UNIVERSITY OF THESSALY

Department of Biochemistry and Biotechnology

"The role of soil microorganisms in the mitigation of the environmental deterioration imposed by the use and dispersal of anthelminthic veterinary drugs."

A thesis submitted by

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Λαγός Ευστάθιος

"Ο ρόλος των μικροοργανισμών του εδάφους στην ανάσχεση της περιβαλλοντικής υποβάθμισης από την χρήση και διασπορά ανθελμινθικών κτηνιατρικών φαρμάκων"

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List of abbreviations

VDs	Veterinary drugs
GINs	Gastrointestinal Nematodes
AHs	Anthelmintics
BZs	Benzimidazoles
MLs	Macrocyclic Lactones
ABZ	Albendazole
ABZ-SO	Albendazole Sulfoxide
ABZ-SO2	Albendazole Sulfone
FBZ	Fenbendazole
FBZ-SO	Fenbendazole Sulfoxide
MBZ	Mebendazole
FLU	Flubendazole
TBZ	Thiabendazole
IVM	Ivermectin
EPM	Eprinomectin
MOX	Moxidectin
AHR	Anthelminthic Resistance
TPs	Transformation Products
SFO	Single First Order
HS	Hockey Stick
FOMC	First Order Multi-Compartment
DFOP	Double First Order in Parallel
TOC	Total Organic Carbon
AOMs	Ammonia Oxidizing Microorganisms
AOA	Ammonia Oxidizing Archaea
AOB	Ammonia Oxidizing Bacteria
MSM(N)	Mineral Salt Medium (amended with Nitrogen)

ΣΥΝΟΨΗ

Τα συνθετικά ανθελμινθικά (σΑΕ), χρησιμοποιούνται ευρέως για την πρόληψη και τη θεραπεία των παρασιτώσεων του γαστρεντερικού σωλήνα στα παραγωγικά ζώα, αλλά όπως οι περισσότερες χημικές ουσίες δεν απορροφώνται τελείως από τον οργανισμό με αποτέλεσμα να απεκκρίνονται με τα κόπρανα των ζώων στο περιβάλλον σε συγκεντρώσεις που κυμαίνονται σε επίπεδα $\mu g \ Kg^{-1}$ έως $mg \ Kg^{-1}$. Κατά συνέπεια, οι επιβαρυμένοι με σΑΕ κοπροσωροί όταν χρησιμοποιούνται ως λίπασμα στην πρωτογενή γεωργική παραγωγή προκαλούν ρύπανση του εδάφους και πιθανά των παρακείμενων φυσικών υδάτινων πόρων, μέσω απορροής. Προηγούμενες μελέτες έχουν δείξει τις ανεπιθύμητες επιδράσεις των σΑΕ σε οργανισμούς μη-στόχους που διαβιούν σε βοσκοτόπους, όπως έντομα που σχετίζονται με την αποσύνθεση των κοπράνων και άλλους οργανισμούς που διαβιούν γενικότερα στο έδαφος. Ωστόσο, λίγα είναι μέχρι σήμερα γνωστά σχετικά με την περιβαλλοντική τους τύχη στο έδαφος και την επίδρασή τους στους μικροοργανισμούς του εδάφους. Ως εκ τούτου, κύριος στόχος της παρούσας διατριβής ήταν η διερεύνηση των αλληλεπιδράσεων μεταξύ των σΑΕ, της ομάδας των βενζιμιδαζολικών, όπως το albendazole (ABZ) και της ομάδα των μακροκυκλικών λακτονών όπως τα ivermectin (IVM) και eprinomectin (EPM) και των μικροοργανισμών του εδάφους αλλά και να αξιολογήσει τη χρήση της βιοαποδόμησης ως μέσου μείωσης της περιβαλλοντικής έκθεσης σε αυτά τα σΑΕ. Για την επίτευξη αυτού του στόχου α) αξιολογήσαμε τον ρόλο των μικροοργανισμών του εδάφους στην αποδόμηση αυτών των σΑΕ στο έδαφος, β) διερευνήσαμε την επίδραση των σΑΕ στη λειτουργία & ποικιλότητα των μικροοργανισμών του εδάφους, και την πιθανή εμφάνιση νέων καταβολικών μηχανισμών από μικροοργανισμούς του εδάφους έναντι αυτών των ενώσεων γ) αξιολογήσαμε την ικανότητα των βακτηρίων που αποδομούν βενζιμιδαζολικά σΑΕ ως παράγοντες βιοαποκατάστασης για την απορρύπανση κοπροσωρών και εδάφους, και δ) απομονώσαμε εδαφογενή βακτηριακά στελέχη ικανά να αποδομήσουν το ABZ.

Αρχικά, πραγματοποιήθηκαν μελέτες σε αποστειρωμένα ή μη εδάφη (μέσω υποκαπνισμού) που συλλέχθηκαν από 12 εκτροφές αιγοπροβάτων με διαφορικό ιστορικό χορήγησης ABZ, IVM ή EPM σε ζώα. Από κάθε μονάδα, συλλέχθηκαν εδάφη από το εσωτερικό των μονάδων (σειρά Α, υψηλή έκθεση) και από τους παρακείμενους βοσκότοπους (σειρά Β, χαμηλή έκθεση). Θέσαμε τα ακόλουθα ερωτήματα: (α) Ποιος είναι ο ρόλος των μικροοργανισμών του εδάφους στην αποδόμηση των σΑΕ; (β) Η επανειλημμένη έκθεση των εδαφών στα σΑΕ οδηγεί σε επιταχυνόμενη βιοαποδόμησή τους; (γ) Ποιες φυσικοχημικές ιδιότητες του εδάφους επηρεάζουν τη αποδόμηση των σΑΕ; Ο υποκαπνισμός του εδάφους μείωσε σημαντικά την αποδόμηση τόσο του ABZ (DT_{50} = 1,9 vs 4,33 ημέρες), όσο και των IVM (34,5 vs 108,7 ημέρες) και EPM (30 vs 121 ημέρες) υποδηλώνοντας το βασικό ρόλο των μικροοργανισμών του εδάφους στη αποδόμηση αυτών των ενώσεων. Δεν ήταν εμφανής καμία σημαντική επιτάχυνση στην αποδόμηση των σΑΕ σε εδάφη από εκτροφές με ιστορικό χορήγησης ή σε εδάφη από τη σειρά Α έναντι της σειράς Β, γεγονός που υποδηλώνει ότι το επίπεδο προηγούμενης έκθεσης δεν ήταν επαρκές για να προκαλέσει επιταχυνόμενη βιοαποδόμησή των υπό μελέτη σΑΕ. Ο μετασχηματισμός του ABZ στα μεταβολικά του προϊόντα, ABZ-SO και ABZ-SO$_2$ παρατηρήθηκε τόσο στα υποκαπνισμένα όσο και στα μη υποκαπνισμένα δείγματα εδάφους αναδεικνύοντας το ρόλο τόσο βιοτικών όσο και αβιοτικών διεργασιών στην οξείδωση του ABZ σε σουλφοξείδια και σουλφόνες. Παρατηρήθηκαν σημαντικές θετικές και αρνητικές συσχετίσεις του ολικού οργανικού άνθρακα του εδάφους (TOC) και της αποδόμησης ABZ και IVM, αντίστοιχα. Η προσρόφηση στο έδαφος των σΑΕ αυξήθηκε με τη σειρά IVM > ABZ > EPM. Το TOC συσχετίσθηκε επίσης με την αυξημένη προσρόφηση του IVM και του EPM στο έδαφος, αλλά όχι του ABZ, κάτι που υποστηρίζει το εύρημα της αντίθετης επίδρασης του TOC στην αποδόμηση IVM και ABZ.

Παράλληλα, διερευνήσαμε την πιθανή ικανότητα μιας βακτηριακής κοινοπραξίας που αποικοδομεί το thiabendazole (TBZ) να αποδομεί επίσης άλλα βενζιμιδαζολικά σΑΕ όπως το ABZ και το προϊόν μετασχηματισμού του, albendazole sulfoxide (ονομάζεται επίσης ricobendazole, RBZ), fenbendazole

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(FBZ), flubendazole (FLU) και mebendazole (MBZ). Σε προκαταρκτικές δοκιμές σε υγρές καλλιέργειες, η κοινοπραξία ήταν πιο αποτελεσματική στην αποικοδόμηση ενώσεων με μικρότερους υποκαταστάτες του βενζιμιδαζολικού δακτυλίου (TBZ, ABZ, RBZ), παρά βενζιμιδαζόλες με πιο σύνθετους υποκαταστάτες (FBZ, FLU, MBZ). Ως επόμενο βήμα, διερευνήσαμε τη βιοαποδομητική ικανότητα της κοινοπραξίας σε περιττώματα προβάτου στα οποία είχε γίνει εφαρμογή ποσοτήτων TBZ, ABZ και FBZ σε επίπεδα συγκεντρώσεων 5 και 50 mg kg^{-1}. Ο εμβολιασμός με την βακτηριακή κοινοπραξία ενίσχυσε την αποδόμηση όλων των ενώσεων, αλλά κυρίως του TBZ, και η αποτελεσματικότητά της βιοενίσχυσης επιταχύνθηκε με τον υποκαπνισμό των κοπράνων και της απουσίας της γηγενούς μικροβιακής κοινότητας. Η γηγενής μικροβιακή κοινότητα συμβάλλει επίσης στην αποικοδόμηση των βενζιμιδαζολικών σΑΕ, όπως υποδηλώνεται από τις σημαντικά χαμηλότερες τιμές DT$_{50}$ στα υποκαπνισμένα έναντι στα μη υποκαπνισμένα μη εμβολιασμένα δείγματα κοπροσωρών.

Το τρίτο μέρος αυτής της διατριβής αφορούσε στην διερεύνηση των πολύπλοκων αλληλεπιδράσεων, ωφέλιμων ή επιζήμιων, μεταξύ των μελετηθέντων σΑΕ και των μικροοργανισμών του εδάφους. Δύο εδάφη που επιλέχθηκαν από τα εδάφη της αρχικής μελέτης, τα οποία αναγνωρίστηκαν ως «γρήγορα» ή «αργά», αναφορικά με την αποδόμηση των ABZ, IVM και EPM, υποβλήθηκαν σε επαναλαμβανόμενες εφαρμογές σε δύο επίπεδα συγκέντρωσης (1 ή 2 mg kg^{-1} και 10 ή 20 mg kg^{-1}). Υποθέσαμε ότι αυτή διαδικασία εφαρμογής των σΑΕ θα οδηγήσει σε ενισχυμένη βιοαποδόμηση στα «γρήγορα» εδάφη και συσσώρευση των υπολειμμάτων των σΑΕ και τοξική επίδραση στην μικροβιακή κοινότητα στα «αργά» εδάφη. Η επαναλαμβανόμενη εφαρμογή του ABZ είχε ως αποτέλεσμα διαφορετικά μοτίβα μετασχηματισμού του σε μεταβολικά προϊόντα στα δύο εδάφη και σαφή επιτάχυνση της αποδόμησης του μόνο στο «γρήγορο» έδαφος. Αντίθετα, υπολείμματα του IVM και του EPM συσσωρεύτηκαν και στα δύο εδάφη χωρίς να παρατηρηθεί η επιτάχυνση της αποδόμησης. Επιπλέον, αξιολογήσαμε τις επιδράσεις των ABZ, IVM και EPM στη δραστηριότητα, την αφθονία και την ποικιλότητα των μικροοργανισμών που οξειδώνουν την αμμωνία (AOMs) καθώς και ευρύτερων μικροβιακών ομάδων (βακτήρια, μύκητες, κρεναρχαία και πρωτιστές). Το ABZ ήταν το μοναδικό σΑΕ που προκάλεσε μια σταθερή μείωση της αφθονίας των μυκήτων και κρεναρχαίων στα εδάφη. Επιπλέον, παρατηρήθηκε αναστολή της νιτροποίησης και μείωση τηςαφθονίας των βακτηρίων (AOB) και αρχαίων που οξειδώνουν την αμμωνία (AOA) ύστερα από την εφαρμογή όλων των σΑΕ, ενώ τα βακτήρια commamox δεν έδειξαν καμία απόκριση στην παρουσία των σΑΕ στο έδαφος. Μετα-ταξινομική ανάλυση των διαφόρων μικροβιακών ομάδων στο έδαφος έδειξε δοσοεξαρτώμενες μεταβολές στην ποικιλότητα βακτηρίων, μυκήτων και πρωτίστων ως απόκριση στην εφαρμογή των σΑΕ. Το ABZ παρουσίασε την πιο σταθερή επίδραση στην αφθονία και την ποικιλότητα των περισσότερων μικροβιακών ομάδων που μελετήθηκαν.

Ως επόμενο βήμα αξιολογήσαμε βιολογικές προσεγγίσεις για την αποτοξικοποίηση κοπροσωρών επιβαρυμένων με ανθελμινθικά φάρμακα με βασικό στόχο τον περιορισμό της διασποράς τους στο περιβάλλον. Στο πλαίσιο αυτό αξιολογήσαμε την ικανότητα μιας κοινοπραξίας βακτηρίων που αποδομούν το βενζιμιδαζολικό μυκητοκτόνο-ανθελμινθικό thiabendazole να αποδομεί και τα υπόλοιπα βενζιμιδαζολικά ανθελμινθικά albendazole, flubendazole, fenbendazole, mebendazole, ricobendazole. Αρχικές δοκιμές σε υγρές καλλιέργειες έδειξαν ότι η αποδομητική ικανότητα της βακτηριακής κοινοπραξίας περιορίζεται σε δομικά παρόμοια με το thiabendazole ανθελμινθικά όπως το albendazole και λιγότερο το fenbendazole. Περαιτέρω μελέτες σε κοπροσωρούς που εμβολιάστηκαν με την βακτηριακή κοινοπραξία έδειξαν ότι η κοινοπραξία ήταν ικανή να αποδομεί με αυξημένους ρυθμούς τα thiabendazole και albendazole, ιδιαίτερα σε κοπρωσωρούς που είχαν αποστειρωθεί καταδεικνύοντας τον ανταγωνιστικό ρόλο που παίζει η γηγενής μικροβιακή κοινότητα στην εδραίωση και δράση εξωγενών μικροβιακών εμβολίων σε κοπροσωρούς. Τα αποτελέσματα αυτά έδειξαν την προοπτική χρήσης του βιολογικού εμπλουτισμού για τον περιορισμό της διασποράς

των ανθελμινθικών στα γεωργικά εδάφη και ίσως καταδεικνύει την χρήση πιο εξειδικευμένων μικροοργανισμών στην αποδόμηση των μελετούμενων ανθελμινθικών φαρμάκων.

Με βάση τα παραπάνω αποτελέσματα, στοχεύσαμε να απομονώσουμε εξειδικευμένους μικροοργανισμούς που να αποδομήσουν ταχύτατα το ABZ. Οι καλλιέργειες εμπλουτισμού από έδαφος που εμφάνισε ταχύτατη αποδόμηση του ABZ, οδήγησαν στην απομόνωση δύο βακτηριακών στελεχών ικανών να αποδομούν ταχύτατα το ABZ τα οποία με βάση της αλληλουχία του 16S rRNA γονιδίου τους ταξινομήθηκαν στο γένος *Acinetobacter*.

