokaryotes	Prokaryotic V4 region of the 16S rRNA gene	515f 806r	0.1 ng/µl	1	(Parada et al., 2016; Walters et al., 2016)
Fungi	ITS2 Fungal genomic Region	ITS7f ITS4r	1 ng/µl	1	(White et al., 1990; Ihrmark et al., 2012)
Protists	The V9 region of the 18S rRNA gene	1391f EukBr	5 ng/µl	1	(Medlin et al., 1988; Stoeck et al., 2010)
Ammonia oxidizing Archaea	Archaeal <i>amoA</i> gene	amoA310f amoA529r	5 ng/µl	2	(Marusenko et al., 2013)
Ammonia oxidizing bacteria	Bacterial <i>amoA</i> gene	amoA-1f amoA-2r	5 ng/µl	2	(Rotthauwe et al., 1997)

Table S4. The primers, sequences and thermal cycling conditions used for amplicon sequencing analysis. Primer site notation: bold letters in primers (forward or reverse) indicate the sample-specific index sequence sites generated with the barcrawl software (Frank, 2009); italics indicate linker sequences designed to assist index sequence detachment in the case of direct amplification of environmental templates.

Primers	Thermocycling conditions	Sequence (5' – 3')	Target
515f 806r	98°C for 10 s, 50°C for 30 s, 72°C for 30 s (28 cycles + 7 cycles) 72°C for 10 min	NNNNNNNNNN <i>GT GTGYCAGCMGCCGCGGTAA</i> <i>GGACTACNVGGGTWTCTAAT</i>	Bacterial V4 region of the 16S rRNA gene
fITS7 ITS4	98°C for 10 s, 55°C for 30 s, 72°C for 15 s (28 cycles + 7 cycles) 72°C for 10 min	<i>GTGARTCATCGAATCTTTG</i> NNNNNNNNNN <i>GA TCCTCCGCTTATTGATATGC</i>	Fungal ITS2 genomic Region
1391f EukBr	98°C for 10 s, 57°C for 30 s, 72°C for 10 s (30 cycles + 10 cycles) 72°C for 10 min	<i>GTACACACCGCCCGTC</i> NNNNNNNNNN <i>CA</i> <i>TGATCCTTCTGCAGGTTACCTAC</i>	Eukaryotic 18S V9 rRNA gene
amoA310f amoA529r	98°C for 10 s, 54°C for 30 s, 72°C for 20 s (30 cycles + 7 cycles) 72°C for 10 min	NNNNNNNNNN <i>GG TGGATACCBTCWGCAATG</i> <i>GCAACMGGACTATTGTAGAA</i>	Archaeal <i>amoA</i> gene
amoA-1F amoA-2R	98°C for 10 s, 54°C for 30 s, 72°C for 20 s (30 cycles+ 7 cycles) 72°C for 10 min	NNNNNNNNNN <i>AA GGGGTTTCTACTGGTGGT</i> <i>CCCCTCKGSAAAGCCTTCTTC</i>	Bacterial <i>amoA</i> gene

Table S5. The dissipation kinetic parameters of TIA, TLM and SMX in two different soils (low of high pH) where the VAs were repeatedly applied directly or through VA-manure. The SFO kinetic model results are presented in case the χ^2 values ≤ 7.8 % (for m of 3) according to FOCUS (2006). In all other cases, the data obtained by the bi-phasic FOCUS model providing the best fit are presented.

Soil	Treatment	Kinetic Model	DT ₅₀ (days)	DT ₉₀ (days)	k _{deg} (days ⁻¹)	k1 alpha k1	k2 beta k2	Tb g	χ^2 (%)
1 st application cycle									
Sulfamethoxazole									
High pH	Direct	SFO	1.14	3.78	0.609				6.41
	Manure	SFO	0.64	2.13	1.082				3.69
Low pH	Direct	HS	2.36	15.91		0.294	0.094	4.000	1.16
	Manure	DFOP	0.99	8.229		1.089	0.132	0.704	0.47
Tiamulin									
High pH	Direct	SFO	13.93	46.26	0.050				9.98
	Manure	FOMC	7.60	139.7		0.636	3.851		6.00
Low pH	Direct	HS	>365	>365		349.3	<0.001	1050	8.54
	Manure	SFO	50.89	169.10	0.014				4.48
Tilmicosin									
High pH	Direct	SFO	97.67	324.40	0.007				3.30
	Manure	FOMC	>365	>365		0.441	<0.001		4.19
Low pH	Direct	FOMC	>365	>365		0.025	0.026		9.52
	Manure	FOMC	>365	>365		0.418	<0.001		16.06
3 rd application cycle									
Sulfamethoxazole									
High pH	Direct	SFO	9.94	33.02	0.070				2.59
	Manure	SFO	5.75	19.11	0.120				7.36
	Direct	DFOP	3.685	50.48		0.529	0.031	0.523	0.38

Low pH	Manure	DFOP	3.21	39.49	0.548	0.039	0.539	1.22
Tiamulin								
High pH	Direct	SFO	7.47	24.81	0.093			0.56
	Manure	SFO	12.14	40.33	0.057			Inf
Low pH	Direct	SFO	70.84	235.30	0.010			5.99
	Manure	SFO	59.00	196.00	0.012			6.74
Tilmicosin								
High pH	Direct	SFO	135.60	>365	0.005			7.80
	Manure	SFO	126.30	>365	0.005			5.51
Low pH	Direct	SFO	122.10	>365	0.006			2.93
	Manure	SFO	115.50	>365	0.006			2.16

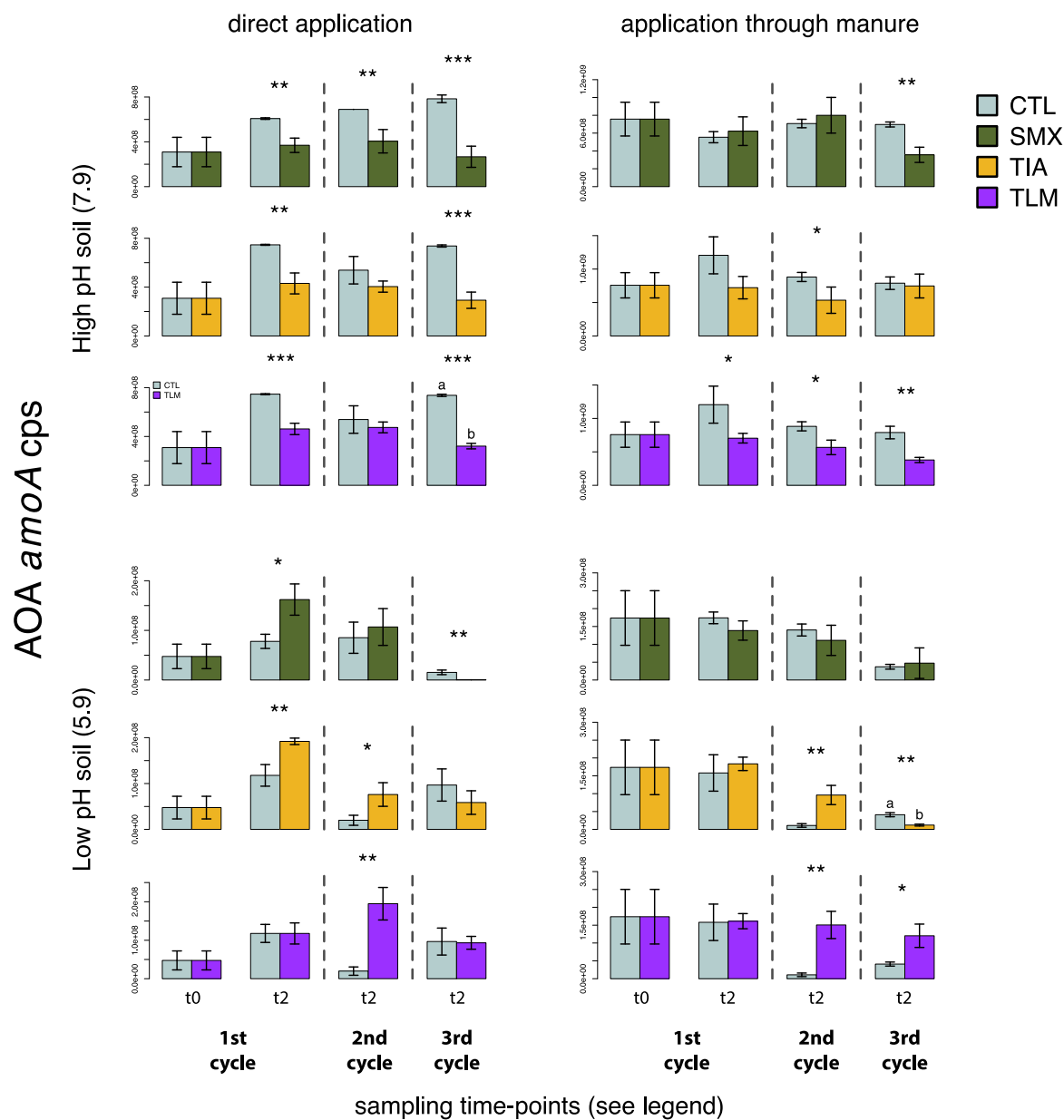


Figure S1. The effects of repeated applications of VAs on the AOA *amoA* abundance in the high and low pH soils receiving VAs directly or via spiked manures. Within each time point significant differences between treated and control samples, as determined by student's t-test, are denoted with asterisks for the Benjamini-Hochberg adjusted P-values for multiple hypothesis testing (sig. codes: '***' 0.001; '**' 0.01; '*' 0.05). CNT: control; SMX; TIA; TLM; t0: time 0 days post application (dpa) within each application cycle; t1: 8 dpa for SMX and 15 dpa for TIA/TLM; t2: 25 dpa for SMX and 50 dpa for TIA/TLM.

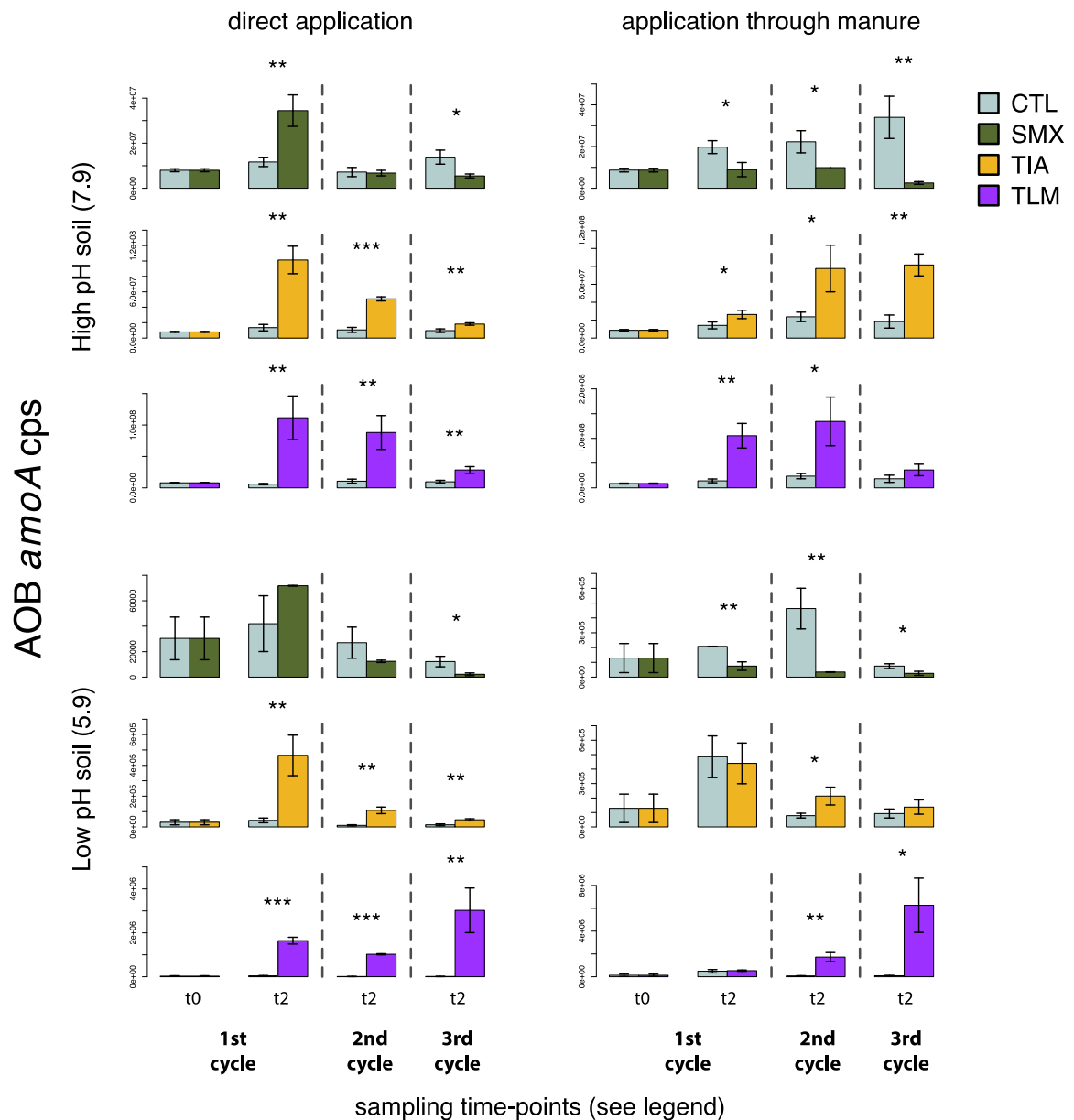


Figure S2. The effects of repeated applications of VAs on the AOB *amoA* abundance in the high and low pH soils receiving VAs directly or via spiked manures. Within each time point significant differences between treated and control samples, as determined by student's t-test, are denoted with asterisks for the Benjamini-Hochberg adjusted P-values for multiple hypothesis testing (sig. codes: '***' 0.001; '**' 0.01; '*' 0.05). CNT: control; SMX; TIA; TLM; t0: time 0 days post application (dpa) within each application cycle; t1: 8 dpa for SMX and 15 dpa for TIA/TLM; t2: 25 dpa for SMX and 50 dpa for TIA/TLM.