Συνολικά, τα ευρήματά μας οδηγούν σε αρκετά σημαντικά συμπεράσματα για την περιβαλλοντική τύχη των σΑΕ στα εδάφη και τις αλληλεπιδράσεις τους με τους μικροοργανισμούς του εδάφους. Συνοπτικά, η παρούσα διατριβή ανέδειξε τον σημαντικό ρόλο των μικροοργανισμών του εδάφους στην αποδόμηση των βασικότερων σΑΕ. Παράλληλα έδειξε ότι η έκθεση της εδαφικής μικροβιακής κοινότητας σε συνθετικά σΑΕ αναμένεται να έχει σημαντικές επιδράσεις στην μικροβιακή κοινότητα του εδάφους, τόσο σε επίπεδο ποικιλότητας όσο και σε επίπεδο λειτουργίας, με πιο χαρακτηριστικό παράδειγμα την αρνητική επίδραση όλων των σΑΕ και ειδικότερα του ABZ στην λειτουργία, ποικιλότητα και αφθονία των AOM. Τα παραπάνω ευρήματα θα πρέπει να ληφθούν υπόψη σε ένα μελλοντικό ρυθμιστικό πλαίσιο που θα αφορά στην εκτίμηση της επικινδυνότητας των σΑΕ στο περιβάλλον. Επιπλέον, επιβεβαιώθηκε η υπόθεση ότι ο βιολογικός εμπλουτισμός θα μπορούσε να αποτελέσει μια πιθανή λύση για την αποκατάσταση κοπροσωρών επιβαρυμένων με βενζιμιδαζολικά σΑΕ.

SUMMARY

Treatment with synthetic anthelmintics (AHs), constitute the main strategy for prevention and treatment of gastrointestinal nematodes (GINs) in livestock. However, as many chemical substances AHs are not totally absorbed by the animals and as a consequence the are excreted with the faeces where they are detected at concentration levels ranging from μg Kg^{-1} to mg Kg^{-1}. The subsequent application of contaminated manures in agricultural settings could lead to the contamination of soils with AHs and their further transport and pollution of natural water resources. Previous studies have demonstrated the undesirable effects of AHs on non-target organisms inhabiting pasture areas, like insects associated with fecal decomposition and other soil-dwelling organisms. However, little is currently known regarding their environmental fate in the soil and their effects on the soil microorganisms. Therefore, the main objective of this thesis was to investigate the interactions of synthetic AHs, with particular focus on the benzimidazoles (BZ) albendazole (ABZ) and the macrocyclic lactones (MLs) ivermectin (IVM) and eprinomectin (EPM) with soil microorganisms and to further evaluate the potential use of microbial degradation as a means for mitigating environmental exposure to AHs. To achieve this goal we (a) evaluated the role of soil microorganisms on the dissipation of AHs in soils, b) investigated the potential effects of AHs on the function and diversity of soil microorganisms with particular emphasis on toxicity effects on the soil microbiota or the potential acclimation of the soil microbiota towards the evolution of novel catabolic mechanisms against these compounds (c) explored the capacity of AH-degrading bacteria as bioaugmentation agents for the detoxification of contaminated manure, and d) isolated specialized soil bacteria capable to degrade the benzimidazole ABZ as a mean for the more efficient implementation of bioaugmentation of contaminated manures.

We first explored the degradation of the selected AHs in fumigated and non-fumigated soils collected from 12 sheep farms with a variable history of administration of albendazole (ABZ), ivermectin (IVM) and eprinomectin (EPM). From each farm, we collected soils from inside small ruminant barn facilities

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(series A, high exposure) and the associated grazing pastures (series B, low exposure). We asked the following questions: (a) What is the role of soil microorganisms in AH dissipation? (b) Does repeated exposure of soils to AHs lead to their accelerated biodegradation? (c) Which soil physicochemical properties control AH dissipation? Our results provided answers to all these scientific questions. First, soil fumigation significantly retarded ABZ (DT_{50} 1.9 and 4.33 days), IVM (34.5 and 108.7 days) and EPM dissipation (30 and 121 days) suggesting a key role of soil microorganisms in AHs dissipation. No significant acceleration in AH dissipation was evident in soils from farms with a history of administration of the studied AHs or in soil series A vs series B, suggesting that the level of prior exposure in our experimental setting was not adequate to induce enhanced biodegradation of AHs. Transformation of ABZ to its transformation products ABZ-SO and $ABZ-SO_2$ was observed in both fumigated and non-fumigated soil samples. Significant positive and negative correlations of soil total organic carbon (TOC) and ABZ and IVM dissipation, respectively, were observed. Soil adsorption of AHs increased in the order IVM > ABZ > EPM. TOC controlled soil adsorption of IVM and EPM, but not of ABZ, in support of the contrasting effect of TOC on IVM and ABZ dissipation.

Following up our first soil dissipation study, we further tried to further shed light into the complex interactions, beneficial or detrimental, between the studied AHs and the soil microbiota. In this quest two soils selected from the soils used in our first dissipation survey (see paragraph above) which were identified as «fast» or «slow», regarding the degradation of ABZ, IVM and EPM, were subjected to repeated applications at two dose rates (1, 2 mg kg^{-1} and 10, 20 mg kg^{-1}). We hypothesized that this application scheme will lead to enhanced biodegradation of AHs in «fast» soils and accumulation of AH residues and toxicity in the «slow» soils. Repeated application of ABZ resulted in different transformation pathways in the two soils and a clear acceleration of its degradation in the «fast» soil only. In contrast residues of IVM and EPM accumulated in both soils. In addition, we evaluated the effects of ABZ, IVM and EPM on activity, abundance, and diversity of functional microbial group of ammonia oxidizing microorganisms (AOMs) as well of broad microbial groups (bacteria, fungi, crenarchaeota and protists). ABZ was the sole AH that induced a consistent reduction in the abundance of total fungi and crenarchaea. In addition, inhibition of nitrification and of the abundance of ammonia-oxidizing bacteria (AOB) and archaea (AOA) by all AHs was observed, while commamox bacteria were less responsive. Amplicon sequencing analysis showed dose-depended shifts in the diversity of bacteria, fungi, and protists in response to AHs application. ABZ presented the most consistent effect on the abundance and diversity of most microbial groups. These results provided first strong evidence for the potential adverse effects of AHs on the soil microbiota at levels expected to be found in soils and hence could be useful in a forthcoming revision of the regulatory framework regarding the environmental risk assessment of AHs.

Considering the harmful effects of AHs on the soil microbiota we explored means to alleviate the dispersal of AHs in soil. In this respect bioaugmentation of contaminated manures with microorganisms able to degrade AHs would be a promising, biobased mitigation measure. In this frame we investigated the potential capacity of a thiabendazole (TBZ)-degrading bacterial consortium to degrade other AHs belonging to the same benzimidazole group like ABZ and its transformation product, albendazole sulfoxide (ABZ-SO, also called ricobendazole, RBZ), fenbendazole (FBZ), mebendazole (MBZ) and flubendazole (FLU). Preliminary liquid culture tests showed that the consortium was more efficient in the degradation of AHs with smaller benzimidazole substituents (TBZ, ABZ, RBZ), rather than benzimidazoles with bulky substituents (FBZ, FLU, MBZ). We further explored the bioaugmentation capacity of the consortium under realistic conditions, in sheep feces fortified with 5 and 50 mg kg^{-1} of TBZ, ABZ and FBZ. Bioaugmentation enhanced the degradation of TBZ and ABZ, and its efficiency was accelerated upon fumigation of feces, in the absence of the indigenous fecal microbial community which competes with the exogenous inoculum.

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In order to further optimize the efficiency of our bioaugmentation approach we aimed to isolate specialized microorganisms able to rapidly and specifically degrade ABZ. Enrichment cultures led to the isolation of two bacterial strains which were able to rapidly degrade ABZ. Sequencing of their 16S rRNA gene and phylogenetic analysis showed that both isolates belonged to the genus *Acinetobacter*.

Overall, our findings lead to several important conclusions for the environmental fate of AHs ABZ, IVM and EPM in soils and the mechanisms driving their interactions with the soil microbiota. Briefly, this thesis highlighted the important role of soil microorganisms in the dissipation of AHs. In addition, we showed the exposure of the soil microbiota to synthetic AHs is expected to have strong effects on the soil microbiota, both at diversity and functional level, with more characteristic example the negative effects of all AHs, but mostly of ABZ, on the function, diversity, and abundance of AOM. These data could be used as benchmarcks for the future adjustment of the regulatory framework regarding the environmental risk analysis of AHs which is currently not considering the soil microbiota as protection goal. Finally, we verified the potential of bioaugmentation of manures as a mitigation mean to alleviate the dispersal of AH residues in the soil environment, although further optimization is needed most probably with the use of tailored-made microbial inocula per compound.

Chapter 1

Introduction

1. Use of Veterinary drugs in animals' health

1.1. Importance of Veterinary Drugs usage and Risks

Veterinary medicinal products (VDs) are pharmacologically active compounds that are administered to animals for the prevention (prophylactic use) and treatment (therapeutic use) of animal diseases, but also for growth-promoting purposes (growth promoters). Other, secondary, uses of veterinary drugs are, preservation and processing of food (food additives), pre-slaughter control of stress (neuroleptic drugs, tranquilizers) and reproduction regulation (analogs of prostaglandins and sex steroids) (Fink-Gremmels, 2014). The global population is constantly increasing, and it is estimated to reach 9.7 billion by 2050 (FAO, 2017). At the same time, there is an increase in global consumption of meat proteins which, over the next decade, is projected to increase by 14% by 2030 compared to the base period average of 2018-2020, driven largely both by the income increasement in developing countries and by this projection of population growth (OECD-FAO, 2020). All the above signify the importance of VDs in veterinary practice (Van Boeckel et al., 2015). Indeed, the global animal health market size was valued at USD 39.9 billion in 2021 and it is expected to grow at a compound annual growth rate (CAGR) of 10.0% from 2022 to 2030 (Grand View Research, 2021).

The excessive use of VDs το support health/welfare and productivity of livestock may entail certain risks for human health and the environment. Several studies have reported the detection of VD residues in meat and meat products although in levels that may pose only limited and on rare occasions health risks for the consumers. These including, the establishment and dispersal of antibiotic resistance bacteria, allergies, reproductive disorders, hypersensitivity reactions, carcinogenicity, and disruption of the indigenous intestinal microflora (Jin Hur et al., 2019; Kuppusamy et al., 2018). In addition, a range of VD residues have been detected worldwide in soils, surface water, and groundwater systems (Boxall et al., 2004; Kwon, 2011; Wei et al., 2016). In recent years preliminary data on the environmental fate and effects of VD on non-target organisms, most notably antibiotics and secondarily anthelminthic (AH) compounds, have become available (Boxall et al., 2006; Horvat et al., 2012; Rath et al., 2019; Thiele-Bruhn, 2005; Zortéa et al., 2017). As a result, authorities have issued regulations regarding the use of VDs (Directive 2001/82/EC of the European Parliament, 2018) and established preliminary guidelines on their environmental risk assessment (European Medicines Agency (EMA), 2016) with the overall goal to reduce the impact of veterinary medicines in the environment and on human health.

1.2. Categories of Veterinary drugs

Of the enormous range of veterinary drugs currently available, antibiotics are used most frequently and in the largest amounts compared with the other groups. Main chemical subclasses of veterinary antibiotics include tetracyclines, penicillins, cephalosporins, sulfonamides, macrolides, etc. Chemotherapeutics and some growth-promoting compounds are also considered as antibiotics. Other subclasses of growth-promoters are hormonal implants, growth hormone (Somatotropins), beta-agonists and probiotics. Another important category of VMPs are anthelmintics which have been the main focus of the current thesis and they will be further discussed, and ectoparasiticides. Ectoparasiticides include phosphoesters, carbamates, pyrethroids, a.o. Other main categories of VMPs are antifungal, coccidiostats (antiprotozoal agents), hormones (glucocorticosteroids, androgenic and estrogenic sex steroids, peptides, thyreostats, a.o.) and anesthetics/tranquilizers (ketamine, benzodiazepine etc.) (O'Keeffe, 2005).

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2.   Anthelminthic veterinary drugs

2.1.   Gastrointestinal Nematodes: Life cycle & infection of animals

Gastrointestinal nematodes (GINs) constitute a major threat for pasture grazing ruminants like cattle or small ruminants (sheep, goats) worldwide (Charlier et al., 2020; Kaplan, 2020; Mavrot et al., 2015). These infections (called gastrointestinal helminthiases) are associated with a range of clinical effects collectively known as parasitic gastroenteritis (Cortés et al., 2020; Zajac & Garza, 2020). Common pathological effects by helminthiases in ruminants (with economic importance) include reductions in weight gain, milk production, wool growth (for sheep), fertility and carcass quality (Charlier et al., 2014a; Forbes, 2021). Most species of GINs which infect small and large ruminants are related to the order *Strongylida*, family *Trichostrongyloidea*. The most prevalent genera of GINs are *Haemonchus, Cooperia, Ostertagia, Teladorsagia, Bunostomum, Trichostrongylus, Chabertia, Nematodirus, Protostrongylus* and *Trichuris* (Belina Kitila et al., 2017; Charlier et al., 2014a; do Amarante & Amarante, 2016). They usually inhabit in the abomasum (the fourth stomach of ruminants), small intestine while some genera can also be detected in the large intestine. In general, the different species of GINs exhibit host specificity. However, exclusions can be found among different ruminants such as *Trichostrongylus axei* and *Haemonchus placei* which are found both in cattle and sheep. The most common species which infect cattle, in temperate climate zones, are *Ostertagia ostertagi* and *Cooperia oncophora* (Charlier et al., 2020) while small ruminants (sheep and goats) are mostly infested by *Haemonchus contortus* (Zajac & Garza, 2020).

Regardless of their host specificity, all economically important GINs of ruminants have a similar direct life cycle (Charlier et al., 2020; Zajac & Garza, 2020). This begins when the adult female lay eggs in the abomasum or intestine of the animal which are then passed to the feces. Within the egg the first-stage larva (L1) is formed and in most cases hatches out of the egg in the environment. After hatching, larvae feed on bacteria and undergo two molts to reach the infective third larval stage (L3). The ruminants become infected when they graze and ingest the infective L3 stage larvae which then is found in the gastrointestinal tract of the animal and gradually mature to male and female adult parasites ready to produce eggs (Charlier et al., 2014b; Vlassoff et al., 2001). A schematic representation of GINs life cycle is presented in Figure 1.1
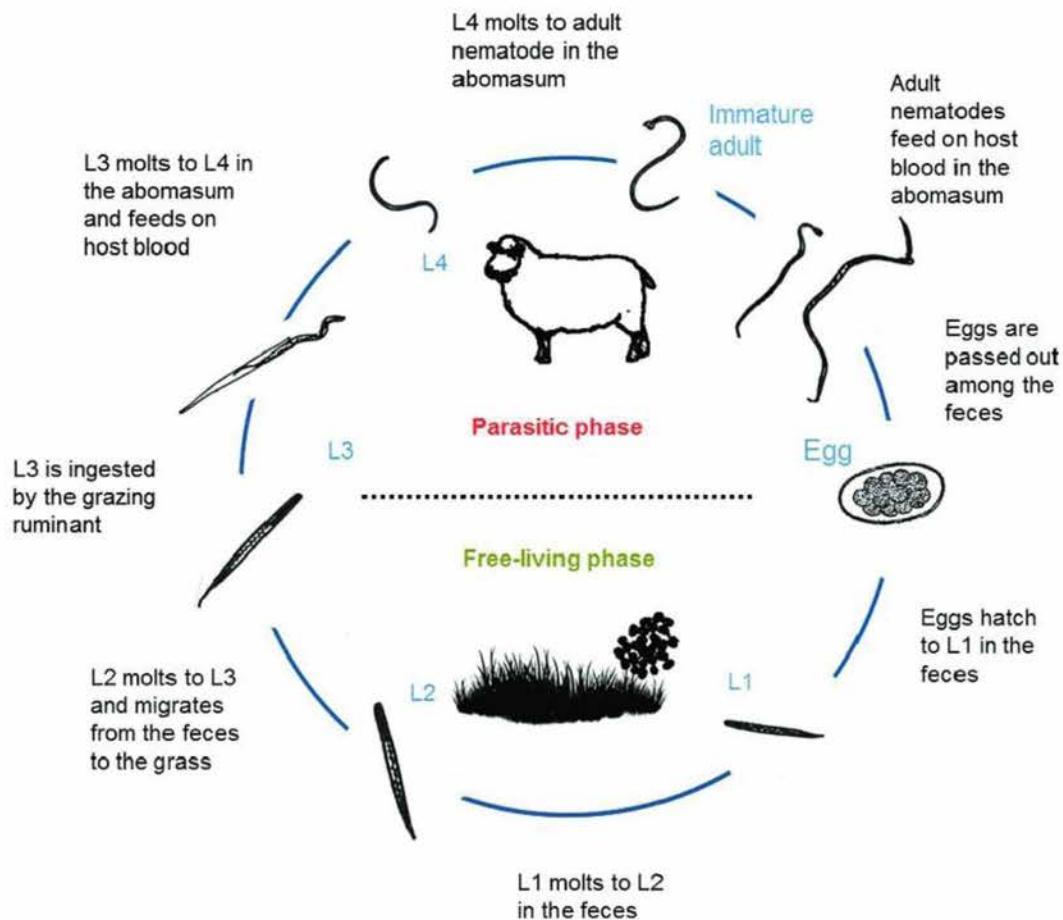
Figure 1.1 The life cycle of GINs in ruminants. (From Engström, Marica T. "Understanding the bioactivity of plant tannins: developments in analysis methods and structure-activity studies.", 2016. Doctoral thesis, University of Turku, Department of Chemistry/Faculty of Mathematics and Natural Sciences.)