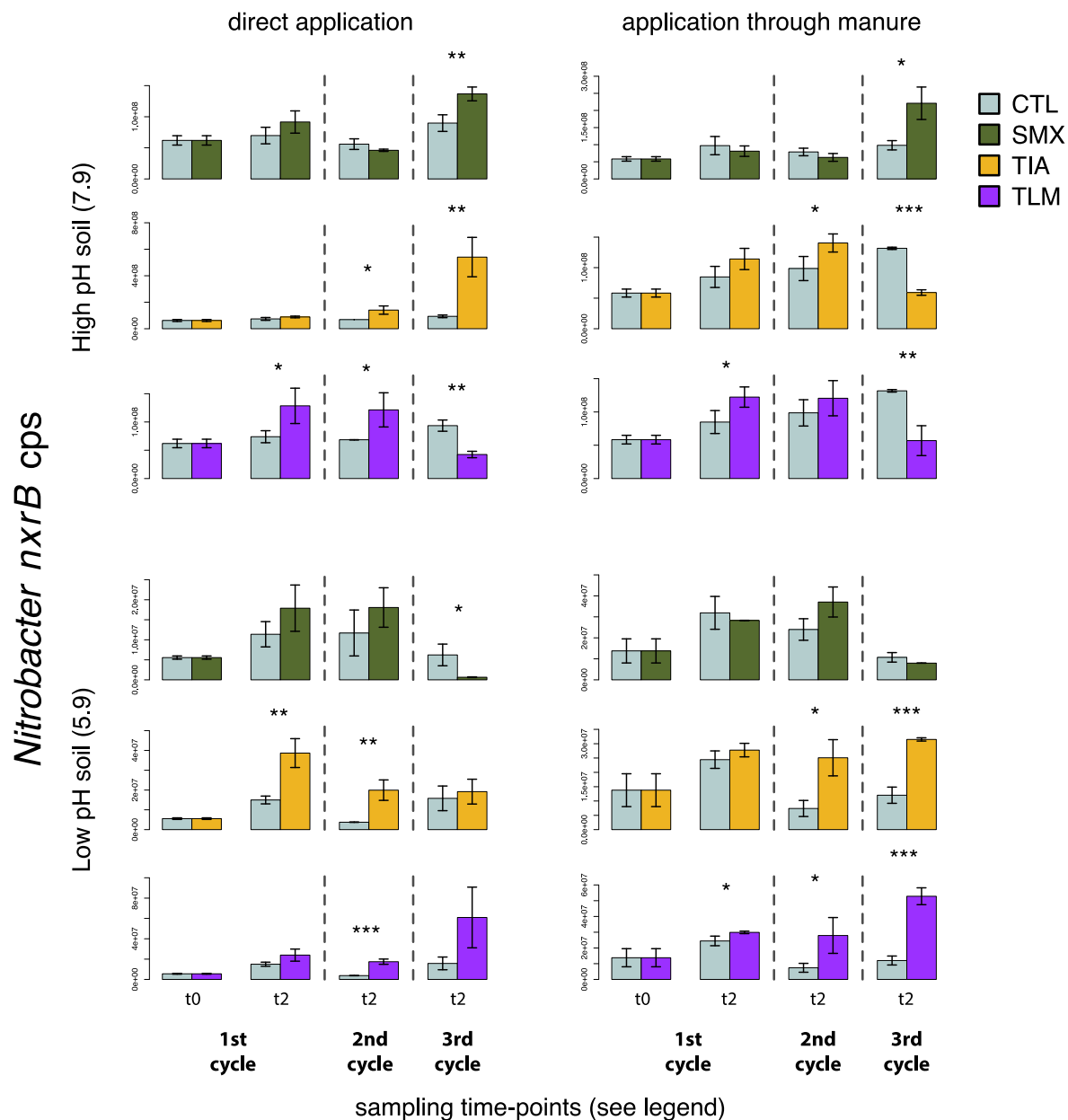


Figure S3. The effects of repeated applications of VAs on the *Nitrobacter nxrB* gene abundance in the high and low pH soils receiving VAs directly or via spiked manures. Within each time point significant differences between treated and control samples, as determined by student's t-test, are denoted with asterisks for the Benjamini-Hochberg adjusted P-values for multiple hypothesis testing (sig. codes: '****' 0.001; '***' 0.01; '**' 0.05). CNT: control; SMX; TIA; TLM; t0: time 0 days post application (dpa) within each application cycle; t1: 8 dpa for SMX and 15 dpa for TIA/TLM; t2: 25 dpa for SMX and 50 dpa for TIA/TLM.

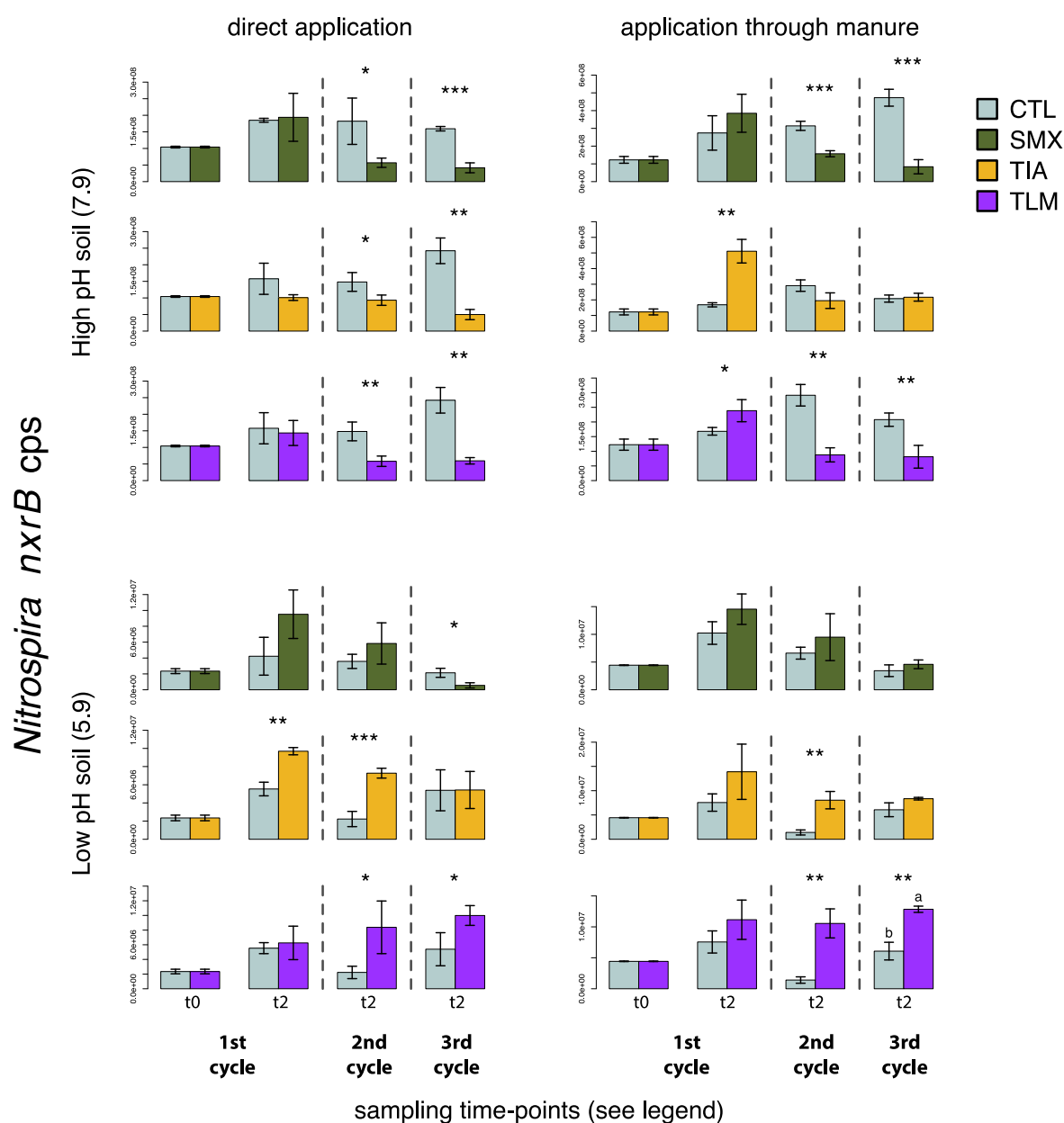


Figure S4. The effects of repeated applications of VAs on the *Nitrospira nxrB* gene abundance in the high and low pH soils receiving VAs directly or via spiked manures. Within each time point significant differences between treated and control samples, as determined by student's t-test, are denoted with asterisks for the Benjamini-Hochberg adjusted P-values for multiple hypothesis testing (sig. codes: '***' 0.001; '**' 0.01; '*' 0.05). CNT: control; SMX; TIA; TLM; t0: time 0 days post application (dpa) within each application cycle; t1: 8 dpa for SMX and 15 dpa for TIA/TLM; t2: 25 dpa for SMX and 50 dpa for TIA/TLM.

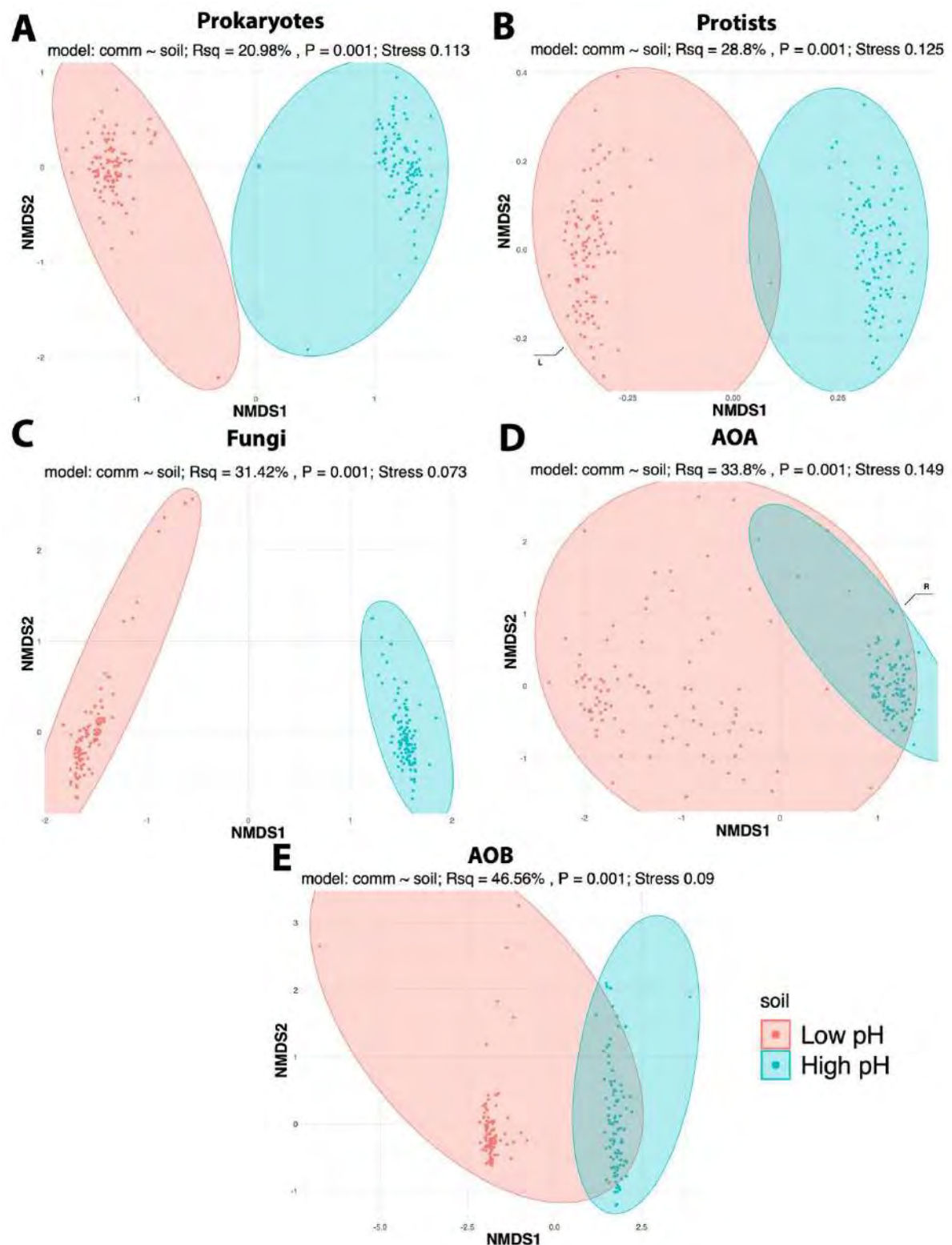
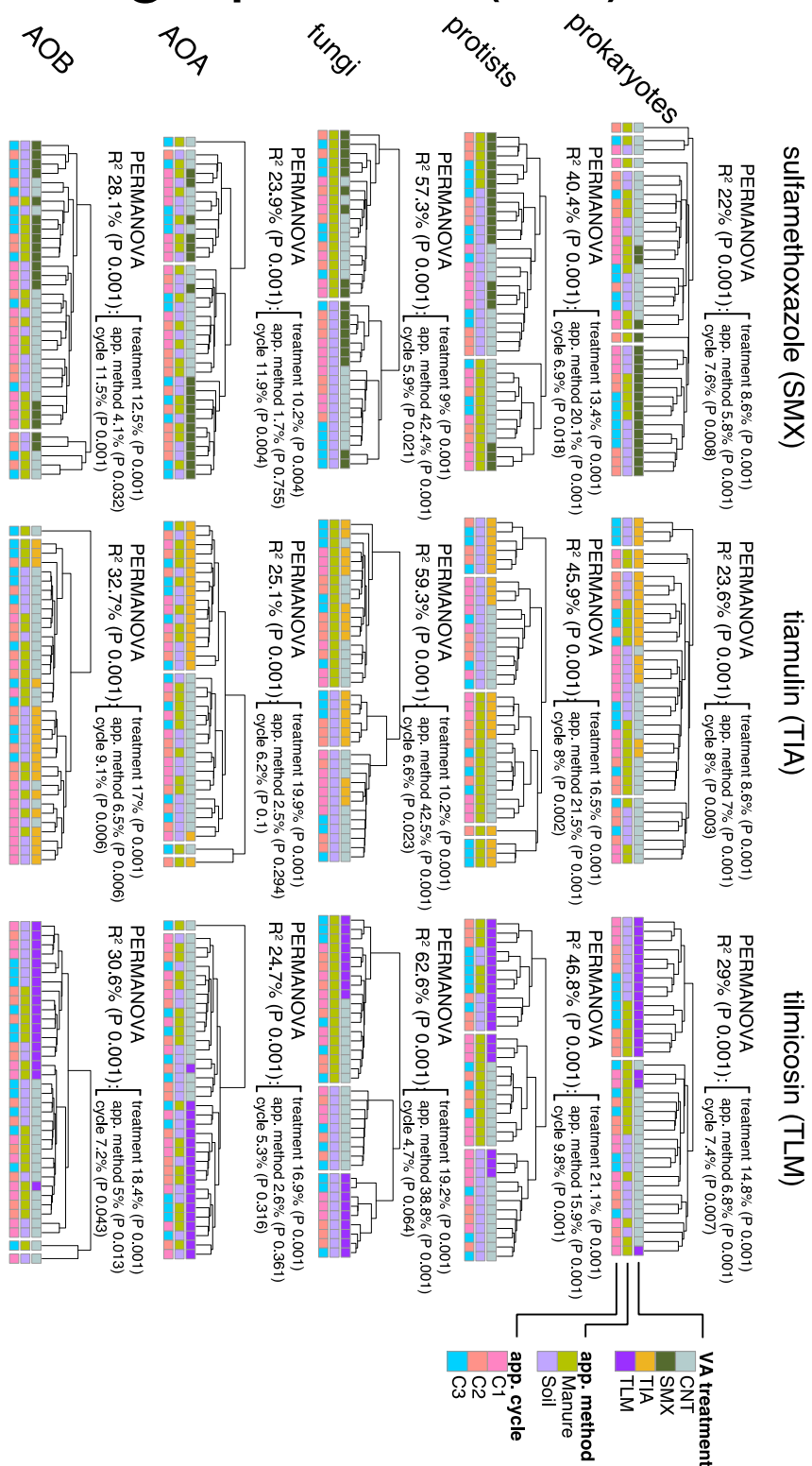