2.2. Anthelmintic veterinary drugs: A brief introduction and main chemical groups with anthelminthic use

As mentioned in Section 1.3.1, helminthiases lead to pathogenic effects in livestock and have an important economic impact to farmers revenue (Charlier et al., 2014b). Countries of the Mediterranean basin (Greece, Italy, Tynisia etc.) constitute a typical example where the main portion of dairy products and meat production comes from small ruminants (sheep & goats) in livestock units where pastoral components are still important (Dubeuf et al., 2016). Therefore, a systematic control and treatment of GIN infections is crucial for the productivity of livestock farms. For more than 50 years now, this is achieved mostly with the use of synthetic AHs. Thiabendazole (TBZ), which belongs to the chemical class of benzimidazoles (BZs) (Mckellar & Scott, 1990), was the first highly efficacious broad-spectrum AH which was introduced in the market in 1961 (Brown et al., 1961). Besides BZs, which continue to represent to date one of the most widely used groups of AHs (Horvat et al., 2012; Romero-González et al., 2014), only a few other chemical classes are being used as AHs including imidazothiazoles, tetrahydopyrimidines, macrocyclic lactones (MLs) and more recently (after 1990) amino-acetonitrile derivatives (AADs), spiroindoles and cyclooctadepsipeptides. Imidazothiazoles act as nicotinic acetylcholine receptor (nAChR) agonists. They bind to nAChRs on body wall muscles, causing paralysis of the worm, and therefore, its expulsion from the host (Aceves et al., 1970).

Tetramisole was the first member of this class. Tetrahydropyrimidines are commonly grouped together with imidazothiazoles as nicotinic agonists (Aubry et al., 1970). Examples of this AH chemical class include pyrantel, oxantel and morantel. The AADs are a new class of AHs with broad spectrum activity against GINs that are resistant to benzimidazoles, imidazothiazoles and macrocyclic lactones (Ducray et al., 2008). Monepantel, is the first member of this class. Derquantel is the first commercial member of the spiroindoles, a group which was introduced in 2010 for use in combination with MLs, under the commercial name STARTECT®, for the control of GINs in sheep (Little et al., 2011). Spiroindoles acts as antagonists of nAChRs. Cyclooctadepsipeptides were discovered in 1992 when PF1022A, the parent compound, was isolated from the fungus *Mycelia sterilia.* The target of these compounds has been suggested to be on the calcium-activated potassium channel (SLO-1). The chemical structure and mode of action of BZs and MLs will be discussed in detail in Sections 2.3 and 2.4, respectively.

2.3. Benzimidazoles: Chemical structure and mode of action

As described at the previous section, BZs was the first chemical class of AHs reaching the market. All BZs are synthetic benzo derivatives of compounds carrying the imidazole ring (Pardeshi et al., 2021). The chemical structure of the scaffold of BZs, the 1H-1,3-Benzimidazole or 1,3-benzodiazole, is presented at Figure 1.2. The benzimidazole ring is considered a highly bioactive heterocyclic moiety that exhibit a range of biological activities including antimicrobial, antiparasitic, antitumor, antiviral, anti-inflammatory, antioxidant and antihypertensive (Bansal & Silakari, 2012; Salahuddin et al., 2017).

Figure 1.2 Chemical structure of 1H-1,3-Benzimidazole (Source: PubChem)

Additionally, this biological active chemical nucleus (5,6-dimethylbenzimidazole) constitutes an integral part of the structure of the vitamin B_{12} (Yadav & Ganguly, 2015). Regarding their antianthelminthic activity, since the synthesis of TBZ in 1961 several other BZs with improved efficacy and broad spectrum against nematodes were developed by adding akyl or aromatic substituents on the benzimidazole ring (Bansal & Silakari, 2012; Mckellar & Scott, 1990). Albendazole (ABZ), Mebendazole (MBZ), Flubendazole (FLU) and Fenbendazole (FBZ) are the most popular of those.

All BZs have a relatively similar mode of action and their differences in efficacy are mainly due to a) their bioavailability within the host and b) enzymatic variations in different GIN species (Mckellar & Scott, 1990; Picanço et al., 2019). Their main mode of action is through binding to β-tubulin, which is a structural protein of cells, leading to suppression of its polymerization. As a result, the formation of microtubules in the GIN cells and the formation of the mitotic spindle, which is a necessary structure for cell mitosis is disrupted. Thus, the development of parasites is prevented by interfering with the process of mitosis and cell division (Mckellar & Scott, 1990; Robinson et al., 2004). In addition, the inhibition of microtubules formation is likely to be associated with metabolic disturbances in various cell functions, such as the uptake and storage of glucose and amino acids as well as protein synthesis, thus inducing GINs death. Also, by binding to β-tubulin, BZs impose changes in the structure of cells, such as for example on the disappearance of cytoplasmic microtubules, resulting in the lysis of the cytoplasm and the degradation of the cells (de Andrade Picanço et al., 2017; Mckellar & Scott, 1990). Effect of BZs is limited only to the cells of GINs while the structure of the host cells is not affected. This is due to the higher affinity of BZs for the β-tubulin of GINs compared to their mammalian homologue (Lacey, 1990; Robinson et al., 2004). Another mode of action of BZs is related to inhibition of the mitochondrial enzyme fumarate reductase (Mckellar & Scott, 1990; Qing-zhang et al., 2007). This enzyme is important for several crucial metabolic functions, anaerobic respiration, citric acid cycle, mitochondrial respiration and production of ATP. Thus, disruption of these processes ultimately leads to the death of GINs.
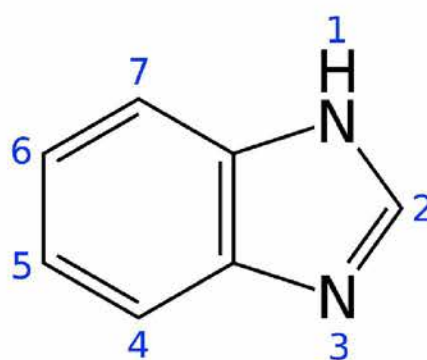
2.3.1. Albendazole

Albendazole (ABZ), [*methyl [5-(propylthio)-1H-benzimidazol-2-yl] carbamate*], is a broad- spectrum anti-parasitic BZ, which was first introduced in 1975 for the treatment of liver flukes, tapeworms, lung and gastrointestinal nematodes (GINs) in sheep and cattle (Theodorides et al., 1976). It was approved for human use as well, in 1982 (Chai et al., 2021). ABZ is administrated to animals (sheep, goats, cattle but also horses, dogs and cats), orally as a tablet or a drench, (Chai et al., 2021). The recommended therapeutic dose for ruminants (cattle, sheep & goats) is 5 to 7.5 mg/kg body weight with a safety margin of 7.5-20 times the recommended dose (European Medicine Agency (EMA), 1997). It is characterized by low absorption from the gastrointestinal tract due to low water solubility (Molina et al., 2007). It is rapidly oxidized in the liver and/or small intestines by flavin-monooxygenases (FMO) to albendazole sulfoxide (ABZ-SO), which is considered the main metabolite. ABZ-SO is further oxidized by cytochrome P-450 to albendazole sulfone (ABZ-SO$_2$) which can be further hydrolyzed to albendazole-2-aminosulfone (Gyurik et al., 1981; Rawden et al., 2000; Zhang et al., 2020). ABZ-SO, the active metabolite of ABZ, presents anthelminthic properties as well. Thus, it is also administered as an anthelmintic agent with the common name ricobendazole (Campbell, 1990; Canton et al., 2018). Regarding its physicochemical properties, it has a

Figure 1.3 Chemical structure of ABZ and transformation and its main metabolic pathway.

chemical formula of $C_{12}H_{15}N_3O_2S$ and a molecular weight of 265.34 g mol^{-1}. It is soluble in dimethyl sulfoxide, strong acids, and bases, while it is slightly soluble in methanol, chloroform, ethyl acetate and acetonitrile. Also, it is practically insoluble in water (Jung et al., 1998). The chemical structure of ABZ and its main transformation products, ABZ-SO and ABZ-SO$_2$ is presented in Figure 1.3.

2.4. Macrocylic Lactones: Chemical structure and mode of action

Macrocyclic lactones (MLs) constitute an important class of AHs. Since their introduction at the early 80's, they have become one of the largest selling class of AHs in the world, widely used in veterinary and human medicine and crop protection due to their broad spectrum of activity and high efficacy as 'endectocides' against nematode and arthropod species (insects and mites) (McKellar & Benchaoui, 1996; Merola & Eubig, 2012; Vardanyan & Hruby, 2016). MLs are divided into two sub-classes: avermectins and milbemycins. The first sub-class includes doramectin, eprinomectin, ivermectin and abamectin while milbemycins include milbemycin oxime and moxidectin (McKellar & Benchaoui, 1996; Prichard et al., 2012; Vardanyan & Hruby, 2016). Avermectins were discovered in 1976 as a mixture of natural products produced by a soil actinobacterium, *Streptomyces avermitilis* (Lasota & Dybas, 1991) while their antihelminthic activity was first described in 1979 (Burg et al., 1979). On the other hand, milbemycins were discovered in 1967 as fermentation products of another actinobacterium, S *treptomyces hygroscopicus* and exhibited very high acaricide activity (Prichard et al., 2012). In 1972, the active compound was elucidated and identified as

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milbemycin and from there, the first AH of this class, milbemycin oxime was derived (Takiguchi et al., 1980).

Both avermectins and milbemycins have a similar chemical structure which consists of a 16-membered macrocylic lactone ring. This ring consists primarily of four major (A_{1a}, A_{2a}, B_{1a}, B_{2a}) components and four homologous minor (A_{1b}, A_{2b}, B_{1b}, B_{2b}) components. Avermectins are designated as A1, A2, B1 and B2, referring to mixtures of the homologous pairs containing at least 80% of the A component and no more than 20% of the B component. From the naturally occurring MLs which have been discovered, compounds of the B series (B_{1a} / B_{1b}, B_{2a} / B_{2b}) of avermectins were found to be the most active against helminths and arthropods. Therefore, all semi-synthetic avermectins are the result of mixture of these homologous components and chemical modifications (Lasota & Dybas, 1991; Lumaret et al., 2012; McKellar & Benchaoui, 1996). The principal structural difference is
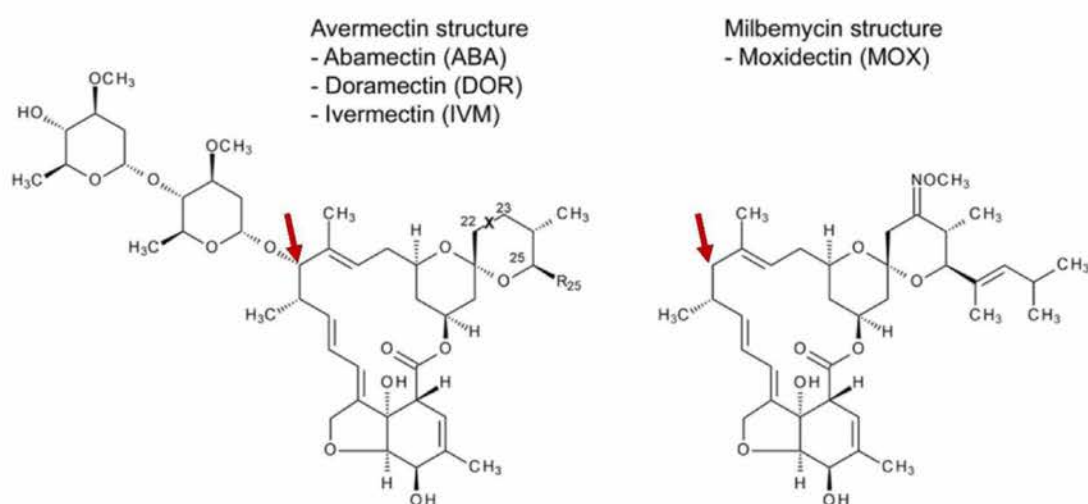
Figure 1.4 Chemical structures of Avermectins and Milbemycins. Red colored arrows indicate the C13 position in macrocylic lactone ring. (Source: Heinrich et al. Environ Sci Eur, (2021), 33:77).

that avermectins have sugar groups at C13 of their macrocyclic ring, whereas the milbemycins are protonated at the C13 position and they also carry a butyl or isopropyl in the C25 position (R. Prichard et al., 2012). Chemical structures of avermectins and milbemycins are presented at Figure 4.

MLs (both avermectins and milbemycins) exert their pharmacological effects acting as allosteric antagonists for ligand-gated chloride channels, particularly those controlled by the neurotransmitters γ-aminobutyric acid (GABA) and glutamate (Holden-Dye & Walker, 1990; Wolstenholme & Rogers, 2005). Some recent findings also propose a potent effect on Nicotinic Acetylcholine Receptors (nAChRs) in combination with the nicotinic antagonist, derquantel (Abongwa et al., 2016). Binding of MLs to ligand-gated chloride channels causes increased chloride entry across the cell membrane, leading to hyperpolarization and paralysis of the nematode and arthropod musculature. Paralysis of pharyngeal muscle cells reduces food intake, while paralysis of body muscles inhibits the ability of the parasite to remain in its preferred site on the host, leading to parasite death. In addition, they affect the muscles of the female reproductive system leading to a reduction in egg production (Fellowes et al., 2000).

Glutamate-gated channels which high sensitivity to MLs are present only in nematodes and arthropods (Wolstenholme, 2011). Whereas the GABA-gated chloride channels of mammals are not impaired by MLs since these compounds cannot cross the blood-brain barrier and enter the brain. GABA-gated chloride channels are found only in the mammalian CNS, from which MLs are excluded through the action of a p-glycoprotein efflux pump. But when the p-glycoprotein transporter is

overloaded, MLs could pass the blood-brain barrier and lead to toxic poisoning of the host (Gwaltney-Brant et al., 2018).

2.4.1. Ivermectin and Eprinomectin

Ivermectin (IVM), a mixture of 22,23-dihydroavermectin B1a (>90%) and B1b (<10%), is the most used member of avermectin AHs. It is a 22,23-dihydro derivative of avermectin B1 (Abamectin) which differs from abamectin by a single methylene group at the position C26 (Campbell & Benz, 1984). Since its introduction to the market at 1981 (McKellar & Benchaoui, 1996), IVM has become one of the best-selling antiparasitic in the world (Merola & Eubig, 2012). It is widely used in agriculture, aquaculture and livestock sectors as an efficient and broad-spectrum antiparasitic agent and acaricide against nematodes and arthropods (González Canga et al., 2009; Prichard et al., 2012). IVM is also approved for the treatment of human onchocerciasis (Aziz et al., 1982), lymphatic filariasis (Fischer et al., 1997), streptocerciasis (Õmura, 2008), and pediculosis (Ameen et al., 2010). During Covid-19 outbreak IVM was tested for its antiviral efficiency against SARS-CoV-2 but evidence on its efficacy is still conflicting (Martin et al., 2021). As an AHs, IVM which is available in several formulations (oral drench for small ruminants, injectable, pour-on and long-lasting boluses) (Prichard et al., 2012). The injectable or pour-on dosage of IVM for cattle is 0.2 and 0.5 mg kg^{-1} respectively (Laffont et al., 2001). Oral administration of IVM in ruminants range between 0.15 and 0.2 mg kg^{-1} (González Canga et al., 2009).

Eprinomectin (EPM), or 4"-epiacetylamino-4"-deoxy-avermectin B1, is an amino-avermectin derived from avermectin B1 with a modified oleandrose moiety, by the addition of an aminosaccharide at the C4 position. It consists of a mixture of two homologous components B1a (not less than 90%) and B1b (not more than 10%). It was synthesized and produced in 1996 by Merck as a novel ML with limited persistence in milk (Shoop et al., 1996). The effectiveness of EPM in combination with the very low value of its concentration ratio in animal milk made its use appropriate for antiparasitic use in milk producing ruminants, especially in goats, even during the lactating period (Mason et al., 2012; Rostang et al., 2020). Its application method is mainly by instillation along the spine of the animal, but it is also administered as injectable and orally. Recommended doses are 0.5 mg/kg for cattle (European Medicine Agency (EMA), 1996), 0.5 mg kg^{-1} for sheep (Kírcalí Sevimli et al., 2011), and 1 mg kg^{-1} for goats (Scheuerle et al., 2009).

Concerning physicochemical properties, IVM and EPM as avermectins are lipophilic compounds, which dissolve in most organic solvents and are practically insoluble in water (0.006-0.009 mg/L) (McKellar & Benchaoui, 1996). They are acid sensitive and treatment with dilute hydrochloric acid results in cleavage of the first of the C13 sugars. Both compounds are also photosensitive and exposure to ultraviolet (UV) light leads to the isomerization of their 8,9 and 10,11 double bonds (McKellar & Benchaoui, 1996).

2.5. Anthelminthic resistance of gastrointestinal nematodes

As described in previous sections, AHs are used for the control and treatment of helminthiases for more than 50 years now. However, the first reports of a phenomenon called anthelminthic resistance (AHR) go back to 1964 (Ahuir-Baraja et al., 2021), only three years after the introduction of TBZ in the market. During the next twenty years AHR against BZs was already a major concern (Prichard et al., 1980). Nowdays, AHR is a serious world-wide problem (Kaplan, 2020; Kaplan & Vidyashankar, 2012; Lanusse et al., 2018). Currently, AHR has been reported for most AH classes, and mostly BZs (Traversa & von Samson-Himmelstjerna, 2016; Charlier et al., 2022), while multidrug resistance has been

reported in USA (Kaplan, 2020), North America (Veríssimo et al., 2012), Oceania (Lamb et al., 2017) and Europe (Geurden et al., 2014; Rose et al., 2015). Introduction in the market, after 2008, of new AHs like AADs and Spiroindoles was an encouraging step on the fight against AHR. However, there are already available some preliminary reports of AHR against novel AH compounds like monepantel (AAD) and derquantel (Spiroindole) (Sales & Love, 2016; Van den Brom et al., 2015).

In broad terms, AHR is a heritable trait (Prichard et al., 1980), and is defined as occurring "when a greater frequency of individuals in a parasite population, usually affected by a dose or concentration of compound, are no longer affected, or a greater concentration of drug is required to reach a certain level of efficacy." (Wolstenholme, 2011). In practical terms, AHR is present in a population of GINs when the reduction related to a treatment is under 95% (Coles et al., 1992). Regarding small ruminants, the most resistant GINs belong to *Haemonchus cortortus,* although there are also resistant strains of *Teladorsagia* and *Trichostrongylus spp.* worldwide (Ahuir-Baraja et al., 2021). In case of cattle, in USA, Oceania and Europe AHR is commonly encountered in pathogenic GINs such as *Cooperia spp., Ostertagia ostertagi* and *Haemonchus spp.* (Geurden et al., 2014; Kaplan, 2020). AHR resistance mechanisms include: (i) mutation or deletion of one or more amino acids in the target genes, (ii) reduced expression of receptors, (iii) decreased affinity of receptors and (iv) absence of bioactivating enzyme (Gilleard, 2006; James et al., 2009). AHR can be delayed or reduced by a range of different strategies including: (a) the introduction of new AH classes with different modes of action compared to the existing AHs (high cost) (b) treatment with compounds from different AH classes, (c) rotation of AHs with different modes of action between dosing seasons, and (d) keeping a part of population in untreated refugia (Kaplan, 2020; Leathwick, 2012).

2.6. Metabolism and pharmacokinetics of benzimidazole and macrocyclic lactone anthelminthic drugs in animals

Upon their administration in animals BZ and ML AHs are subject to various metabolism processes. The pharmacokinetic patterns of these compounds in the body of the treated animas is controlled by their main physiochemical properties like (a) their aquatic solubility, which significantly determines the area under the plasma drug concentration-time curve (AUC), (b) the route of administration and appropriate administration practices (Křížová-Forstová et al., 2011; Lanusse et al., 2018; Mckellar, 1997). Sex, age, amount and composition of animals' feed and animal species are also important parameters which define both a) the effectiveness and b) the metabolic route of BZs and MLs. In case of BZs their metabolism depends mainly on the substituent present on the C5 position of the benzimidazole moiety and involves a wide variety of reactions. Two major enzyme systems, the cytochrome P450 family and the microsomal flavin monooxygenases are primarily responsible for the biotransformation of BZs in animals' body (Gottschall et al., 1990). The parent AH compounds have rather short half-life and transformation products that are produced through hydroxylation (TBZ, parbendazole), S-oxidation (ABZ, FBZ), and reduction (MBZ) predominate in the tissues and excreta of treated animals (Aksit et al., 2015; Gottschall et al., 1990). These primary metabolites usually result from normal oxidative and hydrolytic processes and are more polar and water soluble than the parent compounds (Aksit et al., 2015; Gottschall et al., 1990).

MLs are partially metabolized in mammals (Lee Chiu et al., 1987) and in nematodes (Alvinerie et al., 2001), which contribute to the elimination of these compounds (Prichard et al., 2012). Cytochrome P450 3A and 2B are the predominant enzymatic systems involved in MLs metabolism (González Canga et al., 2009; Prichard et al., 2012). IVM from avermectins and moxidectin (MOX) from milbemycins are the most well studied compounds regarding their metabolism in treated animals. The main identified metabolite of IVM in rats, cattle and sheep is the 24-hydroxy-IVM

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while in swine and goats, the main metabolite detected was 3-O-desmethyl-IVM (González Canga et al., 2009; Junco et al., 2021; Mckellar, 1997; R. Prichard et al., 2012). However, metabolic processes are considered to contribute only little to the elimination of MLs from the treated host (8-10% of the administered MLs is transformed by metabolic processes) (González Canga et al., 2009; Junco et al., 2021; Mckellar, 1997; R. Prichard et al., 2012). MLs are characterized by high lipophilicity which leads to their extensive distribution in the body of mammals and high concentration in adipose tissues regardless of the route of administration (Prichard et al., 2012). Despite that, MLs are effectively removed from animal's body intact, in the form of the parent compound, via efflux transporters (Chen et al., 2016). These proteins belong to the ATP-binding cassette (ABC) transporters family (Chen et al., 2016) and they are found on mammalian barrier epithelium tissues such as intestine, plancenta, mammary gland and the blood–brain barrier. ABC transporters are also found in GINs and some of them are involved in AHR mechanisms (Prichard et al., 2012). Therefore, efflux of MLs via active transport from mammalian and parasite cells strongly influence the pharmaco- and toxico-kinetics of these drugs and contributes extensively to the high disposal of these compounds in the feces.

3.   Environmental fate of benzimidazole and macrocyclic lactone anthelmintics

3.1.   Entry to soil through animal excretions and manure application

Both BZ and ML AHs, due to their physicochemical properties and pharmacokinetic features, are excreted by the treated animals, either in form of parent compound or in the form of active metabolites, through feces and urine. Concerning BZs, the parent compounds have in general short half-life and their metabolites predominate in the tissues and excreta of treated animals. Metabolites have been isolated mainly from urine. However, their limited absorption and biliary excretion can lead to high fecal disposal levels. The metabolic profiles of individual BZs in animals' excreta follow similar patterns across species, but metabolite percentages do vary substantially between different BZ compounds (Gottschall et al., 1990). TBZ, based on experiments in mice (Tsuchiya et al., 1986) and sheep (Tocco et al., 1966), is excreted mainly in urine (60-62%) with a percentage of 24-34% excreted through feces. TBZ in the form of the parent compound was present at levels of 15-20% in feces. ABZ showed a similar excretion profile in cattle, goats, and sheep with 59, 61 and 70 % respectively of the administrated dose excreted through urine and 14-28 % excreted through feces (Gottschall et al., 1990; Hennessy et al., 1989). ABZ generally is excreted in the form of its oxidized metabolite ABZ-SO with low levels of the parent compound also detected (<5%). However, Prhal et al (2016) and Porto et al. (Silveira Porto et al., 2021) in recent studies showed high concentration levels of the parent compound reaching to 7.7 and 10.8 µg g^{-1} in fecal residues 24h post oral administration of a single dose of 10 µg g^{-1}. These high levels of the parent compound may be associated with the fact that in the digestive system of sheep the gastrointestinal microflora is able to transform ABZ-SO back to ABZ (Lanusse & Prichard, 1993; Renwick et al., 1986). Other members of BZs like fenbendazole (FBZ) and MBZ are excreted from animals mostly through feces. FBZ metabolism has been studied extensively in cattle, goats, and sheep (Hennessy et al., 1993; Mckellar, 1997). In cattle when an oral administration was used 36% of FBZ was recovered from feces but no residues of BZ were detected in the urine while 50% was recovered in the form of the main metabolite, fenbendazole sulfone. Oral administration of FBZ to sheep and goats led to 65-80% of the dose excreted in feces and 17-25% in urine mainly in the form of fenbendazole sulfone. On the other hand, MLs and avermectins are mainly excreted intact through feces, with elimination rates ranging between 50 and 90% of the dose applied (Hentz et al., 2019), (See Section 2.6). IVM, which is the most well studied avermectin, is excreted at percentages of up to 90%

in feces and less than 2% was detected in urine in cattle, sheep, and goat (González Canga et al., 2009; Junco et al., 2021). EPM, another representative member of avermectins, showed similar behavior with 80% excretion levels in faces in cattle (Aksit et al., 2016; Halley et al., 2005).

The presence of BZs and MLs in fecal material is determined by several factors besides their chemical properties and pharmacokinetic features in animals. The route of administration, the husbandry systems, stocking densities of the target host animals (Boxall et al., 2004; McKellar & Benchaoui, 1996) and finally the existence or absence of good husbandry practices and therefore incorrect or excessive use of AHs (Goodenough et al., 2019). The method of administration can also be a key factor in determining a compound's excretion profile. Pour-on treatments tend to result in higher and more variable concentrations in feces compared to injectable treatments, while excretion is more rapid following oral treatment (Pope, 2009). On the other hand, sustained release delivery systems lead to lower concentrations but also to a continuous release of AHs (Mckellar, 1997).

An important parameter which strongly affects the environmental fate of BZ and ML anthelmintics, besides their excretion levels, is the persistence and mobility of these compounds in feces, manure and slurry which are applied in agricultural soils. In general, the persistence of these drugs in feces and manure can be influenced by environmental processes like biodegradation and photodegradation, and factors like temperature, moisture, and pH, as well by the method and the duration of storage of the fecal material (Boxall, 2010; Boxall et al., 2004; Halley et al., 1993; Horvat et al., 2012; Pope, 2009). There is limited literature regarding the fate of these compounds in fecal material in contrast with other veterinary drugs like antibiotics. However, available studies have shown that both BZs and MLs can persist in fecal material and manures for extended periods of time, ranging from several days to over a month. Regarding BZs there are only a couple of studies on their persistence in fecal material, while the fate of their transformation products is still unclear. According to the most notable study, Kreuzig et al (2007) monitored the fate of BZs FBZ and FLU in pig manure under laboratory conditions. Both BZs showed a slow dissipation rate. After a 102-day incubation period, extractable fractions contained 72% and 80% of the initially applied FLU and FBZ respectively. These results raise concerns about the possible persistence of BZs in manure even after prolonged storage periods. On the other hand, the persistence MLs and particularly of IVM in fecal material and manure has been explored more extensively. Celestina et. al (2010) monitored the fate of avermectins abamectin and doramectin in feces of treated sheep under different experimental conditions. DT_{50} values of both compounds ranged between 9 and 27 days. In feces of cattle treated with IVM in two dose levels (3 & 0.3 μg g^{-1}), the compound persisted for the whole duration of the study with DT_{50} values of 39 and 88 days for the low and high dose respectively (Iglesias et al., 2018). Further analysis confirmed that IVM moved from feces to the underlying soil as well as to nearby plants raising concerns about its transfer to the trophic chain and also to other environmental compartments. Furthermore, IVM residues in cattle dung released in pastures in Denmark and Tanzania showed no notable degradation after 45 and 14 days, respectively (Sommer & Steffansen, 1993). These findings indicates that IVM could be persistent in dung pats under different climate conditions. Regarding EPM, some preliminary data support that it could be persistent in manure, at levels that could pose environmental risk. Litskas et al., (2013) reported DT_{50} value of 333 days for EPM in cattle manure. This in line with the report of the European Medicinal Agency (European Medicines Agency (EMA), 2018) where high DT_{50} values of EPM in manure were reported (312 to 3922 days).

3.2. Processes that control the environmental fate of anthelmintics in soils

Anthelmintics, as most other veterinary pharmaceuticals, reach grassland soils through direct disposition of fecal and urinary material by animals and agricultural soils through application of

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contaminated manure (Boxall, 2010; Boxall et al., 2004; Horvat et al., 2012). Once in soils, BZs and MLs are expected to undergo processes like photodegradation, biodegradation, hydrolysis, adsorption, leaching to groundwater or surface run-off (Boxall, 2010; Horvat et al., 2012; Mooney et al., 2021). Manure and slurry applications in soil may also alter the behavior and transport of AHs. Studies have reported that the addition of these materials can affect the adsorption behavior of veterinary medicines and that they may affect their persistence (Boxall, 2010; Boxall et al., 2012; Pope, 2009). These effects have been attributed to changes in pH or alterations in the nature of the dissolved organic carbon in the soil/manure system (Boxall, 2010). However, the environmental fate of these compounds is less documented than other pharmaceuticals, like antibiotics. The limited knowledge of their fate, behavior and (eco)toxicological effects and the absence of routine monitoring programmes following their presence in natural water resources (Boxall et al., 2012), resulted in the identification of AHs as emerging pollutants.