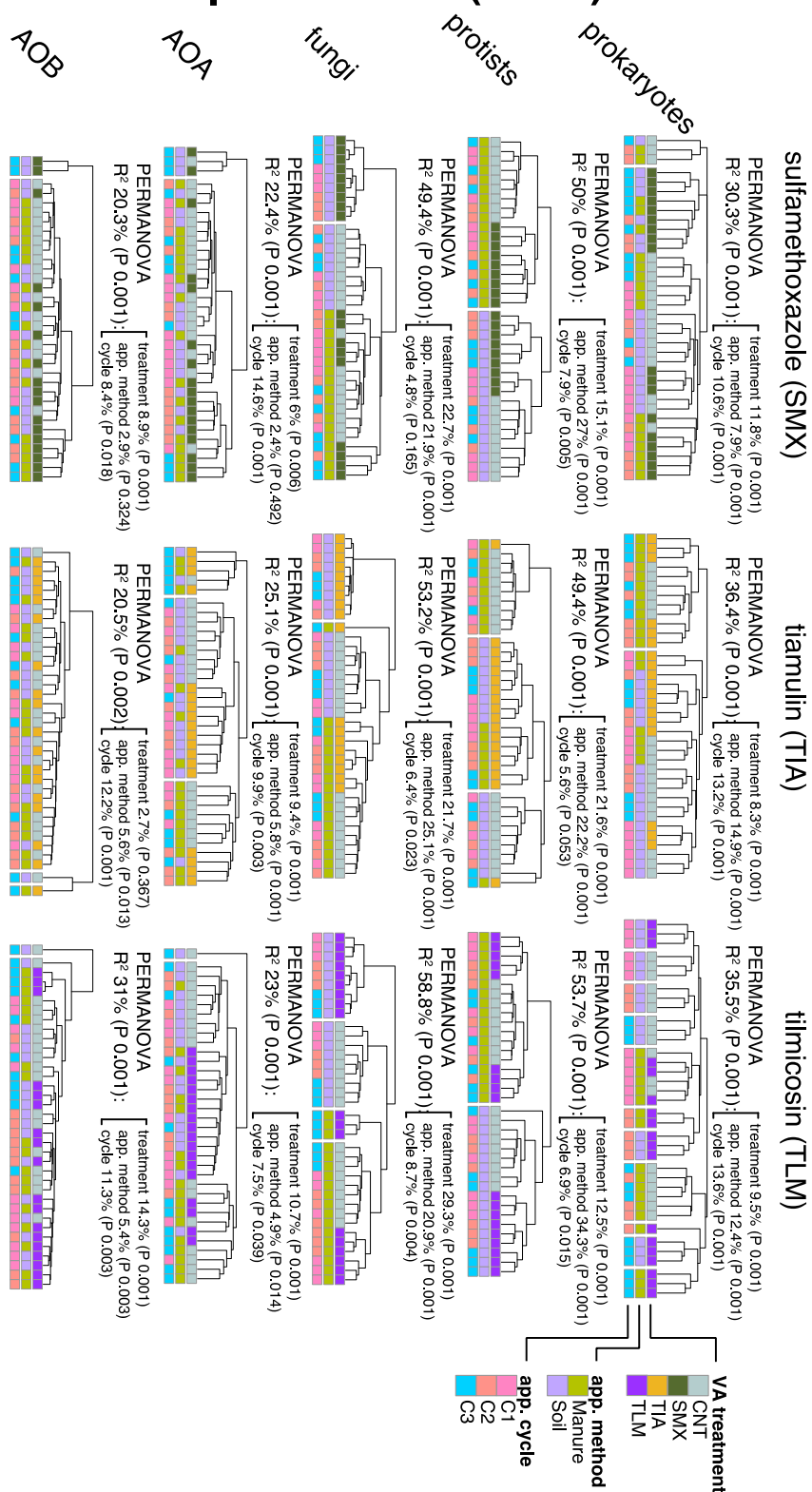
Figure S5 Non-metric multidimensional scaling (nMDS) scatterplots with ellipses representing the two soil samples studied. PERMANOVA results regarding P-values and the level of variance represented by the factor soil are given for prokaryotes (A), protists (B), fungi (C), AOA (D) and AOB (E).

High pH soil (7.9)



A

Low pH soil (5.9)



B

Figure S6 Average linkage hierarchical clustering of samples according to their Bray-Curtis dissimilarities and PERMANOVA output for the analysed molecular markers and antibiotic, treatments, application method and cycle in the high (A) and the low (B) pH soil.

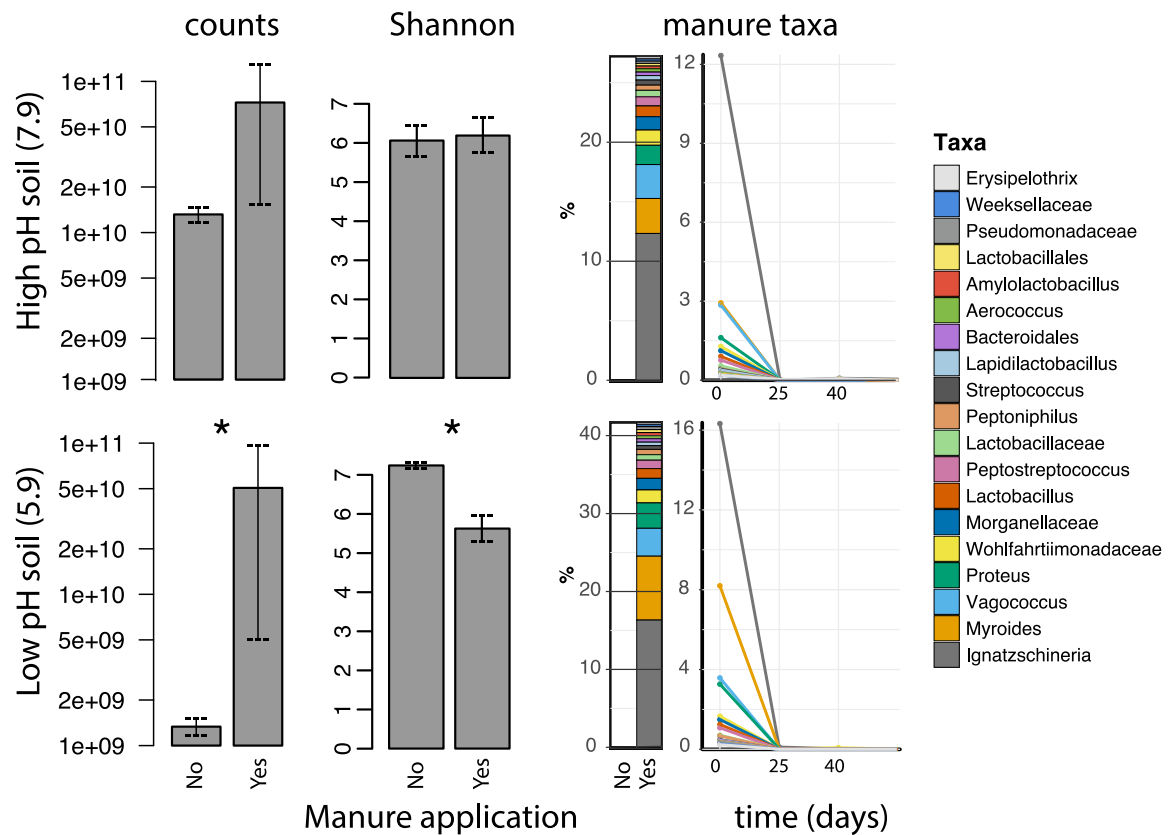
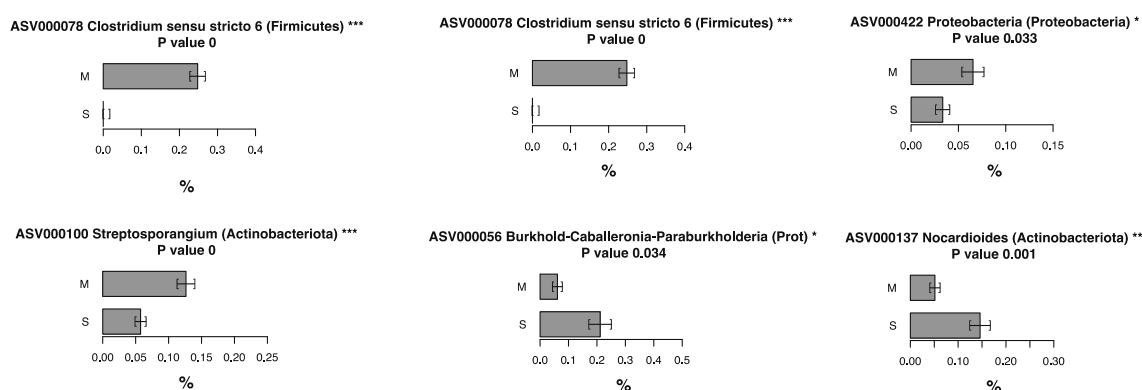


Figure S7. The effect of manure incorporation in the soil bacterial 16S rRNA gene counts and the Shannon diversity index. The fate and persistence of manure-specific bacterial taxa over time in the high and the low pH soils is also presented. Pairwise comparisons of the α -diversity indices between the manure amended and unamended soils were performed in samples retrieved right after the manure application with the Student's t-test or the Wilcoxon rank-sum test (in case the t-test conditions were not met) for $\alpha = 0.05$. Stacked barplots and line graph of manure specific bacterial taxa (with resolution reaching to genus level), as identified with the pime prevalence/random-forest based algorithm followed by the Wilcoxon Rank-sum test after P-value adjustment with the Benjamini-Hochberg method (adj. α of 0.05).

A



B

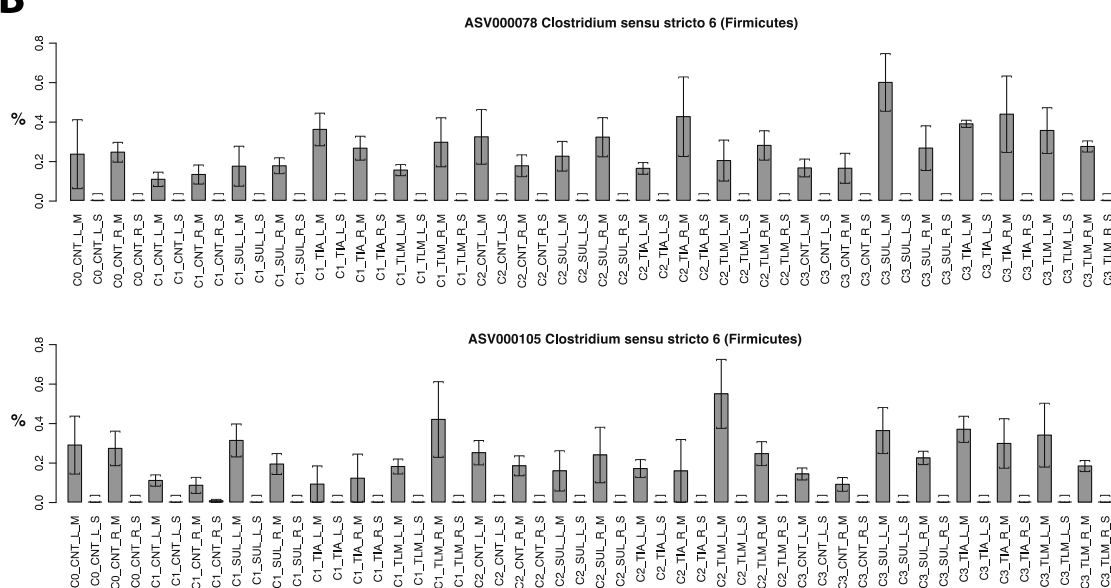


Figure S8. (A) Barplots depicting the mean relative abundances of the significantly different taxa between the manure amended soils (regardless antibiotic treatment or sampling time) and the manure free soils out of the taxa having significantly positive correlations with any of the HGT or ARG tested markers shown in Figure 6. Error-bars represent the standard error values, whereas the significance of the differences according to the performed Wilcoxon rank sum test is reported by the asterisks next to the taxon annotation above the plots in each panel (P-value sig. codes: ‘****’ 0.001; ‘***’ 0.01; ‘*’ 0.05). (B) Barplots showing the means and standard error values (error bars) for the two clostridia of Figure 6 throughout all application cycles (C0-3), all treatments (CNT, SMX, TIA, TLM), the two soils (L: low pH or Livadi; R: high pH or Rodia), and the application methods (M: Manure; S: soil).