Regarding their occurrence in soils, BZs (both parent compounds and transformation products) and MLs are considered moderately persistent (Boxall, 2010; Horvat et al., 2012). Although this is based on a limited number of relevant studies especially in case of BZs. Thiabendazole is considered the most persistent BZs with reported DT_{50} values in soils under field conditions between 833 and 1100 days (European Chemicals Agency (ECHA), 2017). Kreuzig et al. (2007) determined the fate of BZs FLU and FBZ in soils after direct application or via application of contaminated manure. Under field conditions, FLU showed high persistence with DT_{50} values of 126 days, while dissipation of FBZ was more rapid with DT_{50} values of 22 days. On the other hand, ABZ is rapidly dissipated in soils. Wu & Hu, (2014) reported DT_{50} values between 4.95 and 6.3 days without measuring transformation and persistence of its metabolites in soil. However, transformation products of veterinary drugs have reported to be more persistent than the parent compound (Boxall et al., 2004; Horvat et al., 2012; Snow et al., 2019). In a recent study Navrátilová et al., (2023) demonstrated that the transformation products of ABZ, ABZ-SO and ABZ-SO$_2$, were present at high concentrations in soil three months after the application of ABZ-contaminated feces in the field site. More data is available for avermectins, and particularly for IVM. Avermectins and particularly IVM and EPM are photodegraded (Halley et al., 1993) and differences in their dissipation rate is soil have been reported between samples kept in dark and exposed to sunlight (Litskas et al., 2013; Mougin et al., 2003). Oxygen levels in soil also affect the persistence of these compounds. Litskas et al (2013) reported DT_{50} values of 38–53 days for EPM under aerobic conditions while under anaerobic conditions these values increased to over a year. Similar results were provided by Krogh et al (2009) where no dissipation was detected in soil under anaerobic condition while DT_{50} values were ranged between 16.1 and 36.1 days in three different soils under aerobic conditions. These findings combined with the absence of dissipation of IVM and EPM in sterilized soil samples (Krogh et al., 2009; Litskas et al., 2013) highlight the role of the aerobic soil microbial community on the dissipation of avermectins in soils. Climatic conditions could also affect dissipation of MLs under field conditions based on reports on IVM which dissipated rapidly (DT_{50} = 7-14 days) during the summer in soil/feces mixtures. The aerobic dissipation rate of IVM was reduced in the winter, with DT_{50} values of 91-217 days (Halley et al., 1993). In general, MLs have shown a large variability in their persistence across different soils. Reported half-lives range from 14 to 56 days for Abamectin, 61 to 79 days for doramectin and about 60 days for moxidectin (Floate et al., 2005). IVM has showed DT_{50} values in soil ranging from 11.5 up to 240 days, while in most cases these values ranged between 30 to 66 days (Dionisio & Rath, 2016; Halley et al., 1989; Krogh et al., 2009). EPM is also considered moderately persistent with DT_{50} values in soils ranging between 20.8 to 57.9 days (Litskas et al., 2013).

Chemical and biological degradation constitute the major processes driving the dissipation and environmental fate of veterinary drugs. However, their fate is also controlled by adsorption and transportation processes like leaching and surface run-off (Boxall, 2010; Boxall et al., 2004). In general,

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both BZs and MLs are tightly absorbed in soil particles thus they are considered slightly to moderately mobile in contrast to most antibiotics (Boxall et al., 2012; Horvat et al., 2012; Pope, 2009; Snow et al., 2019). One indicator of the mobility of organic pollutants in soil is their adsorption coefficients normalized for the organic carbon content of the soil (K_{oc} values). In general, substances with K_{oc} values >1000 mg L^{-1} are likely to show low mobility in soil. ABZ is not particularly mobile in soil and sediments, due to its relatively high adsorption affinity (Mutavdžić Pavlović et al., 2018) which was further controlled by the organic carbon content, pH and ionic strength of soil particles. Kreuzig et al. (2007) reported similar results for FBZ and FLU (K_{oc} values > 1100 mg L^{-1}). Therefore, BZs are not expected to show high leaching potential as confirmed by Porto et al. (2021) in column experiments with different soils treated with ABZ, FBZ and TBZ. Unlike the parent compounds (ABZ and FBZ) their oxidized transformation products (ABZ-SO, ABZ-SO$_2$ and FBZ-SO), which are more polar compounds, could leach to deeper soil layers. Avermectins also exhibit high soil adsorption affinity with K_{oc} values > 5000 mg L^{-1} as reported for IVM, abamectin (Dionisio & Rath, 2016; Halley et al., 1993; Krogh et al., 2008) and EPM (Floate et al., 2005; Litskas et al., 2016; Litskas et al., 2011). Adsorption behavior of IVM, EPM and avermectins in general is possibly controlled by both Ionic (especially presence of Ca^{2+}) and lipophilic binding (related with organic carbon matter) (Krogh et al., 2008; Litskas et al., 2011). Thus, due to their high adsorption in soils, these compounds are not prone to leaching in soil which has been confirmed in leaching column studies (Litskas et al., 2016; Oppel et al., 2004; Rath et al., 2016). Furthermore, recent studies highlight that when in soils both BZs and MLs can be further transported to plants, moving up in the food chain. Navratilova et al. (2021) reported transportation of ABZ metabolites from sheep excrement to fodder plants and from there through grazing to other sheep in the pasture. This circulation process and continuous uptake of ABZ and its transformation products by animals could stimulate the rapid development of drug resistance in GINs (Dimunová et al., 2022). Similar results are available for IVM (Iglesias et al., 2018; Mesa et al., 2020) where this compound was detected in plants nearby points where feces were deposited and in macrophytes in wetlands located near to cattle pasture areas. Due to their low mobility, low levels of BZs and MLs are expected to be found in drainage waters through surface run-off. The few studies available reported that these compounds can be found in drainage water and run-off particles but at levels significantly lower than antibiotics (Fernández et al., 2011; Weiss et al., 2008). Despite their low solubility and mobility, BZs and MLs have been detected in surface water systems at varying frequency (Sim et al., 2013; Zrnčić et al., 2014). For example, BZs like ABZ, FBZ and FLU have been detected in riverine water at levels between 0.32 to 39.43 ng L^{-1} which could pose a threat for the environment. On the other hand, MLs like IVM are rarely detected in surface waters (probably due to their rapid photodegradation), but they could be highly persistent in sediments with DT_{50} values exceeding 365 days (Horvat et al., 2012; Mesa et al., 2020) and concentration levels between 1.4 and 17 µg Kg $^{-1}$ (Liebig et al., 2010; Mesa et al., 2020; Mesa et al., 2017). In addition, recent studies (Mooney et al., 2021) reported the detection of AHs (with ABZ being the most commonly detected AH) in groundwater. This was associated with agricultural land use and sheep population density. Overall, the results of recent monitoring studies reporting the common detection of AHs in several environmental compartments have raised environmental concerns about their role as emerging environmental pollutants.

3.3. Effects of benzimidazole and macrocyclic lactone anthelmintics on non-target organisms

3.3.1. Ecotoxicity effects of anthelmintics on soil and aquatic organisms

The presence of BZs and MLs AHs in soil and aquatic environments, has been the focus of previous sections. The frequent occurrence of these compounds in different environmental compartments raise

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concerns about the potential risk for effects on to non-target organisms. Indeed, in recent years there is an emerging interest of monitoring these effects. To date most currently ecotoxicity data for AHs relate to dung fauna and earthworms as well as on planktonic aquatic organisms. Goodenough et al. (2019) demonstrated that FBZ and IVM can affect, in environmental realistic levels, the common earthworm, *Lumbricus terrestris*, which has a key functional role in soil ecosystems. Toxic effects of ABZ have been also reported on a different species of earthworm, *Eisenia fetida* (Gao et al., 2007). A review study by Belew et al., (2021) reported toxic effects of ABZ in earthworms (*Eisenia fetida*), in planktonic crustacean organisms (*Daphnia magna*) and in the fish species *Danio rerio*. More studies are available for avermectins, reporting toxic effects (lethal or sublethal) of IVM and EPM in a variety of soil and dung fauna including coprophilous Coleoptera, Diptera and Hymenoptera, coprophilous arthropods and nematodes, other soil dwelling organisms like collembola, mites and dung beetles. (Hempel et al., 2006; Lumaret et al., 2012; Nieman et al., 2018; Verdú et al., 2015; Zortéa et al., 2017). Effects of IVM and EPM were also reported on aquatic fauna including crustacean like *Daphnia magna*, copepods, cladoceran and also mayflies (Ephemeroptera) (Sanderson 2007; de Souza & (de Souza & Guimarães, 2022; Sanderson et al., 2007), 2022). These effects are not limited to acute lethal toxicity. Mortality is reported mostly in cases of larvae of organisms like earthworms and mayflies which hatch in feces and soil. Other sublethal effects including developmental toxicity (morphological dysfunctions), reproductive toxicity (e.g. sperm deformity, decrease in cocoon number), alterations of biochemical parameters (affect ATPase activity, mitochondria and smooth endoplasmic reticulum function) reduced growth rates and effects on gene expression (Belew et al., 2021; de Souza & Guimarães, 2022) have been also reported.

3.3.2. Interactions with the soil microbiota

In contrast with the existing data about effects of BZs and MLs on soil and aquatic biota, little is currently known about the interactions of these compounds with the soil microbiota. Upon their release in the soil ecosystems AHs are expected to interact with soil microorganisms. The exact nature of these interactions and the factors that determine the outcome of these interactions need to be explored. Thus, once in soil AHs will have a toxic effect on soil microbial communities and ecosystem functioning, or the genetic plasticity of soil microbiota will lead to microbial adaptation and possible enhanced biodegradation in soils. Concerning the potential toxicity of these AHs in soil or aquatic microbiota, the available literature is very limited. Toxicity of six BZs was reported on marine bacterium *Vibrio fischeri* via the Microtox assay, with ABZ being the most toxic amongst the compounds tested (Jin et al., 2006). Moreover, da Rocha et al. (2020) reported possible toxicity of abamectin to soil microbial communities based on qCO_2 increase, which reflects a lowered microbial biomass once microorganisms spend more energy in detoxification processes than in microbial growth. Previous studies with other BZ compounds showed contrasting results. For example, Papadopoulou et al., (2016) showed that TBZ would not affect the composition of the soil microbial community at concentration levels equivalent or even higher than the ones used in the current study. Whereas carbendazim reduced the α-diversity of bacteria and fungi in soil (Ma et al., 2021; Wang et al., 2009). Limited studies have explored the potential effects of avermectins on the soil microbial diversity. In a recent study abamectin, applied in soil at concentrations of 0.1 and 1 mg kg^{-1}, induced temporary effects on soil microbial diversity as determined by shotgun metagenomics (Qiu et al., 2022). Whereas Konopka et al. (2015) reported that IVM applied in soil as a mixture with the antibiotics monensin, and zinc bacitracin at a concentration of 10 mg kg^{-1} suppressed the abundance of ammonia oxidizing microorganisms (AOM), and more specifically ammonia oxidizing bacteria (AOB).

As for the second hypothesis, microbial degradation is considered a possible important environmental process controlling the dissipation of AHs in soil (Boxall et al., 2004; Horvat et al., 2012).

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However, only a few studies have so far demonstrated the role of microorganisms in the degradation AHs and most of them have focused on MLs. Krogh et al. (2009) and Litskas et al. (2013) exhibited a halting of degradation of IVM and EPM in sterile soil samples. Similarly, the degradation of abamectin was strongly inhibited in sterile vs non-sterile soil samples (Dionisio & Rath, 2016). For other organic compounds like pesticides (Arbeli & Fuentes, 2007) and antibiotics (Topp et al., 2013, 2016) the continuous exposure of soils to them have occasionally resulted into the evolution and spread amongst the soil microbiota of novel catabolic traits that lead to the enhanced biodegradation of these compounds, in a phenomenon called accelerated or enhanced biodegradation. The relevance of this phenomenon for AHs has not been investigated to date and worth exploring.

Soils exhibiting enhanced biodegradation of organic pollutants have been used as source for the isolation of microorganisms specialized in the degradation of the studied pollutants. Such microbes could be used as tailored-made inocula in the removal of pollutants, like AHs, from environmental matrices. To date, a limited number of studies have reported the isolation of single microorganisms or consortia of soil microorganisms capable to degrade BZ and ML compounds. In case of BZs, a bacterial consortium has isolated from a wastewater disposal site, capable to degrade efficiently TBZ, which is used mostly as a fungicide (Perruchon et al., 2018). In case of avermectins, a bacterium characterized as *Aeromonas taiwanensis* has isolated from contaminated soil and pig feces with the ability to degrade efficiently IVM (Wang et al., 2020). Previously, Wang et al. (2015) had reported the isolation of a *Stenotrophomonas maltophilia* bacterium capable to degrade another member of this group, abamectin, and characterized its abamectin-tolerance mechanism.

4. Reducing the environmental impact of benzimidazole and macrocyclic lactone anthelmintics

4.1. Treatment of manures prior to their soil application and AHs removal

As mentioned in the preceding sections the main route of entrance of BZs and MLs into the environment is through the direct deposition of feces by animals and to a large scale in agricultural settings through the application of AH-contaminated manures. Thus, decontamination of manures (liquid or solid) during storage and certainly prior to their environmental release is crucial to prevent the release of AHs in agricultural soils. The main methods of manure, solid (greater than 15% dry matter), slurry (5 to 10% dry matter), or liquid (less than 5% dry matter) (Janni & Cortus, 2020), storage is described in detail below. Manure storage systems are categorized based on the prevalence of aerobic or anaerobic conditions. In the USA and Canada, manure or slurry is generally stored in anaerobic lagoons. In those systems only the surface of the manure pile is exposed to air and sunlight, limiting the extent of photodegradation and aerobic biodegradation in its larger mass and maintaining anaerobic conditions in its inner mass (Pope, 2009). In Northern and Central Europe, where the available area is limited, solid or liquid manure is stored mostly in large tanks -anaerobic digesters. In these tanks manure is exposed to anaerobic conditions, stable temperature and moisture (DeRouchey, 2014; Janni & Cortus, 2020). On the other hand, in Asia, Africa and in the Mediterranean region outdoor stock-pilling and composting under aerobic or semi-aerobic conditions are the most commonly used handling strategies of manure and fecal material (DeRouchey, 2014; Janni & Cortus, 2020; Wohde et al., 2016) As manure is produced throughout the year and the slurry tanks are emptied periodically, e.g. in Europe, February to April for cereals and September for rapeseed (Lopez-Ridaura et al., 2009), storage time may be anything from five months to a year. As demonstrated in earlier sections, the transformation of AHs is faster under aerobic conditions compared to anaerobic conditions. In addition, high temperatures promote the degradation of

compounds in liquid manure (Pope, 2009; Wohde et al., 2016). Other parameters such as redox reactions, organic matter content and pH will vary according to storage methods and will affect the degradation rates of AHs during storage. If oxygen, water content and carbon to nitrogen rations are managed (which is therefore more costly) stored fecal material could be composted, accelerating the rate of AH degradation (Khan et al., 2008). In conclusion, the transformation process of AH compounds in manure is affected largely by the storage practices. There is increasing research activity regarding the transformation of VMPs under laboratory conditions, but field data are very limited up to date (Wohde et al., 2016). Current guidance, (European Medicines Agency (EMA), 2011), takes transformation of VMPs in manure into account. However, there is no standardized experimental test protocol available to examine the transformation of VMPs in manure. The EMA guideline on transformation in manure (European Medicines Agency (EMA), 2011) only contains basic regulatory requirements. To allow for a consistent assessment of studies within regulatory frameworks, a harmonized internationally accepted and validated test method is needed.

4.2. Bioremediation

The treatment of fecal material via composting or anaerobic digestion prior to their environmental release and during storage aims to reduce the load of VMPs in the manures that will be applied in agricultural settings. These approaches have shown variable results, and they have only been tested for the removal of antibiotics (Berendsen et al., 2018; Selvam et al., 2013). While in the case of AHs there is limited data and those have been produced under laboratory conditions (Kreuzig et al., 2007; Litskas et al., 2013). One interesting, promising, low-cost and non-invasive mitigation approach which can support the existing treatments is bioremediation of fecal material, contaminated manure or even contaminated soils with microorganisms capable of degrading BZ and ML AHs. First attempts (Hirth et al., 2016; Hong et al., 2020) reported interesting results on the removal of the veterinary antibiotics sulfamethazine and tetracycline from soil. In general, bioremediation is any method that uses microorganisms or the enzymes they produce, for the decontamination and restoration of polluted soils, aquifers and other ecosystem. During bioremediation, microorganisms are used to break down hazardous and toxic substances to form less toxic or non-toxic products. Indigenous or exogenous bacteria and fungi can be used. Its effectiveness depends on several factors such as the physicochemical characteristics of the contaminated substrate, temperature, oxygen availability, concentrations of available nutrients and others (Vidali, 2001). In general, the use of microorganisms and the enzymes they produce to detoxify drugs is considered an environmentally friendly decontamination method (Azubuike et al., 2016; Juwarkar et al., 2010; Patel et al., 2022). Depending on the degree of substrate saturation and aeration, different bioremediation strategies are applied and they are generally classified as *in situ* and *ex situ* bioremediation techniques.

In situ techniques involve treatment of the contaminated material in its area and are generally more desirable because they cause minimal disturbance to the soil ecosystem, due to fewer interventions which are achieved at lower costs (Azubuike et al., 2016; Juwarkar et al., 2010; Patel et al., 2022). The most important *in situ* interventions in soils are biostimulation and bioaugmentation. Biostimulation involves the addition of nutrients and oxygen that enable the indigenous microbiota to produce the necessary enzymes to dissipate the pollutants. On the other hand, bioaugmentation involves the addition of native or exogenous microorganisms (axenic strains or microbial consortiums) with the desired catabolic capacities to contaminated sites. Bioaugmentation has several advantages which are listed below: (i) it is characterized by low implementation cost, (ii) it does not require transfer to another place for its application, (iii) it is considered as a desirable "green" remediation method (iv) it produces minimal harmful effects on the environment and (v) it allows other physical dissipation processes such as evaporation, dissolution, dispersion and photooxidation to be operative and

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contribute to the removal of AH residues. On the other hand, its disadvantages are: (i) the need for optimum environmental conditions for the introduced microbiota to thrive which are not always easy to control and maintain (ii) the potential failure of exogenous inocula to establish and proliferate due to intense competition with the indigenous microbiota (Azubuike et al., 2016; Juwarkar et al., 2010; Patel et al., 2022).

Aim of the study

Treatment with synthetic anthelmintics (AHs), constitute the main strategy for prevention and treatment of gastrointestinal nematodes (GINs) in livestock. However, as many chemical substances AHs are not totally absorbed by the animals and as a consequence they 365are excreted with the faeces where they are detected at concentration levels ranging from $\mu g\ Kg^{-1}$ to mg Kg^{-1}. The application of AH-contaminated manures, produced by animal feces, leads to soil pollution and possibly through transport processes to the pollution of associated natural water resources and possibly to uptake and translocation up in the trophic chain. Previous studies have demonstrated the undesirable effects that AHs can have on non-target organisms inhabiting pasture areas, like insects associated with fecal decomposition and other soil-dwelling organisms. However, little is currently known regarding their fate in the soil but mostly about their effects on the soil microorganisms that have a pivotal role in ecosystem functioning. Two hypotheses for the nature of these interactions have been put forward (a) the input of AHs will have a toxic effect on soil microbial communities and ecosystem functioning, or (b) the genetic plasticity of soil microorganisms will lead to microbial adaptation and accelerated biodegradation of AHs in soil. Therefore, extensive study of these interactions is of utmost importance to determine whether biodegradation of these compounds can be a realistic approach in the future to halt the environmental degradation they can cause.

Thus, the main objective of the present study is to investigate the interactions between synthetic AHs like the BZ compound ABZ and the MLs IVM and EPM, and soil microorganisms and to evaluate the use of biodegradation as a means of decreasing environmental exposure to these AHs. This main objective is going to be achieved through a series of specific scientific objectives like: (1) to investigate the role and contribution of biodegradation and adsorption, as basic dissipation processes, in the environmental fate of AHs in pasture soils collected from livestock units of Lesvos island (Chapter 2), (2) to explore the effects of AHs on the function and diversity of soil microorganisms (ecotoxicity), and the potential emergence of new catabolic mechanisms by soil microorganisms constantly exposed to these compounds facilitating their rapid degradation (biodegradation) (Chapter 3), (3) the isolation of bacteria that have the ability to degrade AHs (Chapter 5), (4) the evaluation of AH-degrading bacteria as bioaugmentation agents for the detoxification of contaminated manures (Chapter 4).

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Chapter 2

Biodegradation of anthelmintics in soils: does prior exposure of soils to anthelmintics accelerate their dissipation?

1.   Introduction

Worldwide, gastrointestinal nematode (GIN) infections remain among the main parasitic threats for grazing ruminants, as they affect both health and welfare of the animals (Kaplan 2020). Ruminant GIN control is heavily reliant on the prophylactic use of anthelmintic (AH) products to maintain infections below levels that can cause clinical and sub-clinical disease (Stear et al. 2007; Hoste et al. 2016). Benzimidazoles (BZs) and macrocyclic lactones (MLs) are the two main groups of AHs (Horvat et al. 2012) with their market share reaching a value of €470 million (Morgan et al. 2013). BZs include a wide range of AH compounds like albendazole (ABZ), ricobendazole (sulfoxide derivative of ABZ), fenbendazole (FBZ), flubendazole (FLU), mebendazole (MBZ) and thiabendazole (TBZ) (McKellar and Scott 1990). MLs are disaccharide derivatives of avermectins isolated from cultures of *Streptomyces avermitilis* including very popular AHs like abamectin, ivermectin (IVM), eprinomectin (EPM) and doramectin (Omura 2008).

Upon their administration (orally for BZs and intramuscularly injected or through the skin as pour-ons for MLs), AHs are excreted by the animals, intact or transformed depending on their mode of administration, through feces and urine to levels varying from 60 to 90% of their administered dose (Halley et al. 1989; Aksit et al. 2015). Previous studies have reported ABZ concentrations in sheep and lamb excreta of 12.8 and 7.7 mg kg^{-1}, respectively, while active transformation products (TPs) of ABZ like albendazole sulfoxide (ABZ-SO) and inactive ones like albendazole sulfone (ABZ-SO$_2$) were also detected at lower (Prchal et al. 2016; Porto et al. 2020) or higher levels than the parent (Navratilova et al. 2021). Similar studies for IVM and EPM have reported concentrations of 0.3–3 mg kg^{-1} (Iglesias et al. 2018) and 0.4–3.34 mg kg^{-1} (Halley et al. 2005), respectively.

In feces, AHs can be particularly persistent with DT$_{50}$ values of 89 days for IVM (Iglesias et al. 2018) and 333 days for EPM (Litskas et al. 2013). Based on their slow dissipation in feces, AHs could be released in grassland soils, through direct deposition of feces and urine during grazing, and in agricultural soils, through the application of feces as manures. Monitoring studies have verified the transportation of AHs from dung piles and manures to soils (Oppel et al. 2004; Fernandez et al. 2011; Iglesias et al. 2011). From there, they can be further transported to plants, moving up in the food chain (Iglesias et al. 2018; Mesa et al. 2020), and to receiving water bodies (Sim et al. 2013; Petrovic et al. 2014). Navratilova et al. (2021) showed that subcutaneous sheep application of ABZ resulted in undesirable chronic environmental exposure to ABZ/ABZ-SO which move from feces to plants and through grazing back to the animals at sublethal levels which favors the development of drug resistance in helminths. In addition, Mooney et al. (2021) showed that the regular detection of AHs in groundwater systems in Ireland was associated with agricultural land use and sheep population density. The presence of AHs in terrestrial and aquatic ecosystems entails a high environmental risk (Liebig et al. 2010; Belew et al. 2021). This is based on their high toxicity of ABZ, IVM and EPM to terrestrial, dung (i.e., earthworms, springtails) (Verdu et al. 2018; Serafini et al. 2019; Barron-Bravo et al. 2020) and aquatic (e.g., invertebrates, fish) (Garric et al. 2007; Hal- ley et al. 1989; Carlsson et al. 2011) organisms, reinforcing their characterization as emerging contaminants.

To date, few studies have investigated the fate of AHs in soil compared to other soil contaminants like pesticides and antibiotics. This is further highlighted by a non-curated literature search in Scopus using the terms ((pesticides/ antibiotics/anthelmintics)) AND ((soil)) AND (sorption OR degradation) which identified 22,594 records for pesticides, 11,288 records for antibiotics and only 234 records for AHs. ABZ is known to oxidize to ABZ-SO and to ABZ-SO$_2$ with DT$_{50}$ values ranging from 4.95 to 6.3 days (Wu and Hu 2013). It is not particularly mobile in soil, due to its relatively high adsorption affinity (Pavlovic et al. 2018), unlike its two oxidized TPs which could leach to deeper soil layers (Porto et al. 2020). IVM is considered moderately persistent with DT$_{50}$ values ranging from 11.5 to 39.0 days (Krogh

et al. 2009; Levot 2011; Rath et al. 2016). It exhibits high soil adsorption affinity (Krogh et al. 2008), in accordance with its low mobility in soil (Oppel et al. 2004; Rath et al. 2016). EPM is also moderately persistent in soil with DT_{50} values ranging from 38 to 53 days (Litskas et al. 2013). It is not prone to leaching in soil (Litskas et al. 2021), as dictated by its relatively high affinity for soil adsorption (Litskas et al. 2016).

Microorganisms play a pivotal role in the degradation of organic pollutants like pesticides (Rousidou et al. 2017; Wirsching et al. 2020) and antibiotics (Topp et al. 2016; Billet et al. 2021) in agricultural soils. This is also presumed for AHs; however, hard evidence is still lacking. Previous studies have isolated a bacterial consortium able to degrade thiabendazole (TBZ) (Perruchon et al. 2017), and two bacteria able to degrade abamectin (Ali et al. 2010; Wang et al. 2015), all derived from soils previously exposed to these compounds. Repeated exposure of soils to pesticides (e.g., carbamates, triazines, organophosphates) and veterinary antibiotics (e.g., sulfonamides) under certain soil conditions could lead to their accelerated biodegradation by a fraction of the soil microbial population which is able to utilize these compounds as an energy source (Arbeli and Fuentes 2007; Topp et al. 2013; Billet et al. 2021). In this frame, a similar exposure regime of soils to AHs could also trigger similar microbial responses, leading to accelerated biodegradation of AHs, that have not been explored yet.
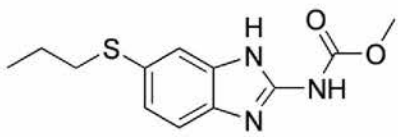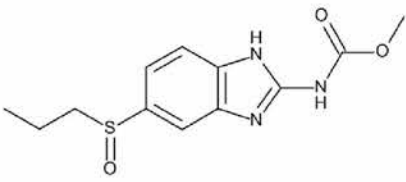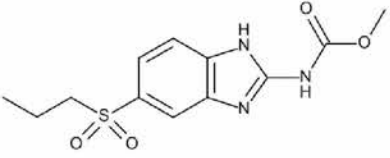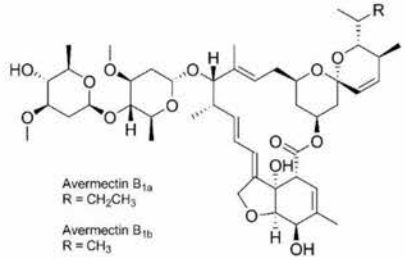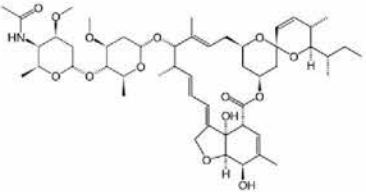
Our study aimed to provide answers to the following scientific questions: (a) What is the role of soil microorganisms in the dissipation of AHs? (b) Does repeated exposure of soils to AHs lead to their accelerated microbial degradation? (c) Which soil physicochemical properties control the dissipation of AHs? To address these questions, we employed a series of microcosm studies with three major AHs currently used in livestock farming, ABZ, IVM and EPM, in soils collected from sheep farms and their devoted grazing pastures. To further explain the dissipation patterns of AHs, we undertake complementary adsorption studies.

2. Materials and methods

2.1. Anthelmintics

Analytical standards of ABZ (98% purity) and ABZ-SO (98% purity) were purchased from Tokyo Chemical Industry (Zwijndrecht, Belgium), while $ABZ-SO_2$ (97% purity) was purchased from Santa Cruz Biotech (Heidelberg, Ger- many). Analytical standards of IVM (99% purity) and EPM (99% purity) were purchased by Sigma-Aldrich (St Gallen, Switzerland). Stock solutions of IVM and EPM in methanol (1000 mg L^{-1}) and of a mixture of ABZ, ABZ-SO and $ABZ-SO_2$ in acetonitrile (1000 mg L^{-1}) were used for analytical purposes. Analytical standards of the studied AHs were also used for the preparation of methanol solutions that were applied in the soil study. The chemical structures and main physicochemical properties of the AH compounds and their TPs studied are presented in Table 2.1.

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Table 2.1 The chemical structures and main physicochemical properties of the anthelmintic (AH) compounds and their transformation products (TPs) studied.

Anthelminthic	Chemical structure	Molecular weight (g mol⁻¹)	Water solubility (mg L⁻¹)	Log K$_{ow}$	pKa
Albendazole		265.3	41.0[a]	3.1[b]	3.37, 9.93[c]
Albendazole sulfoxide		281.3	62.0[d]	1,2[e]	3,45, 9.82[d]
Albendazole sulfone		297.3	N/A	N/A	N/A
Ivermectin		875.1	4.0[f]	3.2[g]	No pKa between pH 3-10
Eprinomectin		914.1	3.5[h]	5.4[h]	No pKa between pH 3-10

(a) PubChem [Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; 2004-. PubChem Compound Summary for CID 2082, Albendazole; [cited 2022 Mar. 21], **(b)** Tomasz et al. (2010), cTakacs-Novak (1995), **(d)** Wu et al. (2004), **(e)** Mottier et al. (2003), **(f)** Fent (2014), gHeinrich et al. (2021), **(h)** Ivomec (1996)

*N/A: no data are available

2.2. Soils

The soils used were collected in March 2018 from 12 sheep farms in the island of Lesvos, Greece. All farms had been systematically using AHs for the control of GINs following a traditional chemoprophylactic approach which involves regular dosing to all animals inside the barns regardless of their infection status. In contrast, veterinary antibiotics were rarely used and only to symptomatic animals. The data for the prior use of AHs in the studied sheep farms were collected through personal interviews with the farm owners. AHs were administered once or twice a year (first time in October and if needed a second dosing in May), orally (e.g., ABZ), injectable or subcutaneously (e.g., IVM and EPM) with the dose rates varying according to the mode of application. From each farm, samples were collected from two distinct places: (a) inside fenced farm premises adjacent to barns where animals spent most of their day (soil series A) and (b) nearby pastures where animals graze during the day (soil series B). Considering that the administration of AHs to animals is performed inside the barns, we hypothesized that samples from series A would have a higher exposure to AHs since animal excreta collected immediately or close to drug application contain higher levels of AHs (high exposure soils) (Navratilova et al. 2021). In contrast, samples from series B represent soils with potentially lower exposure to AHs occurring through their disperse deposition of urine and excreta during grazing. For a limited number of small sheep farms, samples were composite, as the farm prem- ises around the barn and relevant pastures cannot be distinguished. Details about the sampling sites and record of AHs administration are given in Supplementary Table S2.1, while the location of sampling sites is shown in Supplementary Fig. S2.1.