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Chapter 4

Bioaugmentation of manures by a tiamulin-degrading *Sphingomonas* as a mean to alleviate environmental dispersal of antibiotic residues

The work presented in Chapter 4 is included in the scientific paper:

Katsivelou E., Perruchon C., Lithourgidis A.A., Kotsopoulos T. A., Karpouzas D.G., Vasileiadis S., 2024 Bioaugmentation of manures by a tiamulin-degrading *Sphingomonas* as a mean to alleviate environmental dispersal of antibiotic residues (Submitted for publication).

1. Introduction

VAs are used in animal husbandry for disease treatment and prevention (Song and Guo, 2014; Martin, et al., 2015; European Medicines Agency (EMA), 2019). Upon their administration ca. 90 % of the dose could be released through animal excreta in the form of the parent compound or their transformation products (Zhao et al., 2010; Song and Guo, 2014; Pan and Chu, 2017; Perruchon et al 2022). Animal excreta are then used as manures for soil organic fertilization facilitating the release of VAs in agricultural soils. Hence, long-term overuse and misuse of VAs may have detrimental effects on the environment appearing either as potential toxicity on off-target soil organisms or evolution and dispersal of ARGs as a result of the selection pressure imposed to the soil microbial community by the presence of VA residues in soil (Katsivelou et al 2023; Castro-Sánchez., 2016; Martin, et al., 2015).

Before being used as fertilizer, faecal matter can be subject to different treatments with the most common being: (i) stockpiling under ambient conditions (Jorgensen and Halling-Sorensen, 2000; Van Epps and Blaney, 2016) or (ii) anaerobic digestion for biogas production (Karim et al., 2005; Bauer et al., 2014). The potential reduction of the VA load of the faecal material by the implementation of such treatments could avert the dispersal of VA residues in agricultural soils. Previous studies have investigated the efficiency of these treatments in removing VAs from the faecal material with the results being contradictory. For example, Visca et al., (2021) showed that SMX was completely removed during anaerobic digestion while, Perruchon et al., (2022) reported limited degradation of TIA and TLM during anaerobic digestion. Gaballah et al., (2023) showed that degradation of antibiotics during stockpiling varied from 61.4% for sulfadiazine to 94.8% for norfloxacin. Bioaugmentation has been a long-known strategy for the removal of xenobiotics from environmental matrices (Miglani et al., 2022). However, its use for the environmental

removal of VAs has been so far limited. To date only a few recent studies have used bioaugmentation for the environmental dissipation of tetracyclines (Hong et al., 2020), SMX (Nguyen et al., 2018), and cephalixin (Gheethi et al., 2016).

TIA is a semi-synthetic derivative of pleuromutilin used to treat enteric and respiratory infections in pigs (EMA, 2014). According to EMA (2021), pleuromutulins account for 2.6% of antibiotic sales for food-producing animals in Europe. Additionally, the World Health Organization categorized TIA as one of the important antibiotics with the intention of prioritizing and protecting the most essential antibiotics for human health (WHO, 2018). Upon its administration in pigs, a significant amount of TIA is excreted through urine (35%) and faeces (65%) (EMA, 1999). Once in faeces TIA tends to be persistent with its DT_{50} exceeding 200 days (Schlüsener et al., 2006; Berendsen et al., 2018). Little is known about the biodegradation of TIA. Nguyen et al., (2017) isolated a white-rot fungus capable of degrading TIA. In a follow up study, the same group (Nguyen et al., 2019) obtained a TIA-degrading bacterial consortium from swine wastewater composed of *Achromobacter*, *Delftia*, *Flavobacterium*, *Pseudomonas*, and *Stenotrophomonas*, although the exact involvement of each of these bacteria in the degradation of TIA was not verified. Our group has isolated, from a soil repeatedly treated with TIA, a *Sphingomonas* strain, belonging to a novel species *Candidatus Sphingomonas perruchonii* (strain DDB INV C78), that was able to rapidly degrade TIA (Perruchon et al., 2023)

The aim of this study was to investigate the efficiency of this TIA-degrading *Sphingomonas* strain for the bioaugmentation of TIA-contaminated manures, comparatively to other commonly used decontamination treatments like anaerobic digestion and stockpiling. To address this we employed a lab study where pig faecal material fortified with TIA at 5 and 50 $\mu\text{g g}^{-1}$ was subjected to bioaugmentation, anaerobic digestion and stockpiling under ambient conditions and the dissipation of TIA was monitored. In addition, the impact of TIA on the performance of the anaerobic

digestion was monitored and the dynamics of the prokaryotic microbiota was determined via amplicon sequencing.

2. Materials and Methods

2.1. Chemicals and faecal material

The analytical standard of TIA fumarate (Vetranal® Sigma-Aldrich, purity > 98%) was utilized for all experimental purposes (treatment of faecal material, analysis and microbial inoculum growth). The faecal material used was collected from a commercial pig farm in the area of Karditsa, Greece (HOIROPAL, Palamas, Thessaly, Greece) from 30-45 days old piglet which had not been treated with TIA (as verified by HPLC analysis). Any possible previous treatment with other antibiotics was curative and stopped ~10 days before any faecal matter collection. The faeces were collected and stored at 4°C in the dark for 2 weeks until downstream use.

2.2. Stockpiling and bioaugmentation of faecal material

2.2.1. Microbial inoculum preparation

Ca. *Sphingomonas perruchonii* DBB INV C78 was isolated from an agricultural soil exhibiting enhanced degradation of TIA (Perrouchon et al 2023). The bacterium was grown in Minimal Salt Medium plus Nitrogen (MSMN) supplemented with 50 µg ml⁻¹ of TIA as the sole carbon source. The flasks were incubated at 25°C for 36 hours at 180 rpm and the density of cells at the time of inoculation (mid log phase of growth) was 3.13 x 10⁶ cells ml⁻¹ as determined by q-PCR according to Perruchon et al (2017a).

2.2.2. Experimental Setup

The faecal material was divided into two portions of 1200 g each. Each portion was treated with a methanol solution of TIA leading to final concentrations of 5 and 50 µg

g⁻¹. These two concentration levels were selected based on the levels of VAs commonly detected in pig faeces and our earlier feeding studies with TIA (Perruchon et al., 2022). Each 1200 g portion was further divided into four 300 g portions. The first two were amended with 60 ml of the *Ca. S. perruchonii* DBB INV C78 inoculum each serving as bioaugmentation stockpiling treatment (BS) and the other two received the same amount of MSMN without bacterial cells to serve as non-bioaugmented stockpiling treatment (nBS). All 300 g portions were divided into triplicates and were placed in aerated plastic bags which were stored at ambient conditions (November 2022-December 2022; mean temperature 14.1°C in November and 10.3°C in December Annex III: Table S1) simulating stockpiling conditions. Overall, the specific experimental setup included the following treatments: BS 5µg g⁻¹, BS 50µg g⁻¹, nBS 5µg g⁻¹, nBS 50µg g⁻¹. Triplicate samples were collected from each treatment at 0, 5, 15, 30, and 60 days and were analysed via HPLC for VA residues. During storage, the moisture content of the faecal material was maintained constant at 70% of the water holding capacity with regular additions of ddH₂O when needed.

2.3. Anaerobic digestion of the faecal material

2.3.1. Experimental Set up

Another portion of the collected faeces was treated with TIA at two concentration levels as described above (5 and 50µg g⁻¹) and was subjected to anaerobic digestion. The specific experiments were performed in collaboration with the Laboratory of Agricultural Structures and Equipment, Aristotle University of Thessaloniki, Greece. The inoculum used for the anaerobic digestion was obtained from a previous anaerobic digestion experiment and pre-incubated at 37 ± 0.50C until no significant methane production was detected to deplete eventual residual biodegradable organic material (Angelidaki et al., 2009). For each concentration level, triplicate batch anaerobic digesters were prepared along with triplicate controls containing VA-untreated faeces

(VA-free controls) and triplicate inoculum controls (no faeces). Methane produced from the inoculum controls was subtracted from the methane production in all faeces-containing samples. Batch anaerobic digestion tests were conducted in glass bottles (batch reactors) of 0.3 L vol. The ratio of TS to VS of the faeces and the inoculum was determined as described by APHA (2005). The feces loading factor was 1.5 g VS and the feed to inoculum ratio (F/I) was 1 in all batch reactors. The F/I ratio was based on the initial VS of the faecal material and the inoculum. Upon addition of the designated amounts of inoculum and faeces in each batch reactor, their working volume was adjusted to 0.15 L with addition of deionized water. The headspace of each batch reactor was purged with nitrogen gas (99.99% purity) for approximately 2 min to ensure anaerobic conditions. Subsequently, all batch reactors were sealed and placed in a heat chamber at $37 \pm 0.50^\circ\text{C}$ and they were daily manually mixed. Headspace gas samples were obtained from the reactors for biogas composition analysis on a regular basis (for the first 11 days daily, and every few days after). Parallel triplicate batch reactors were set for each treatment and sampling timepoint (0, 7, 17, 27 and 37 days) to monitor both VFA concentrations and VA dissipation. Regarding VA residue analysis, samples were centrifuged for 15 min at 10000 rpm. The solid phase was extracted and analysed in HPLC as previously described while the liquid phase was diluted 1:2 with water, filtered to remove suspended solids and directly analysed in HPLC.

2.3.2. Biogas, methane and VFA measurements

Biogas samples (0.15 mL) were analysed in a gas chromatograph (GC-2010plusAT, Shimadzu, Japan) equipped with a thermal conductivity detector (GC-TCD) and two columns: (a) GC-Column, FS, Valco-PLOT HayeSep D and (b) VP- Molesieve Column, FS, ValcoBond, 5AFused Silica. Helium gas was used as a carrier gas and the GC was calibrated by using a certified gas containing 60% methane- 40% carbon dioxide (Kalamaras et al., 2020). CH_4 concentration was used to calculate the cumulative

production. The measured methane volumes were adjusted to the volumes at standard temperature (0°C) and pressure (1 atm). The experiment was terminated when methane production during three consecutive days was <1% of the accumulated volume of methane, as established by Holliger et al. (2016). For VFA measurement, 5 ml samples from each batch reactor were extracted with 0.1 ml of H₃PO₄ and the pH was measured. Samples were centrifuged twice for 10 min at 12000 rpm and then analysed in a gas chromatograph (GC-2010plusAT, Shimadzu) equipped with a flame ionization detector (GC-FID) and a ZB-FFAP column (Zebron-Phenomenex). Column oven temperature was set at 60°C for the first 3.5 min, then increased at 25°C/min rate to 130°C and then at 10°C/min to 235°C, with a final hold of 5 min. The injection sample volume was 1 µL and helium was used as carrier gas (M.L. Christou et al., 2021).

2.3.3. Amplicon sequencing and bioinformatic analysis

Faecal samples collected at days 7 and 27 along the anaerobic digestion were subjected to amplicon sequencing analysis to identify changes in the prokaryotic microbiota that would explain the potential effects of TIA to the performance of anaerobic digestion (biogas production, VFAs etc). DNA from faecal samples were extracted with the DNeasy® PowerSoil® Pro kit (Hilden, Germany). The DNA extract aliquots were shipped to the Integrated Microbiome Resource (IMR) sequencing center (Dalhousie University, Halifax, NS, Canada) and amplicon sequencing was performed according to their Illumina MiSeq 2x300bp in-house protocol for amplicons generated with primers V4 515FB - 806RB (Walters et al., 2015; Comeau et al., 2017) (see Annex III: Table S2 for primer sequences and thermocycling conditions). The retrieved sequences were subjected to quality assessment and control with the dada2 v1.24.0 (Callahan et al., 2016) pipeline using the R software v4.1.3 (R Core Team, 2023), and ASV matrices were obtained as follows. Sequence reads were trimmed at the first instance of very low bases (Phred Q values of 2) while screened from the read error prone end towards the start. The remaining parts were rejected if the expected

error rates were at most 2 or if the remaining read parts were shorter than 150 bp. Moreover, read-pairs where the reconstruction of the amplicon of origin via merging (allowing no mismatches) was not possible, were removed. Finally, chimeric, non-specific or off-target amplicons (non-prokaryotic, unclassified, microchondrial, or chloroplast) were also rejected from downstream analysis. Classification of the ASVs into taxa was performed with the Bayesian classifier (Wang et al., 2007) version of dada2 against the Silva v138 database using an 80 % bootstrap cutoff value (Yilmaz et al., 2014).