From each sampling site, topsoil samples (0–10 cm layer) were taken from the selected points by following a W non-systematic pattern of sampling, according to the ISO 10381–1 and ISO 10381–2 guidelines (ISO 2002). Subsamples were mixed thoroughly to provide a single bulk soil sample per site. Immediately after sampling, soils were placed in portable refrigerators at 4°C, and they were transferred to the laboratory within 12 h. All soils were partially air-dried overnight and sieved to pass a 3-mm mesh. Measurements of the levels of AH in randomly selected soil samples showed that AH levels were below the limit of detection, in line with the 6-month lapse between the last AH dosing (October 2017) and the collection of soil samples (March 2018). Subsamples from each soil were taken for the determination of their physicochemical properties (Table S2.2). We focused our soil analysis on properties that are mostly known to affect the degradation and adsorption of organic pollutants, including AHs, in soil like total organic carbon (TOC) content and soil pH (Popova et al. 2013; Rath et al. 2019). Soil TOC content was measured according to Walkley and Black (1934). Soil pH was determined by pH meter in a 1:2.5 w/v air-dried soil/deionized water ratio. Moisture content was determined by the oven drying of subsamples at 105 °C for 24 h. Water-holding capacity was measured gravimetrically following saturation of the soil (20 g) with distilled water in a funnel with filter paper and allowed to drain for 24 h.

2.3. Dissipation of anthelmintics in soils

2.3.1. Experimental set-up

Each bulk soil sample was divided into samples of 720 g (one subsample for each AH) which were further split into two 360-g portions. The first was fumigated with chloroform, while the second was retained at ambient temperature. Regarding fumigation, soil samples were placed in a polyester fine mesh net and incubated with chloroform under vacuum in a glass desiccator at 37 °C for 7 days. At the end of this period, soils were removed, aerated well to remove residues of chloroform, and incubated at 37 °C for 5 days to allow the growth of any microbial propagules that survived during the first fumigation period. This was followed by a second 7-day fumigation period. The efficiency of the

fumigation process was verified by the absence of growing bacterial and fungal colonies in Potato Dextrose Agar and Luria–Bertani agar plates inoculated with suspensions of fumigated soil samples.

Soil subsamples, both fumigated and non-fumigated, were spiked with solutions of ABZ, IVM or EPM to achieve a soil concentration of 1 mg kg^{-1}. This concentration level was selected as an average value of (a) measured concentrations of AHs in the feces of animals dosed with the studied AHs (Prchal et al. 2016; Iglesias et al. 2018; Porto et al. 2020; Halley et al. 2005) and (b) our own monitoring in the feces of animals treated with ABZ and EPM, where their levels being in the range of 0.13–10.35 and 0.27–3.40 mg kg^{-1}, respectively (Madikas 2019). After AH spiking, soil samples were left for 1 h to allow methanol evaporation, and they were then mixed by hand to ensure a homogeneous distribution of the chemicals. Finally, an appropriate amount of water was added to adjust soil moisture levels to 40% of the water holding capacity. Each sample (fumigated or non- fumigated) was further divided into 18 subsamples (20 g), which were placed in aerated plastic bags and incubated at 25 °C for 40, 60 or 90 days in case of ABZ, IVM and EPM, respectively. The moisture content of all soil samples was maintained throughout the incubation with regular additions of deionized water. Immediately after application (0 days) and at regular intervals thereafter, triplicate samples from each treatment were removed from the incubator and analyzed for AH residues.

2.3.2. Dissipation kinetics

The four models proposed by the FOCUS workgroup on pesticide degradation kinetics (FOCUS 2006) were used for calculating dissipation kinetics. The single first-order (SFO) kinetic model and the biphasic models: hockey-stick (HS), first order multi-compartment (FOMC) and double first order in parallel (DFOP) model were used. The χ^2 test as well as visual inspection and the distribution of the residuals were used as criteria to assess the agreement between calculated and observed data for a given fit. In all cases, the kinetic model selected to describe the degradation data was the one with the lower χ^2 value and the best fitted residuals to the calculated curve. Kinetic analysis was carried out in the R (R Core Team 2018) Studio version 4.1.0, utilizing the package mkin (Ranke 2018) version 0.9.47.1.

2.4.   Anthelmintics adsorption in soils

2.4.1. Soils and manure

The adsorption affinity of ABZ, IVM and EPM was determined in 5 soils with contrasting physicochemical properties. Three of the soils studied were collected from the island of Lesvos including soil 1B (soil 1) and two composite samples constituting a mixture of samples 2A, 2B, 3A (soil 2) and 4B, 6A (soil 3). The other two samples were collected from fallow agricultural fields in the region of Thessaly, Greece (Hellenic Agricultural Organization-Demeter (HAO-Demeter) farm, Larissa, 39°38'02.0" N 22°22'26.5"E as soil 4 and Livadi village, 40°08'29.7"N 22°10'13.5"E as soil 5). Each soil was divided into two portions. The first was amended with 2% (to soil dry weigh basis) sheep manure obtained from HAO-DEMETER, Greece, while the second remained as is. All soils were left to equilibrate for 24 days before used in the adsorption studies. The physicochemical properties of the soils used in the adsorption study are presented in Supplementary Table S2.3.

2.4.2. Experimental setup

The adsorption of the studied AHs was determined with the standard batch equilibrium method according to the OECD guideline 106 (OECD, 2000). Stock solutions of each AH (10000 and 5000 µg mL^{-1}) in methanol or DMSO (in case of ABZ) were prepared using analytical standards. Preliminary

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kinetic studies at a single concentration level (1 mg L^{-1}) were performed to determine the most appropriate soil:solution ratios and equilibration times for each AH. Based on these preliminary experiments (a) 1:10 soil:solution ratio and 4 h equilibration time were used for ABZ (b) 1:25 and 1:50 soil solution ratios and equilibration time of 24 h were selected for EPM and IVM respectively. For the determination of the adsorption parameters appropriate amounts of the AH stock solutions were dissolved in 0.01M CaCl$_2$ for the preparation of a series of solutions with AH concentrations of 0.5, 1, 2.5 and 5 mg L^{-1}. In all cases the amount of organic solvent in the solution phase did not exceed 0.1%. At each concentration level triplicates samples of soil (2 g d.w.) were mixed with appropriate volumes of the different solutions of AHs in glass flasks to achieve the selected soil:solution ratios. Blank samples containing only solution and no soil were also included to assess the stability of AHs and the absence of potential adsorption on the glass surfaces. All samples were shaken in an orbital shaker (200 rpm) in the dark at room temperature until equilibrium was reached. The supernatant was collected by centrifugation at 7500 rpm for 5 min and used for the determination of AH residues by HPLC.

2.4.3. Anthelmintics extraction from aqueous samples

Extraction of AHs from the aqueous phase was performed by a liquid-liquid extraction method. In case of IVM and EPM, 2 mL of the aqueous samples were mixed with 2 mL of methanol, while for ABZ 2 mL of the aqueous samples were mixed with 2 mL of acetonitrile. The mixtures were vortex vigorously for 30 s and the extract was passed through a 0.45 μm syringe filter (PTFE Syringe Filter) before analyzed by HPLC.

2.4.4. Adsorption isotherms and statistical analysis of data

The linear forms of the Freundlich equation (Eqn 1) was used to describe the adsorption of the studied anthelmintics in soil:

$$\log{}^{ads}C_s\,(eq) = \log{}^{ads}K_F + \frac{1}{n} * \log{}^{ads}C_{aq}\,(eq) \tag{1}$$

where $^{ads}C_s\,(eq)$ is the amount of the test substance adsorbed (μg g^{-1}) in equilibrium, $^{ads}C_{aq}\,(eq)$ is the adsorbate equilibrium concentration (μg g^{-1}), $^{ads}K_F$ is the Freundlich adsorption coefficient and 1/n is the Freundlich equation exponent (OECD, 2000).

3. Results

3.1. Dissipation of albendazole in soils

The dissipation patterns of ABZ in most of the soils studied, fumigated or not fumigated, were adequately described ($\chi 2 < 15\%$) by either the SFO or the HS model, although the FOMC model was also used in some cases (Table 2.2). The dissipation of ABZ in all soils and treatments was followed by the formation of ABZ-SO and ABZ-SO$_2$. The dissipation of ABZ and the formation of its TPs in soil series A and B are presented in Figs. 2.1 and 2.2, respectively. ABZ DT$_{50}$ values in the non-fumigated samples ranged from 0.1 to 4.2 days with an average of 1.9 days. Comparatively, the DT$_{50}$ of ABZ in fumigated samples were significantly higher ($p < 0.001$) and ranged from 0.22 to 13.7 days with an average of 4.33 days. In soils collected from units with a record of ABZ administration, DT$_{50}$ values ranged from 0.1 to 2.3 days, compared to soils from units with No history of ABZ administration where the DT$_{50}$ values were higher (not statistically significant, $p > 0.05$) and ranged from 1.19 to 4.2 days (Table 2.2). Similarly, the DT$_{50}$ values of ABZ in soil series A ranged from 0.1 to 2.47 days compared to 1.2 to 3.21 days in soil series B, not significantly differing ($p > 0.05$). Considering the biocidal activity of ABZ-SO

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and the large amounts of ABZ-SO and $ABZ-SO_2$ formed, we calculated DT_{50} values for the total residues of ABZ (sum of residues of ABZ, ABZ-SO and $ABZ-SO_2$) using in most cases the HS model which provided the best fit to the data (Table S4). The dissipation patters of the total residues of ABZ in soil series A and soil series B are presented in Supplementary Fig. S2.2 and S2.3, respectively. The calculated DT_{50} values for ABZ total residues in the non-fumigated samples ranged from 1.12 days to over a year and they were significantly lower ($p < 0.001$) from the corresponding DT_{50} values in the fumigated soil samples which ranged from 3.8 to over a year (Supplementary Table S2.4). In addition, the DT_{50} values of ABZ total residues in soil collected from units with a record of ABZ administration ranged from 1.8 to 4.5 days and did not significantly differ ($p > 0.05$) from the DT_{50} values observed in soils from units with no record of ABZ administration where values ranged from 1.12 to > 365 days.

Finally, the DT_{50} values of ABZ total residues in soil series A ranged from 1.8 to 365 days and were not significantly different ($p > 0.05$) from the corresponding DT_{50} values in soil series B which ranged from 1.12 to 114.8 days. Correlation analysis suggested a significant negative correlation ($r = -0.49$, $p < 0.05$) between soil TOC content and the DT_{50} values of ABZ. This correlation was significant only when the DT_{50} values of ABZ in the non-fumigated samples were considered.

Table 2.2 The kinetic parameters for the dissipation of albendazole (ABZ) as described by the FOCUS kinetic models in the studied soils.

Soil sample	Treatment	Kinetic model	DT_{50} (days)	DT_{90} (days)	χ^2 (%)
1A	Non-fumigated	SFO	1.7	5.5	6.8
	Fumigated	SFO	3.8	12.5	6.7
1B	Non-fumigated	SFO	1.9	6.3	5.3
	Fumigated	SFO	3.6	11.1	6.3
2A	Non-fumigated	FOMC	1.9	6.3	5.3
	Fumigated	SFO	3.6	11.8	13.2
2B	Non-fumigated	SFO	2.3	7.5	7.4
	Fumigated	SFO	1.9	6.3	5.3
3A	Non-fumigated	FOMC	0.1	2.1	7.9
	Fumigated	SFO	3.4	11.4	9.2
3B	Non-fumigated	SFO	1.2	4.0	1.4
	Fumigated	FOMC	0.22	7.2	12.8
4A	Non-fumigated	SFO	1.5	5.1	2.3
	Fumigated	SFO	5.2	17.2	10.5
4B	Non-fumigated	SFO	2.2	7.4	7.9
	Fumigated	FOMC	2.2	17.5	7.8
5	Non-fumigated	SFO	1.5	5.05	3.9
	Fumigated	SFO	1.5	4.91	8.9
6A	Non-fumigated	SFO	2.2	7.3	1.0
	Fumigated	SFO	2.3	7.7	9.7
6B	Non-fumigated	SFO	1.6	5.2	2.6
	Fumigated	SFO	2.9	9.7	10.1
7A	Non-fumigated	HS	1.2	3.9	3.3
	Fumigated	HS	2.2	28.0	4.1
7B	Non-fumigated	HS	1.8	13.1	2.7
	Fumigated	SFO	13.7	45.6	7.3
8A	Non-fumigated	SFO	2.2	7.1	12
	Fumigated	SFO	5.1	16.7	8.4
8B	Non-fumigated	HS	1.7	13.4	3.4
	Fumigated	HS	3.6	22.4	11.5
9A	Non-fumigated	SFO	1.4	4.7	14.3
	Fumigated	HS	4.1	37.6	6.5
9B	Non-fumigated	SFO	3.2	10.7	13

	Fumigated	SFO	12.6	41.8	8.8
10A	Non-fumigated	SFO	2.5	8.2	9.7
	Fumigated	HS	2.9	21.8	2.5
10B	Non-fumigated	HS	2.3	15.1	4.8
	Fumigated	SFO	8.1	26.7	4.3
11	Non-fumigated	SFO	2.2	7.2	8.7
	Fumigated	SFO	2.7	8.9	14.4
12	Non-fumigated	SFO	1.5	4.9	6.7
	Fumigated	SFO	1.5	4.9	6.7

Figure 2.1 The dissipation of albendazole (ABZ) and formation of its oxidized transformation products, albendazole sulfoxide (ABZSO) and albendazole sulfone (ABZSO2), in non-fumigated (non fum., blue captions and lines) and fumigated (fum., red captions and lines) samples of soils collected from inside small ruminant barn facilities(series A). Each value is the mean of three replicates with error bars representing the standard deviation of the mean. The data of the concentrations of ABZ, ABZSO, and ABZSO2 in soil are presented as % of the initial concentration of ABZ recovered in soils at 0 days.

3.2. Dissipation of ivermectin in soils

The dissipation patterns of IVM in the studied soils are shown in Fig. 2.3. The SFO model provided the best fit to IVM dissipation data in most cases, while the HS model was used to describe dissipation kinetics of IVM in a few fumigated soil samples (Table 2.3). The DT_{50} of IVM in the non-fumigated soils ranged from 15.7 to 66.1 days, with an average of 34.5 days.