2.4. Determination of tiamulin residues in faecal material

TIA was extracted from faeces as described by Perruchon et al. (2022). Briefly, 1g of faeces was weighed in a 50 ml Teflon tube (Nalge Nunc International, Oak Ridge Centrifuge Tube) and 10 ml of water: methanol: acetone 50:25:25 v/v/v with 25 mM EDTA, 0.6M NaCl and 2% ammonia (extraction solution) were added. After 1 min vortex and 15 min of sonication in an ultrasound bath, the sample was centrifuged at 10.000 g for 5 min at 4°C and the supernatant was collected. The sample was extracted once more and the combined supernatant was passed through a Poly-Sery HLB cartridge (CNW Technologies) preconditioned with 5 ml of methanol followed by 6 ml of water. Afterwards, the cartridge was washed with 5 ml of water, vacuum-dried for 3 min, and the antibiotic eluted once with 7 ml of 9:1 methanol:acetonitrile (v/v), containing 0.2% formic acid and once with 7 ml of 1:9 methanol:acetonitrile (v/v), containing 0.2% formic acid. After vacuum-dried at 35°C the samples were resuspended in 0.5 ml of a water: acetonitrile (50:50 v/v) solution, filtered through a 0.22µm filter (PTFE Hydrophobic Syringe Filter, ALWSCI), and analysed via a Prominence Modular HPLC-PDA (Shimadzu, Kyoto, Japan) system equipped with a CNH Athena RP C18-150 mm column (CNW Technologies, Düsseldorf, Germany). Elution and detection conditions were also described by Perruchon et al. (2022).

2.5. Statistical analysis

2.5.1. Dissipation kinetics of TIA

The SFO and three biphasic kinetic models (HS, FOMC and DFOP) were used to calculate the dissipation kinetics for TIA in the faecal material (FOCUS, 2006). The χ^2 test as well as visual inspection were used to assess the agreement between curve fitting and observed data. Data analysis was performed with the mkin v1.0.3 package (Ranke, 2020) of the R software v4.0.2 (R Core Team, 2021).

2.5.2. Statistical analysis of amplicon sequencing data

The retrieved ASV matrices were used for a series of statistical analysis tasks. α -diversity indices representing members of the studied microbial communities of various dominance levels were calculated with the Vegan v2.6-4 (Dixon, 2003) and the Entropart v1.6-11 (Marcon and Hérault, 2015) R packages. Specifically, the observed richness (representing all community), the Shannon index (representing the, at least, low in dominance community members), the Inverse Simpson index (representing the, at least, intermediate in dominance community members), and the Fisher's α index (representing the highly dominant community members) were calculated. Permutational multivariate analysis of variance (PERMANOVA) and Redundancy analysis (RDA) were performed with the vegan package of R, for assessing the effects of the antibiotic treatment, the antibiotic dose or the interaction of these two parameters. Analysis of variance with the Tukey's post hoc test or their non-parametric equivalents (Kruskal-Wallis and the Wilcoxon rank sum analysis) were used for comparing α -diversity indices, while analysis for differentially abundant taxa between treatments and time was performed with the Kruskal-Wallis (k test-factor levels, with $k > 2$) and the Wilcoxon rank sum (2 test-factor levels) analysis.

3. Results

3.1. TIA dissipation in the faecal material upon bioaugmentation and stockpiling

The dissipation of TIA in the faecal material was best described by the SFO model, with the exception of the bioaugmented samples treated with the low rate of TIA, where the biphasic HS model provided the best fit to the dissipation data (Table 1, Figure 1). In the stockpiling treatment, the dissipation of TIA proceeded at slow rates with DT_{50} values of 113.8 and 95.4 days in the faecal material initially treated with $5 \mu\text{g g}^{-1}$ and $50 \mu\text{g g}^{-1}$ respectively. Comparatively the dissipation of TIA in the bioaugmented samples proceeded at a faster rate with DT_{50} values of 66.2 and 32.3 days respectively.

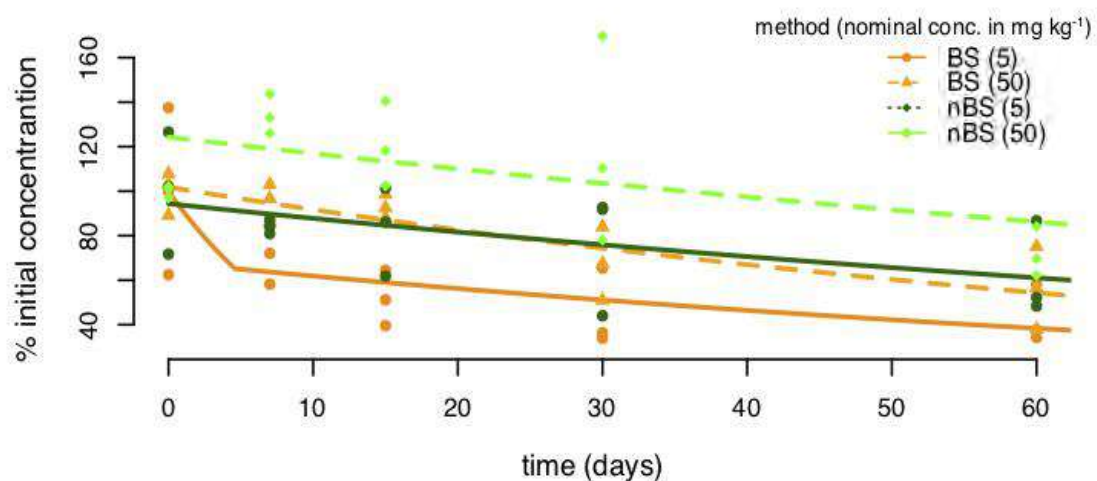


Figure 1: The dissipation of TIA in faecal material treated with 5 and 50 mg kg^{-1} of the antibiotic and either stockpiled (incubated at ambient temperature) (nBS) or amended with the TIA-degrading bacterium *Ca. Sphingomonas perruchoni* (BS). Each point is the mean of three replicates with error bars denoting the standard deviation.

Table 1: The dissipation kinetic parameters of TIA in pig faecal material at two dose rates (5 and 50 $\mu\text{g g}^{-1}$) during stockpiling (nBS) and bioaugmentation (BS). The kinetic model fitting the dissipation data were the SFO and the biphasic HS.

Treatment	Kinetic Model	DT ₅₀ (d)	DT ₉₀ (d)	K _{deg} (d ⁻¹)	k ₁ (d ⁻¹) /alpha	k ₂ (d ⁻¹) /beta	t _b (d) /sigma	χ^2 (%)
nBS 50 $\mu\text{g g}^{-1}$	SFO	113.8	>365	0.006				11.9
BS 50 $\mu\text{g g}^{-1}$	SFO	66.21	219.9	0.010				4.184
nBS 5 $\mu\text{g g}^{-1}$	SFO	95.38	316.9	0.007				3.643
BS 5 $\mu\text{g g}^{-1}$	HS	32.29	200.6		0.094	0.010	4.570	11.05

3.2. Anaerobic digestion

3.2.1 Tiamulin dissipation

The dissipation of TIA during anaerobic digestion was best described by the SFO model. TIA was particularly persistent with DT_{50} values of 103 days and 126.7 days in the samples treated with the 5 and the 50 $\mu\text{g g}^{-1}$ dose rate respectively (Table 2, Figure 2).

Table 2: The dissipation kinetic parameters of TIA in pig faecal material in two different dose rates (5 and 50 $\mu\text{g g}^{-1}$) during anaerobic digestion. The kinetic model fitting the dissipation data was the SFO.

Dose rate ($\mu\text{g g}^{-1}$)	Kinetic Model	DT_{50} (days)	DT_{90} (days)	k_{deg} (days $^{-1}$)	χ^2 (%)
5	SFO	126.7	>365	0.005	5.622
50	SFO	103	342.1	0.007	10.63

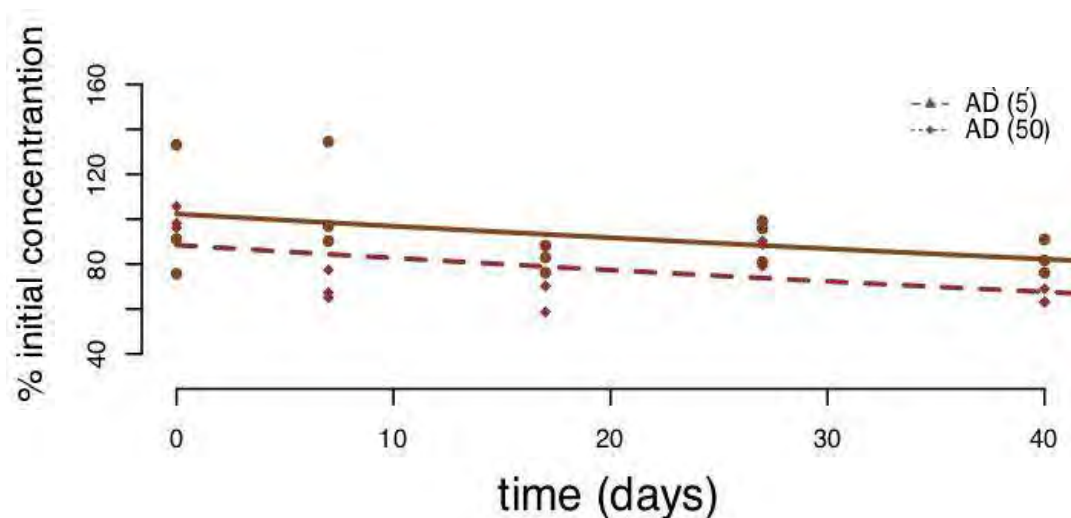


Figure 2: The dissipation pattern of TIA during anaerobic digestion of faecal material fortified with 5 and 50 mg kg^{-1} of the VA. Each point is the mean of three replicates with error bars denoting the standard deviation.

3.2.2. Biomethane and VFAs production profile

In addition to TIA dissipation, we monitored the production of biomethane during anaerobic digestion of the faecal material. TIA-treated samples exhibited significantly higher methane production compared to the untreated control group. Interestingly the highest methane production was observed in the samples treated with the low TIA dose rate (5 mg kg^{-1}) (Figure 3a and 3b). Regarding VFAs, we observed a rapid reduction in their total concentration during the anaerobic digestion process, regardless of TIA presence. The VFA concentrations in the beginning of the process were more than three times higher in the TIA-treated samples (598 and 698 mg L^{-1} for $50 \text{ } \mu\text{g g}^{-1}$ and $5 \text{ } \mu\text{g g}^{-1}$, respectively) compared to the untreated group (184 mg L^{-1}). However, VFA quantities were substantially reduced by day 7 (183 and 162 mg L^{-1} for $50 \text{ } \mu\text{g g}^{-1}$ and $5 \text{ } \mu\text{g g}^{-1}$, respectively) and were completely depleted by day 27, not significantly differing with the untreated control samples (Figure 4a). A total of nine VFAs were detected during the study amongst all treatments, with acetic acid, propionic acid, butyric acid, isobutyric acid, and isovaleric acid being the most abundant at the beginning of the anaerobic digestion process. The first three of those VFAs were produced at significantly higher concentrations at the onset of digestion in the TIA – treated samples compared to the control samples (Figure 4b).

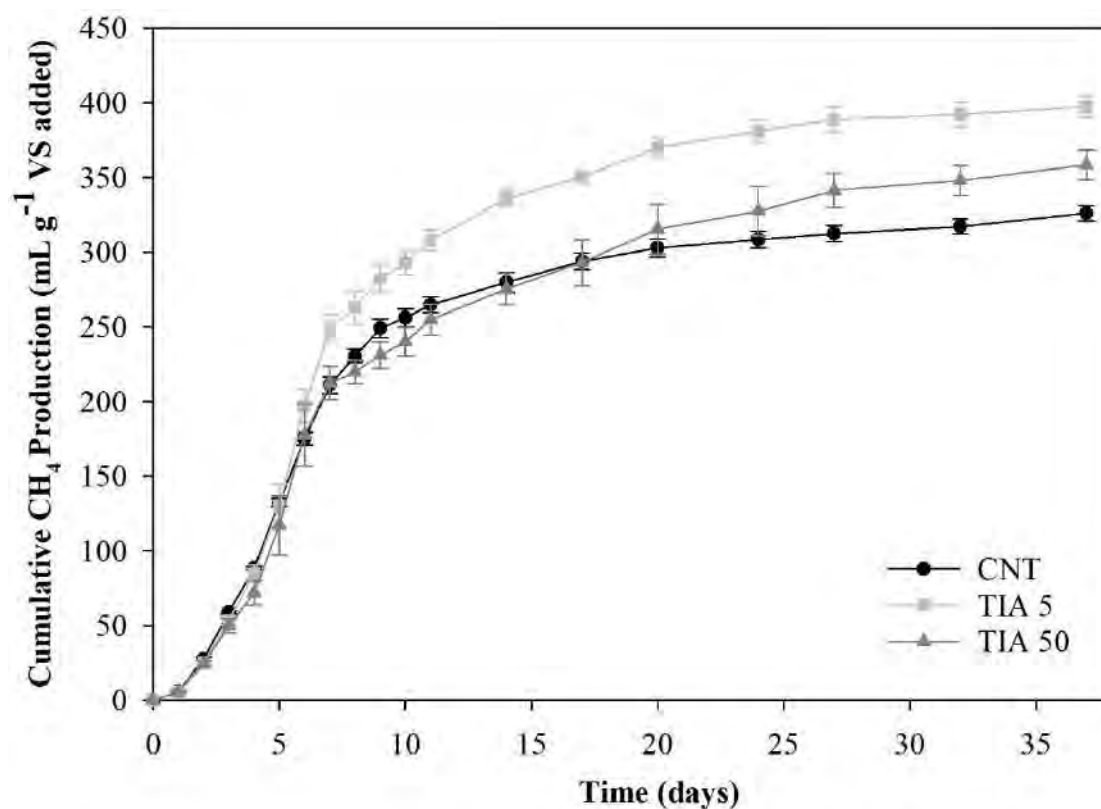
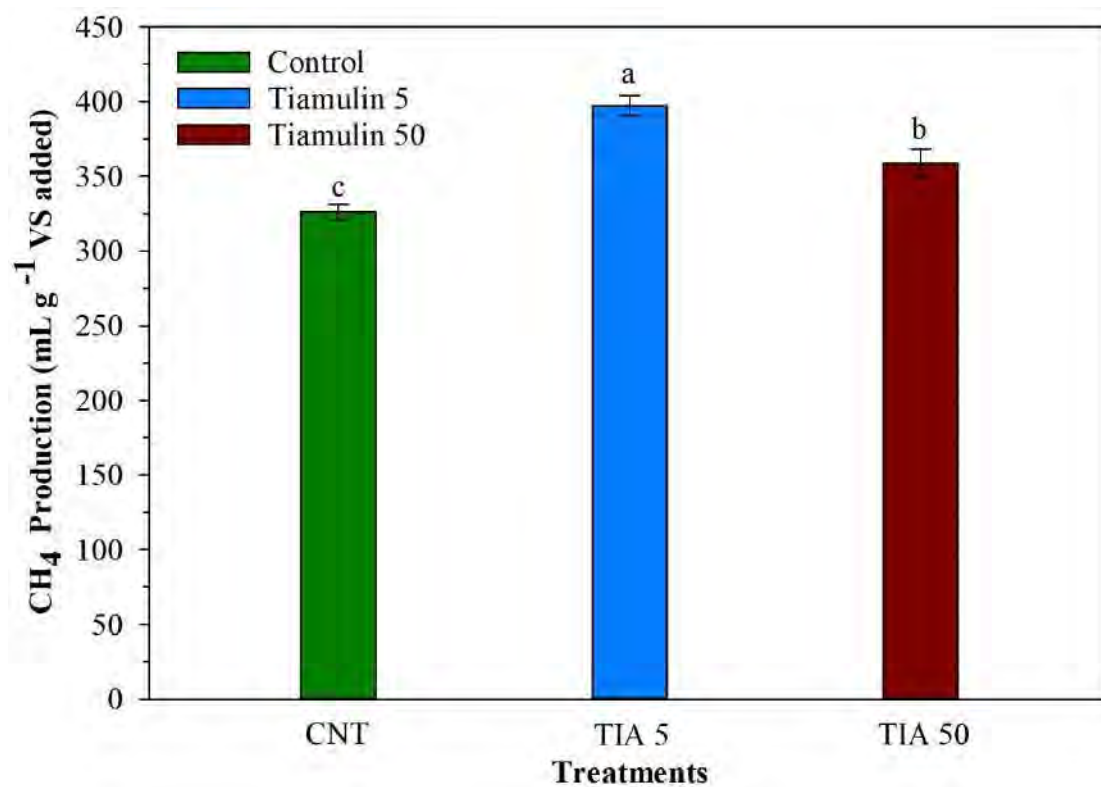


Figure 3: Total (a) and cumulative (b) production of methane during anaerobic digestion of faecal material unamended (CNT) or amended with 5 and 50 mg kg⁻¹ of TIA. Error bars denote the standard deviation of three biological replicates.

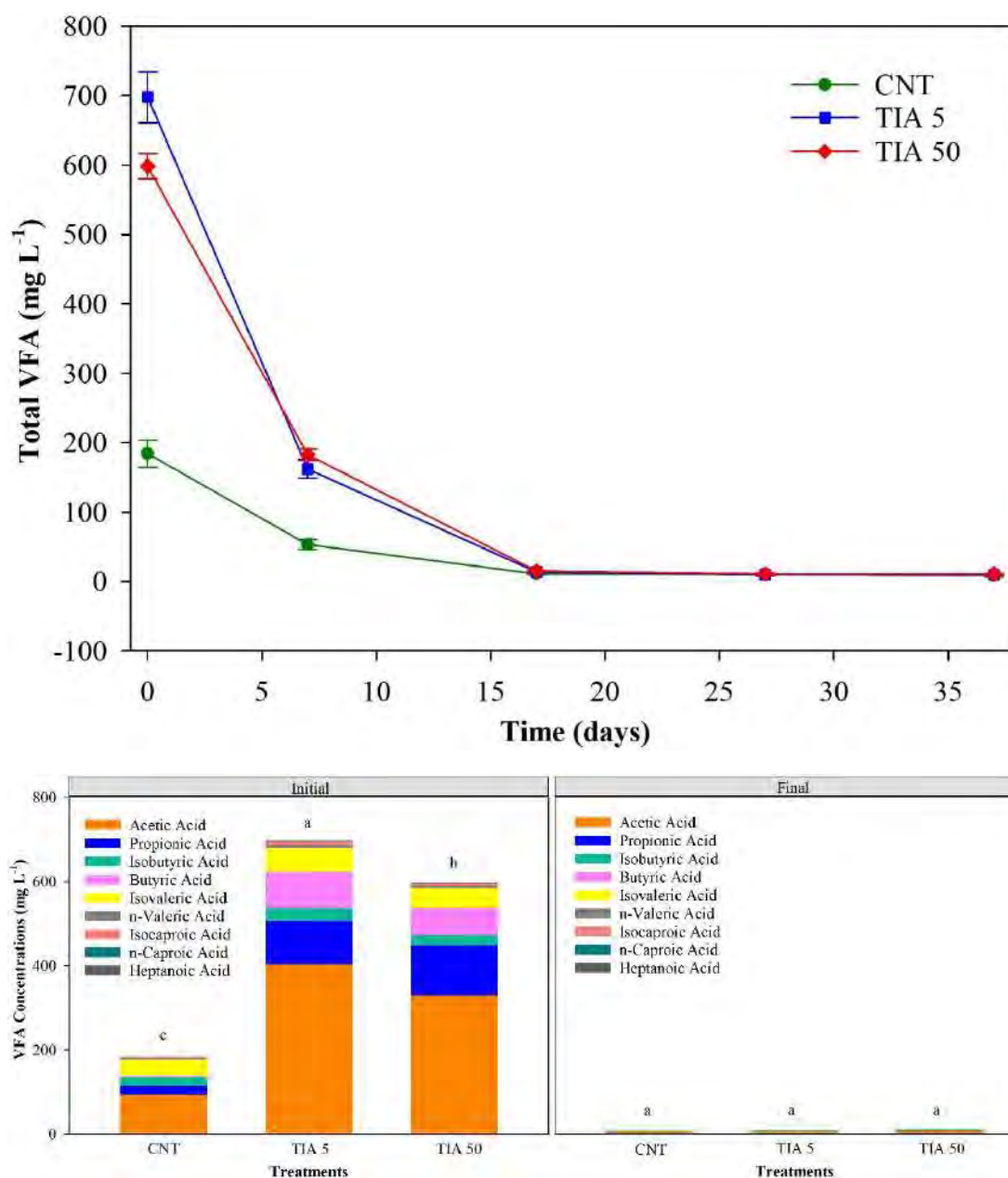


Figure 4: The concentration of total (a) and individual (b) VFAs produced during anaerobic digestion of faecal material unamended (CNT) or amended with 5 and 50 $\mu\text{g g}^{-1}$ of TIA. Each point is the mean of three replicates with error bars denoting the standard deviation.

3.2.3. Analysis of microbial succession during anaerobic digestion

We initially looked at the diversity of archaea in the prokaryotic community as they are considered key contributors in the biomethane process. We noted that methanogenic archaea, of the genus *Methanosarcina* and, only in isolated samples, of *Methanosaeta*, were abundant only at 7 days in the samples treated with the 5 $\mu\text{g g}^{-1}$ TIA, while their relative abundance was below 0.1% in the samples from the other treatments at 7 days and also in all treatments at day 27 (Annex III: Figure S1).

We further explored the impact of TIA treatment on the diversity of prokaryotes. Significantly higher alpha-diversity values were observed in the faecal samples treated with the 5 $\mu\text{g g}^{-1}$ TIA compared to the untreated samples at day 7, while this difference was still significant at day 27 only in the inverse Simpson (Figure 5).

The structure of the prokaryotic community showed significant differences according to time (PERMANOVA R^2 of 23.14 % and p 0.001), whereas no significant differences (p 0.152) according to the antibiotic treatment were evident (Figure 6). When the interaction of these two parameters was tested alone, highly significant differences were observed (PERMANOVA R^2 of 45.09 % and p 0.001), mostly due to the observed high between-treatment-group differences of the samples at day 7 (Figure 6A and 6B). An overall shift of the microbial communities towards an increase of Bacteroidetes was observed over time (Figure 6C), while treatment-wise differences within each timepoint were observed only at day 7 for *Methanosarcinaceae* which showed a significantly higher relative abundance in the samples treated with 5 $\mu\text{g g}^{-1}$ TIA compared with the rest treatments (Figure 6D). Statistically significant differences in the relative abundance of specific ASVs between the two time points were observed for *Marinilabiliaceae* and MgMjR-022 (Bacteroidetes) which showed a significantly higher relative abundance at day 27, unlike *Methanosarcinaceae*, *Clostridiaceae* and *Peptostreptococcaceae* whose relative abundance decreased from day 7 to day 27 (Figure 6E).

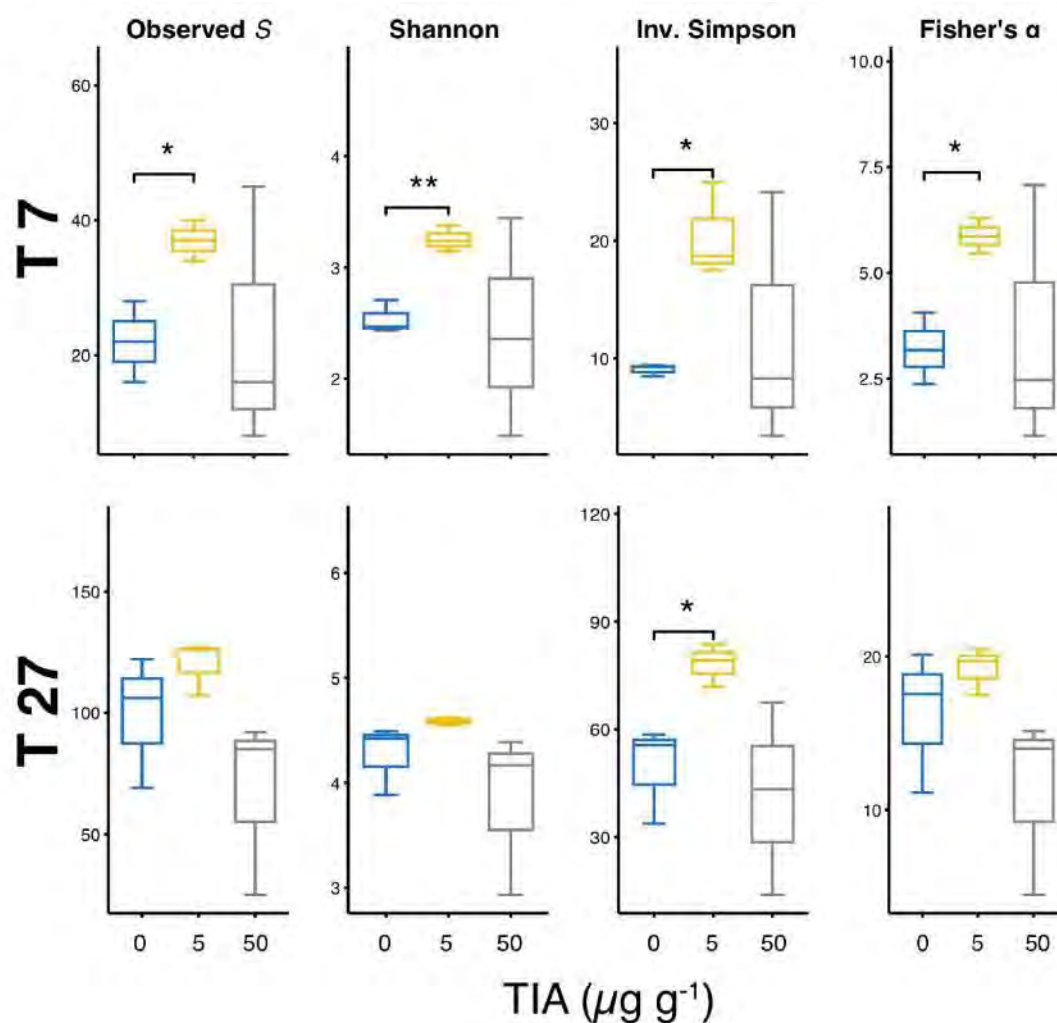


Figure 5: Boxplots comparing the differences between treatments for each of the analysed alpha-diversity indices at each of the two time-points of the study (7 and 27 days). Asterisks show statistically significant differences at various significance levels (significance level notation: * α of 0.05; ** α of 0.01; *** α of 0.001) according to the performed ANOVA and Tukey's HSD post-hoc analysis, or the non-parametric equivalents of Kruskal-Wallis and the Wilcoxon rank-sum tests in case the ANOVA conditions were not met.

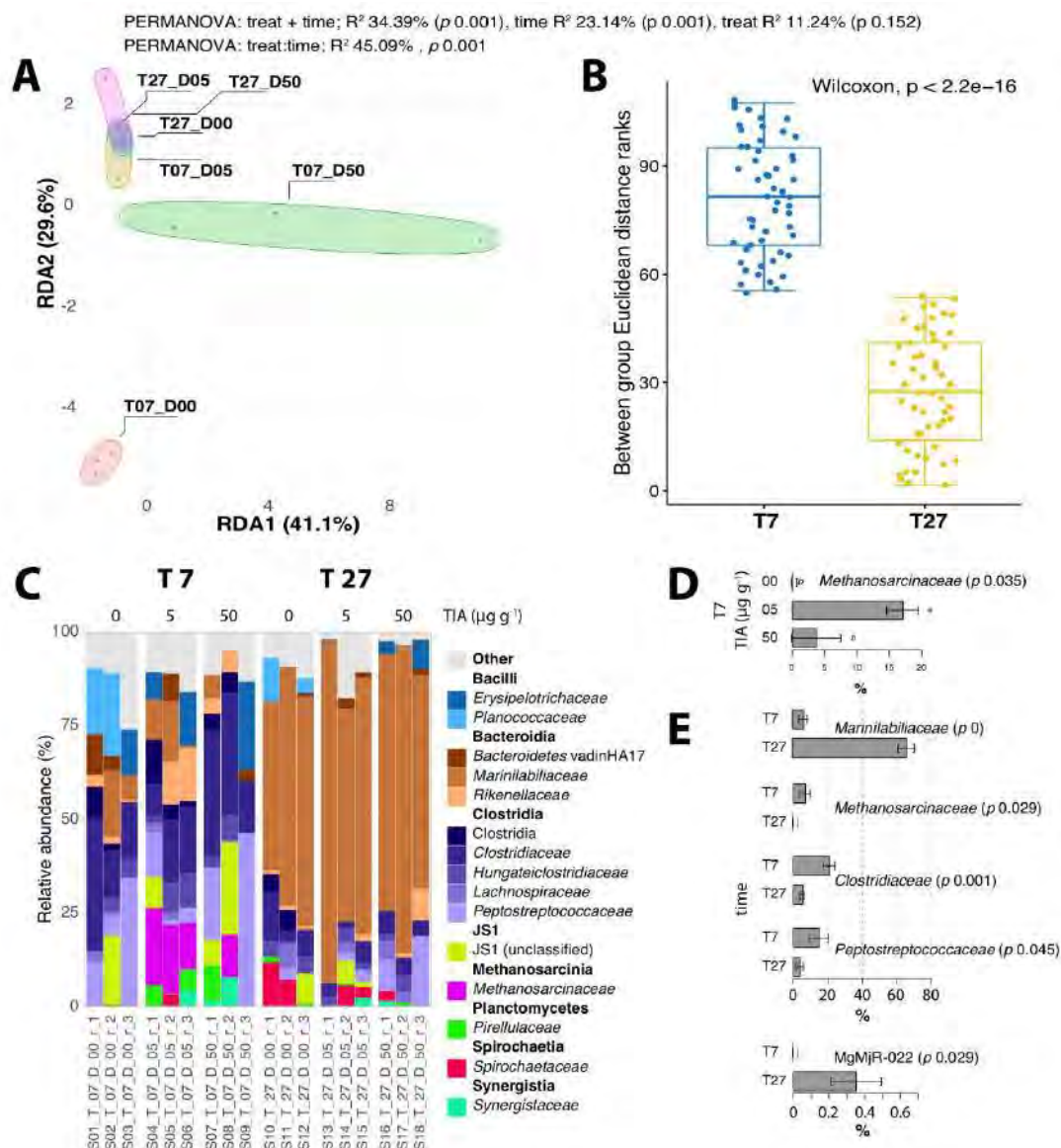


Figure 6: (A) PERMANOVA outcomes for the ASV matrices and hypothesis testing about the time/treatment or treatment:time interaction effects and redundancy analysis (RDA) scatter plot of the ASV matrices using the treatment:time interaction as constraining factor. (B) Boxplots of the between-RDA-group distance ranks for sampling day 7 (T7) and sampling day 27 (T27). (C) Stacked barplot showing the relative abundance of the main prokaryotic taxa per treatment at 7 and 27 days. (D) Barplots showing variation in the relative abundance of Methanosarcinaceae in the different VA treatments at day 7 (no significant differences were observed at day 27). (E) Barplots showing taxa whose relative abundance significantly varies between 7 and 27 days (according to Kruskal-Wallis and/or Wilcoxon rank-sum tests; p values are provided in parentheses).

4. Discussion

The use of faecal material for manuring agricultural soils facilitates the dispersal of VA residues and poses undesirable risks for the environment and human health. In this frame we aimed to test the efficiency of bioaugmentation of TIA-contaminated faecal material with a TIA-degrading *Sphingomonas* isolate as a mitigation measure to block the dispersal of VAs in the agricultural environment. The efficiency of bioaugmentation was compared to established treatment methods like anaerobic digestion or stockpiling.

Bioaugmentation was the most efficient method in the removal of TIA residues from the faecal material with the DT_{50} values of the tested VA drastically reduced (32.3 to 66.2 days) compared to stockpiling (DT_{50} 95.4 to 113.8 days) and anaerobic digestion (DT_{50} 103 to 126.7 days). Previous studies by Perruchon et al., (2022), following a similar experimental setup, reported shorter DT_{50} values for TIA during stockpiling of pig faeces at ambient temperatures (5.95 to 35.9 days), though their study was performed under substantially higher ambient temperature (ca. 25°C) compared to the temperature profile in this study. The use of tailored-made inocula for the bioaugmentation of VA-contaminated matrices has attracted little attention so far. Shi et al., (2021) showed that bioaugmentation of faecal material and soil with a tetracycline-degrading *Arthrobacter* sp., strain was efficient in removing oxytetracycline under alkaline conditions in both matrices. Other studies have either focused on the bioaugmentation of agricultural soils contaminated with VAs (Hirth et al., 2016; Hong et al., 2020) or of other veterinary medicines (e.g. anthelmintics) from faecal material, with the results in all cases being promising. The limited efficiency of stockpiling or anaerobic digestion in dissipating TIA adds to the long list of studies providing contradictory results about the removal efficiency of these methods (Spielmeyer et al., 2015; Widyasari-Mehta et al., 2016; Berendsen et al., 2018; Sui et

al., 2018; Kasumba et al., 2020). For example, Visca et al., (2021) reported 84% to complete degradation of enrofloxacin, ciprofloxacin and SMX during anaerobic digestion and Taleghani et al. (2020) observed complete degradation of tylosin. On the other hand, Perruchon et al., (2022) reported for TIA a DT_{50} of >365 days during anaerobic digestion of pig faecal material and Berendsen et al., (2018) identified TIA as one of the most persistent VA during stockpiling suggesting that this method would not be able to efficiently remove TIA residues before release of manures in agricultural soils. Overall, bioaugmentation of faecal material with tailored-made bacterial inocula could be an efficient and so far overlooked method for mitigating the dispersal of VA residues in the agricultural environment. The application of this method could be further optimized (e.g. formulation of inocula or use of fecal-derived inocula) to drastically reduce the pressure of the soil and plant microbiota for the selection of novel antibiotic resistance traits.

Beyond VA dissipation, we evaluated the overall effects of TIA on the performance of anaerobic digestion via measurements of biomethane and VFA production. In addition, microbial succession of the prokaryotic community during anaerobic digestion was determined via amplicon sequencing. TIA induced a significant increase in methane production with the most beneficial effect being evident at the lowest tested concentration. Several studies have explored the potential effect of antibiotics on anaerobic digestion with the majority of them concluding on a negative effect on biomethane production which was attributed to inhibitory effects of VAs on the various microbial components of the anaerobic digestion process (e.g. hydrolysis, acidogenesis, acetogenesis, methanogenesis) (Xiao et al., 2021; Wu et al., 2022). However, a considerable number of studies deviate from this trend and showed, that depending on the antibiotic and concentration level tested, the presence of VAs in anaerobic digestates might stimulate biomethanation. For example, Zhi et al. (2019), reported an increased production of methane during anaerobic digestion in the

presence of 100 mg L⁻¹ of oxytetracycline, norfloxacin and sulfadimethoxine, while Yin et al (2016) showed that the presence of low levels of chlortetracycline and oxytetracycline enhanced methane production in anaerobic digesters. The reason for the stimulatory effects of antibiotics on biomethane production is still little understood and could be the outcome of complex microbial interactions between the different microbial players in anaerobic digestion.

VFA measurements were consistent with methane production results. The addition of TIA induced a strong and significant increase in the concentration of total VFAs, mainly driven by acetic acid production, at the early stages of anaerobic digestion which was followed by their rapid consumption in the next 17 days. VFAs constitute reliable performance indicators in the methane formation pathway (Merlin Christy et al., 2014), and their production and consumption profile convey with an optimum anaerobic digestion process in the presence of TIA. The biological mechanism behind the stimulatory effect of TIA remains to be defined in a follow up study.

Next to the methane and VFA measurements we looked into the dynamics and composition of the prokaryotic community during anaerobic digestion of the faecal material. We initially focused on archaea which constitute key components of methanogenesis. Overall archaea constituted minor components of the prokaryotic microbiota, with the sole exception of the high relative abundance of methanogens belonging to *Methanosarcina* at the early stages of anaerobic digestion (day 7) in the samples treated with the low TIA rate. The high abundance of *Methanosarcina* coincided with the increasing production of methane in this particular treatment (Figure 3B), suggesting their active role in the enhanced methane production. *Methanosarcina* are the only known anaerobic methanogens that can produce methane using all known metabolic pathways for methanogenesis (acetoclastic, carboxydrotrophic, hydrogenotrophic, methylotrophic, and methyl respiration) (Song and Zhang, 2015). The proliferation of *Methanosarcina* concurred with the rapid consumption of acetic

acid, the main VFA component, suggesting the dominance of the aceticlastic biomethanation pathway during anaerobic digestion of the faecal material. Previous studies with a range of antibiotics (oxytetracycline, norfloxacin, sulfadimethoxine, SMX, enrofloxacin, ciprofloxacin, famethoxazole, ofloxacin) showed a similar stimulatory effect of VAs on biomethanation associated with the proliferation of *Methanarcina* (Zhi et al. 2019; Zhi and Zhang 2019), reinforcing the important role of these methanogens in the anaerobic digestion of faecal material contaminated with VAs.

When looking at the effects of TIA on the diversity of the total prokaryotic community we noted a significant increase in the alpha-diversity and significant structural changes that were limited to the earlier days of the anaerobic digestion. It should be stressed that time, and not the antibiotic treatment, was the most important determinant of the alpha-diversity and prokaryotic community composition. In accord with this, we did not observe any other ASV beyond *Methanosarcinaceae* to be significantly affected by TIA. Whereas we identified certain Bacteroidetes ASVs belonging to the family *Marinilabillaceae* as dominant members of the bacterial community in all treatments at the later stages of anaerobic digestion. Members of this family are common encounters in anaerobic digestion systems encompassing syntrophic acetate oxidizing bacteria (Ziganshina et al., 2015), in line with their late proliferation during anaerobic digestion. *Clostridium* and *Peptostreptococaceae* ASVs showed the exact opposite temporal pattern with significantly higher abundance at the earlier stages of digestion, in line with their key role in VFA production (Talbot et al., 2020).

5. Conclusion

Bioaugmentation with a tailored-made bacterial inoculum was the most efficient remediation processes accelerating the removal of TIA residues from contaminated

faecal material compared to stockpiling and anaerobic digestion which failed to efficiently remove TIA. In addition, the presence of low levels of TIA appeared to be beneficial to methane production driven by the early proliferation of the versatile methanogenic archaea *Methanosarcina*. Our findings highlight the high risk of contamination of agricultural soils by the use of TIA-contaminated manures that have been either stockpiled or anaerobically digested before their use in agricultural settings. Further studies will explore the potential optimization of bioaugmentation of faecal material as a valuable biobased treatment step for the mitigation of environmental dispersal of VAs.

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Annex III – Chapter 4 Supplementary Data

Table S1: The daily and monthly average temperatures recorded during the stockpiling/bioaugmentation experiment in November and December 2022. Data were derived from the local meteorological station (see meteo-thessalia.gr).

Day	November 2022 (°C)	December 2022 (°C)
1	17.7	10.7
2	17.7	10.0
3	17.8	11.1
4	16.8	12.0
5	17.0	12.0
6	15.3	12.6
7	16.2	12.6
8	14.6	12.1
9	14.8	12.2
10	15.1	14.5
11	14.5	14.4
12	13.5	12.9
13	12.7	11.6
14	13.6	11.0
15	13.4	13.6
16	12.2	11.7
17	15.6	12.4
18	17.5	12.8
19	19.3	10.4
20	18.1	7.5
21	14.9	6.0
22	11.2	5.2
23	12.8	6.1
24	12.3	7.0
25	12.1	8.8
26	9.8	9.0

27	9.7	8.4
28	8.4	7.8
29	9.0	9.4
30	8.7	8.5
31	-	5.5
Average	14.1	10.3

Table S2: The primers, sequences and thermal cycling conditions used for amplicon sequencing analysis. Primer site notation: bold letters in primers (forward or reverse) indicate the sample-specific index sequence sites generated with the barcrawl software (Frank, 2009); italics indicate linker sequences designed to assist index sequence detachment in the case of direct amplification of environmental templates.

Primers	Thermocycling conditions	Sequence (5' – 3')	Target
515f	98°C for 10 s, 50°C for 30 s, 72°C for 30 s (28 cycles + 7 cycles) 72°C for 10 min	NNNNNNNNN <i>GT</i>	Bacterial V4 region of the 16S rRNA gene
806r		GTGYCAGCMGCCGCGGTAA GGACTACNVGGGTWTCTAAT	

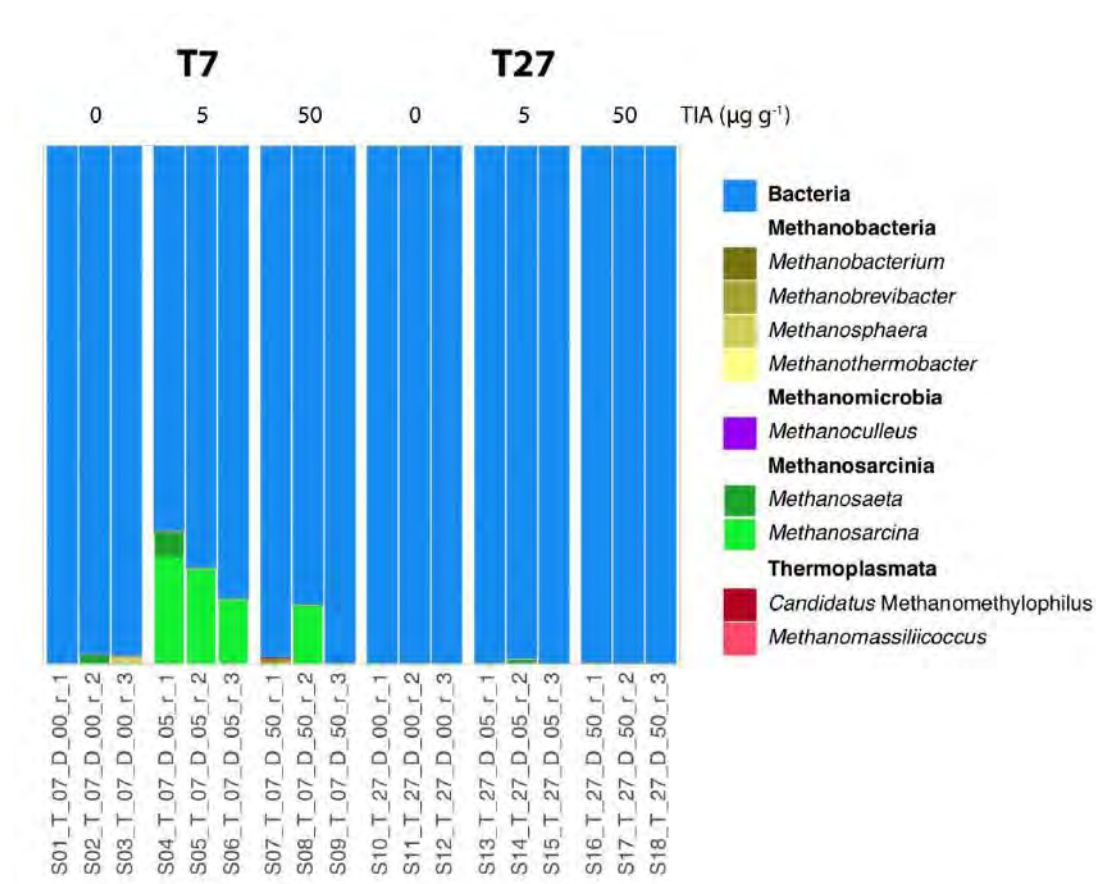


Figure S1. Stacked barplot showing the relative abundances of the archaeal taxa (vs Bacteria) during anaerobic digestion in samples untreated and in samples treated with 5 and 50 mg/kg of TIA.

Chapter 5

General Discussion

1. General Discussion

Intensive livestock farming relies on heavy VA use resulting in pollution of the rural environment with VAs and entails high risks for antibiotic resistance dispersal threatening public health (Ma et al., 2021). A significant portion of the administrated VAs is excreted intact from animals (Pan and Chu, 2017). Animal excreta are then subjected to different intermediate treatments like stockpiling, anaerobic digestion or composting prior to their application as manures in agricultural soils. These treatments aim to reduce the microbial and residual VA load of manures before their release in agriculture, though their level of success has been questioned (Jorgensen and Halling-Sorensen, 2000; Kemper, 2008; Zhao et al., 2010). Organic fertilization of agricultural soil with VA/pathogen/ARG containing manures may result in: (i) selection of novel antibiotic resistance traits (Heuer, & Smalla, 2007); (ii) enrichment of the pathogen potential of agricultural soils; (iii) undesirable toxic effects on the soil microbial community affecting ecosystem functioning and, hence, ecosystem services (Falkowski et al., 2008) or (iv) selection for microbes able to rapidly degrade VAs and use them as an energy source (Topp et al., 2013). Among the methods used to remove the selective pressure imposed by VAs on the soil microbiota, bioaugmentation through antibiophy is an attractive approach (Martin-Laurent et al., 2014; Martin-Laurent et al. 2019).

In this context, we investigated the environmental fate of an array of antibiotics (TIA, a pleuromutilin; TLM a macrolide; and SMX, a sulfonamide) with large use in pig farming, studied their interactions with the soil microbial communities, and explored the bioremediation potentials of VA-degrading bacteria to mitigate environmental exposure to VAs. The experimental section of this thesis was segmented into three main parts which constituted the three main research chapters of this thesis. First in Chapter 2, we investigated the fate of TIA and TLM along the chain, from animal administration to manure application in an array of agricultural soils varying in their physicochemical

traits. Following in Chapter 3, we assessed the potential of accelerated dissipation of VAs in soils, and explored their potential to impose soil microbial toxicity or stimulate the dispersal of AMR in selected soils from Chapter 2 after repeated exposure to VAs. Finally, we utilized a TIA-degrading isolate to evaluate its potential for use in bioaugmentation strategies aiming to curate VA-contaminated manures (Chapter 4).

In **Chapter 2**, the environmental fate of TIA and TLM starting from pig administration, up until manure incorporation in several agricultural soils was studied. We first explored the excretion pattern of TIA and TLM from pigs under two administration regimes: via water or via feed. Residue analysis of TIA and TLM in pig feces for 23 days after VAs administration showed that the mode of administration had a major effect on the excretion amounts and excretion patterns of the two VAs. TLM excreted in lower amounts in feces when administered through water, a result most probably attributed to its higher water solubility which favors its excretion through urine. Regarding TIA, it was detected at higher levels during the administration period when it was administered through feed but its peak was delayed until after the administration period when it was administered through water. These differences could be linked to variances in the distribution of antibiotics in the animal organs as suggested by Halling-Sørensen et al. (2001) and Qiu et al. (2016). In contrast to similar studies with other VAs which have shown limited persistence during the withdrawal phase (Peng et al., 2016; Qiu et al., 2016), our study is the first to follow the excretion patterns of TIA and TLM in pig farming and showed that residues of these VAs can be detected in faeces up until the end of the collection period. These results have major practical implications and suggest that longer withdrawal periods should be implemented for TIA and TLM in pig farming units to minimize the risk for carryover of VA residues in agricultural soil.

We further explored the persistence of TIA and TLM in faeces during standard pre-treatment processes like stockpiling and anaerobic digestion that are employed before the application of faecal material in agricultural soils. Both VAs showed high

persistence in the faecal material during the 60-day stockpiling period and the calculated DT_{90} values suggested that a longer stockpiling beyond 4-6 months should be essential to ensure a complete removal of TIA and TLM from faecal material. Similarly, both VAs showed high persistence in faecal material during anaerobic digestion highlight the limited efficiency of this process in reducing the antibiotic load of manures. Instead the presence of VAs negatively affected the bimethanation process resulting in reduced methane production. Overall, our findings suggest that stockpiling and anaerobic digestion are not expected to reduce the levels of TIA and TLM in faecal material, highlight the considerable risk for dispersal of their residues in agricultural soils by the use of faecal material for manuring and stress the need for alternative mitigation measures to avert the environmental dispersal of VA residues.

Since our earlier experiments indicated a high potential of TIA and TLM persisting in faecal material up until their application to agricultural soils, we explored the persistence of TIA and TLM in the last leg of their route from livestock farms to the environment, in agricultural soils. We determined the persistence of these VAs in fumigated and non-fumigated samples from four agricultural soils with variable physicochemical properties and under two application regimes: direct application in soil (inherent persistence potential) and indirect application through fortified manures (true exposure scenario). Despite the general antimicrobial activity of VAs, the soil microbiota was a major player in the dissipation of TIA and TLM in all agricultural soils, as depicted by the substantial retardation of their dissipation upon fumigation, in line with previous studies for TLM (Berendsen et al., 2021) and several other antibiotics (Topp et al., 2013, Topp et al., 2016). In addition, application of both VAs through fortified manures, a realistic exposure scenario, extended the persistence of TIA and TLM in agricultural soils, in accordance with recent studies by Xie et al. (2023). Overall our data from Chapter 2 showed that the administration of TIA and TLM in pig farms and the subsequent use of faecal material for manuring of agricultural soils entail a

high risk for dispersal of VA residues in soil with yet unexplored consequences for environmental contamination and human or animal health.

Following up on the results of Chapter 2, in **Chapter 3** we explored potential interactions and effects of VAs on the soil microbial community. Hence, we investigated the potential response of the microbiota of two soils, exhibiting in Chapter 2 high and low dissipation capacities against VAs, to repeated applications (direct or through fortified manure) of TIA, TLM and SMX. The latter was included as a model VA for which several studies are available regarding its soil microbial degradation, compared to TIA and TLM. We hypothesized that subjecting the “fast” soils (exhibiting rapid degradation of VAs in Chapter 2) to repeated exposure to VAs could potentially lead to acclimation of the soil microbiota selecting for microbes that could rapidly degrade the applied VA. On the contrary, we assumed that the repeated application of VAs in the “slow” soils (exhibiting slow dissipation of VAs in Chapter 2) will result in accumulation of VA residues and hence toxicity on the soil microbiota. TIA was the sole VA that showed enhanced degradation in the studied soils (in the “fast” soil) and only in the case of its direct soil application, a result reported previously for other VAs as well (Topp et al., 2013; Topp et al., 2016) and questioning the practical significance of this phenomenon in agricultural settings. In all other cases the application of VAs resulted in retardation of degradation (SMX) or accumulation of their residues (TLM) in soil. This pattern induced inhibitory effects on nitrification and the abundance of ammonia oxidizing bacteria (AOB) and archaea (AOA) by SMX and TIA but not by TLM, reinforcing the widely suggested sensitivity of this functional microbial group to abiotic stressors like antibiotics (Karpouzas et al., 2022; Lagos et al., 2023). The application of VAs had a strong structural effect on prokaryotic communities, including bacteria, AOA and AOB, as expected according to the biological activity of the studied VAs. On the contrary, eukaryotic microbial communities like fungi and protists were more affected by the application of manure rather than VAs. An interesting observation,

was the incorporation of a large mass of enteric microorganisms in soils treated with manures, a microbial carriage of the faecal material which did not persist in soils for more than 15 days but entails a serious risk for dispersal of enteric pathogenic microorganisms (Lopatto et al., 2019; Peng et al., 2021). Beyond biodegradation or ecotoxicity we explored the potential of VAs and/or manure to stimulate AMR, HGT and enhance the pool of microbial pathogens in the soil. As expected, SMX stimulated *sul1* (a SMX resistance gene) (Cheng et al., 2020) and *dfrA1* (trimethoprim resistance conferring). Trimethoprim and SMX are usually combined, therefore in manure-treated soils is not unusual to associate a SMX-mediated boost of *dfrA1* abundance. (Carmo et al., 2017; Haulisah et al., 2021). Additionally, manure application by itself boosted ARGs abundance and stimulated HGT, as indicated by the significant increase in the abundance of the marker gene *int11*. Finally, correlation studies identified several opportunistic pathogens like *Clostridia*, *Burkholderia-Caballeronia-Paraburkholderia*, and *Nocardioide*s that might act as AMR reservoirs in soils exposed to VA-contaminated manures. Overall, our findings suggest that the regular exposure of agricultural soils to VAs could trigger toxic effects on key soil functional microbial groups and stimulation of AMR and HGT with unknown reciprocal effects for soil ecosystem functioning and consumers health.

Following the isolation of a *Sphingomonas* strain able to degrade TIA from the “fast” soil from Chapter 3 which exhibited accelerated biodegradation of TIA, in **Chapter 4** we explored the potential of this bacterium for the bioaugmentation of TIA-contaminated faecal material and compared the results with other established treatment methods such as stockpiling and anaerobic digestion. Bioaugmentation was the most efficient method for removal of TIA from the faecal material, in line with the few other studies available on this area (Hirth et al., 2016; Hong et al., 2020; Shi et al., 2021). Furthermore, when investigating the effects of TIA on the performance of anaerobic digestion by assessing the biogas and VFA production, we stumbled

upon a significant increase in methane production at the lowest concentration tested, resembling a hormetic response reported before in previous studies with antibiotics (Yin et al., 2016; Zhi et al., 2019). In an attempt to explain the stimulatory effect on methane production, we delved into the dynamics and composition of the prokaryotic community during anaerobic digestion. We identified the abundance of the methanogen *Methanosarcina* as a key factor in the enhanced methane production in response to exposure to low levels of TIA in line with previous studies (Zhi et al., 2019; Zhi and Zhang 2019). Overall, bioaugmentation with tailored-made microbial inocula specialized in the degradation of VAs could be a valuable method to alleviate the environmental dispersal of VAs and hence reduce the potential contamination of terrestrial ecosystems with VAs.

2. Conclusions

Overall, in this thesis we have provided a comprehensive analysis of the route of VAs heavily used in pig farming (TIA, TLM and SMX) from livestock farms to agricultural soils along with their persistence in each of the studied compartments (animal, faecal material, soil), their reciprocal effects and interactions with microbiota once in soil, and the potential of biosolutions like bioaugmentation for mitigation of the environmental dispersal of VAs. Our work can be summarized in the following conclusive bullet points:

- TIA and TLM are persistent along their route from animals, to faecal material and agricultural soils. Hence their application in livestock farms and the currently applied downstream handling of faecal material from administered animals entails a high risk for environmental contamination

- The regular exposure of agricultural soils to VAs under realistic application regimes (through contaminated faecal material) is expected, depending to the VA applied, to (i) negatively affect key soil microbial processes and microbial diversity (prokaryotic and eukaryotic) (ii) impose selection pressure for ARGs, their HGT, and support opportunistic pathogens as AMR reservoirs in soils.
- Bioaugmentation with tailored-made bacterial inocula specializing in the degradation of VAs has high potential to be used, instead of anaerobic digestion or stockpiling for the decontamination of VA-contaminated faecal material prior to their application in agricultural settings, hence reducing the environmental dispersal of VAs and their reciprocal effects on the environment and human health

3. Future perspectives

This current thesis has provided vital information on the environmental fate of TIA and TLM while highlighting their strong persistence in nature. In addition, it has presented a deep investigation of the mechanism underlying VA - soil microbe interactions. Finally, it has provided promising tools to achieve decontamination of faecal material prior to their application in agricultural soils. According to these findings new research challenges have risen that can be addressed in the future:

- Set up long-term field studies under realistic application regimes to verify under full scale agricultural conditions the practical relevance of our findings
- Optimize the bioaugmentation of faecal material for TIA and extend its application to other VAs known to entail high environmental risk.
- Address the interactive effects of VAs with other soil contaminants, veterinary medicines (e.g. anthelmintics), agrochemicals (pesticides, nitrification inhibitors) or microplastics, on the soil microbiota and assess the implications

for agricultural production, environmental integrity and human health in an one health concept approach

- Define the role of soil mobilome on the evolution and dispersal of AMR traits, and the associated health risks using advanced omic tools (e.g. shotgun metagenome and plasmidome analysis) combined with methods (e.g. HiC) that enable the identification of the microbial carriers of AMR traits in soil.

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