Fumigation significantly ($p < 0.001$) extended its DT_{50} with values ranging from 42.0 to 245.7 days and an average of 108.7 days. Regarding the history of administration, the DT_{50} values of IVM ranged from 22.1 to 66.2 days in soils collected from units with a recent record of IVM administration, which did not significantly differ ($p > 0.05$) with its DT_{50} values in soils collected from units without a

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history of IVM administration (15.7–52.9 days). Finally, we did not observe significant differences ($p > 0.05$) in the DT_{50} values of IVM in the samples from series A (24.3 to 66.2 days) and series B (19.1 to 53.8 days). Correlation tests showed a significant positive correlation ($r = 0.53$, $p < 0.05$) between the DT_{50} values of IVM and soil TOC content, which was valid only when non-fumigated soil samples were considered. In addition, we identified a significant positive correlation ($r = 0.60$, $p < 0.01$) between the DT_{50} of IVM in the fumigated soil samples and soil pH.
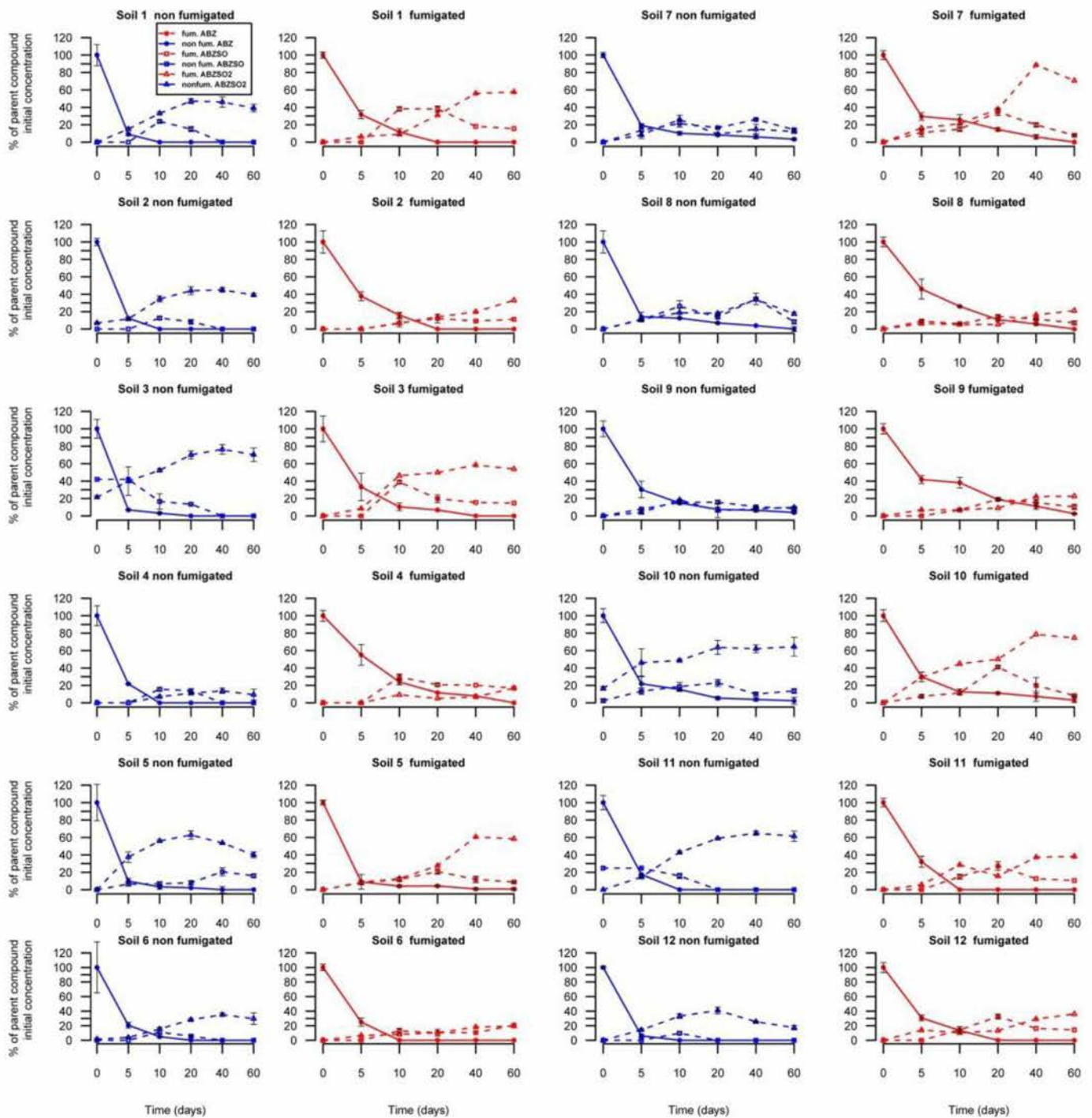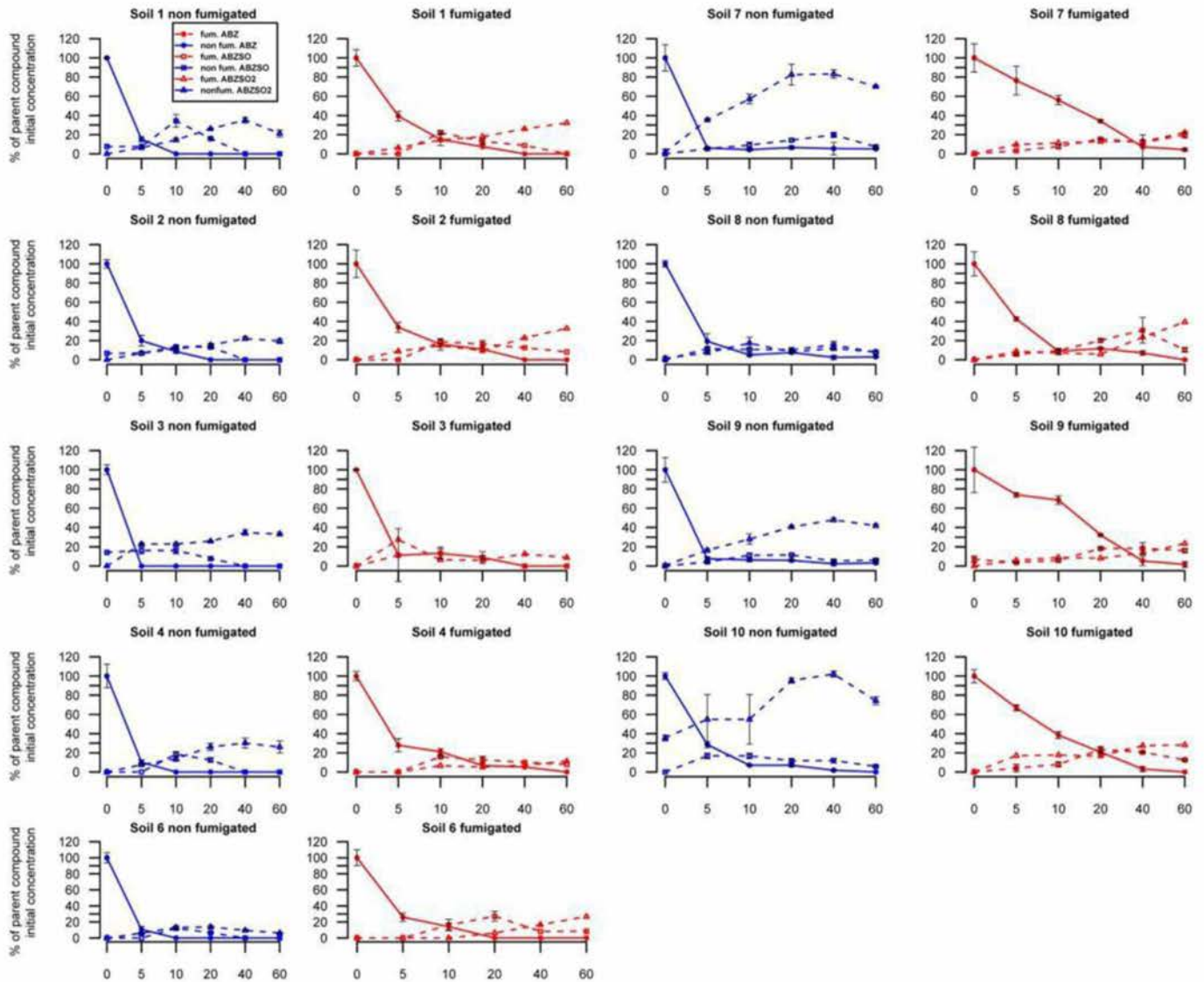

Figure 2.2 The dissipation of albendazole (ABZ) and formation of its oxi- dized transformation products, albendazole sulfoxide (ABZSO) and albendazole sulfone (ABZSO2), in non-fumigated (non fum., blue captions and lines) and fumigated (fum., red captions and lines) samples of soils collected from animal grazing pastures (series B). Each value is the mean of three replicates with error bars representing the standard deviation of the mean. The data of the concentrations of ABZ, ABZSO, and ABZSO2 in soil are presented as % of the initial concentration of ABZ recovered in soils at 0 days.

3.3. Dissipation of eprinomectin in soils

The dissipation of EPM in the studied soils is presented in Fig. 2.4. The SFO and HS models described adequately the dissipation of EPM in the studied soils with the sole exception of non-fumigated soil 1A, where the FOMC model was used (Table 2.4). The DT_{50} values of EPM in non-fumigated soils ranged from 20.8 to 57.9 days with an average of 30 days, while fumigation extended significantly ($p < 0.001$) the DT_{50} of EPM which ranged from 59.2 to over 365 days, with an average of 121 days.

EPM dissipation did not significantly differ ($p > 0.05$) between samples collected from units with a history of administration of EPM, with DT_{50} values ranging from 20.8 to 41.9 days, compared to the

corresponding samples from units without a history of EPM use where DT_{50} values ranged from 20.9 to 57.9 days. Finally, EPM dissipation in soil series A, with DT_{50} values ranging from 21.2 to 57.9 days, did not significantly differ ($p > 0.05$) with the corresponding DT_{50} values in soil series B which ranged from 20.8 to 41.8 days. Correlation testing identified a significant ($r = 0.58$, $p < 0.05$) positive correlation between the DT_{50} values of EPM in the fumigated soil samples and soil pH.

Figure 2.3 The dissipation of ivermectin (IVM) in fumigated (fum.) and non-fumigated (non fum.) soil sample collected from inside small ruminant barn facilities (series A) and from their associated animal grazing pastures (series B) Each value is the mean of three replicates with error bars representing the standard deviation of the mean.

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Table 2.3 The kinetic parameters for the dissipation of ivermectin (IVM) as described by the FOCUS kinetic models in the studied soils.

Soil sample	Treatment	Kinetic model	DT$_{50}$ (days)	DT$_{90}$ (days)	x^2 (%)
1A	Non-fumigated	HS	52.9	240.7	5.8
	Fumigated	HS	181.5	>365	3.7
1B	Non-fumigated	SFO	33.3	110.8	5.3
	Fumigated	SFO	77.9	259.0	2.9
2A	Non-fumigated	HS	32.9	140.0	4.9
	Fumigated	SFO	122.1	>365	2.7
2B	Non-fumigated	SFO	38.6	128.1	8.1
	Fumigated	SFO	112.7	>365	4.7
3A	Non-fumigated	HS	53.8	210.5	4.1
	Fumigated	SFO	107.6	357.5	2.3
3B	Non-fumigated	SFO	26.1	86.6	2.4
	Fumigated	SFO	42.0	139.5	2.7
4A	Non-fumigated	SFO	43.2	143.4	2.7
	Fumigated	HS	71.3	273.5	1.7
4B	Non-fumigated	SFO	34.7	115.4	3.5
	Fumigated	HS	63.2	216.8	4.4
5	Non-fumigated	SFO	21.6	71.6	5.1
	Fumigated	SFO	56.3	187.1	4.9
6A	Non-fumigated	SFO	19.0	63.1	6.9
	Fumigated	HS	79.2	224.9	1.9
6B	Non-fumigated	SFO	24.3	80.7	4.6
	Fumigated	HS	76.8	183.6	2.0
7B	Non-fumigated	SFO	26.6	88.4	4.3
	Fumigated	HS	67.3	194.6	3.9
8A	Non-fumigated	SFO	22.8	75.6	5.1
	Fumigated	HS	72.6	213.8	3.5
8B	Non-fumigated	SFO	28.7	95.2	7.5
	Fumigated	HS	65.8	218.4	4.8
9A	Non-fumigated	SFO	45.0	149.5	6.7
	Fumigated	SFO	146.7	>365	3.2
9B	Non-fumigated	SFO	36.1	119.9	1.7
	Fumigated	SFO	123.4	>365	1.5
10B	Non-fumigated	SFO	66.2	219.7	3.4
	Fumigated	SFO	245.7	>365	1.3
11	Non-fumigated	SFO	44.4	147.5	3.1
	Fumigated	SFO	175.0	>365	1.4
12	Non-fumigated	SFO	28.5	94.8	5.8
	Fumigated	SFO	127.8	>365	3.9

Table 2.4 The kinetic parameters for the dissipation of eprinomectin (EPM) as described by the FOCUS kinetic models in the studied soils.

Soil sample	Treatment	Kinetic model	DT$_{50}$ (days)	DT$_{90}$ (days)	x^2 (%)
1A	Non-fumigated	FOMC	38.5	107.9	3.0
	Fumigated	HS	259.7	>365	5.6
1B	Non-fumigated	SFO	20.9	91.8	1.4
	Fumigated	SFO	157.4	>365	2.4
2A	Non-fumigated	SFO	40.3	134.0	2.5

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	Fumigated	HS	221.9	>365	4.1
2B	Non-fumigated	SFO	41.9	139.1	4.6
	Fumigated	SFO	59.2	196.6	2.5
3B	Non-fumigated	HS	20.9	93.1	1.1
	Fumigated	HS	80.0	288.5	4.1
4A	Non-fumigated	HS	44.9	231.5	2.9
	Fumigated	HS	203.9	>365	5.1
4B	Non-fumigated	HS	34.5	117.9	2.3
	Fumigated	HS	197.3	>365	2.9
5	Non-fumigated	SFO	22.7	75.4	3.5
	Fumigated	SFO	116.7	>365	2.7
6A	Non-fumigated	SFO	21.2	70.4	9.4
	Fumigated	SFO	240.4	>365	2.3
6B	Non-fumigated	HS	30.0	89.2	7.2
	Fumigated	HS	235.7	>365	1.3
7B	Non-fumigated	SFO	31.9	106.1	10.7
	Fumigated	SFO	151.0	>365	1.0
8B	Non-fumigated	SFO	29.8	98.9	5.6
	Fumigated	HS	128.1	>365	2.0
9A	Non-fumigated	SFO	32.5	108.0	2.1
	Fumigated	HS	170.0	>365	0.47
9B	Non-fumigated	SFO	41.2	136.8	5.3
	Fumigated	HS	374.9	>365	2.9
11	Non-fumigated	HS	57.9	355.9	2.9
	Fumigated	SFO	126.8	>365	8.3

3.4.   Adsorption of anthelmintics in soils

Adsorption isotherms for all three AHs in manured and non manured soils are shown in Fig. 2.5. The adsorption of AHs was adequately described by the Freundlich equation which was used for the calculation of adsorption coefficients (Table 2.5). All AHs showed moderate to high adsorption in both manured and non manured soils. In particular, ABZ K_f values were not significantly affected by manuring and ranged from 43.63 to 153.5 mL g^{-1} in the manured soils and from 43.02 to 131.7 mL g^{-1} in the non manured soils. Regarding EPM and IVM, the latter showed higher adsorption affinity compared to EMP. Similar to ABZ, we did not observe any increase in the adsorption affinity of the two MLs in the manured soils. Specifically, IVM K_f values ranged from 46.1 to 384.6 mL g^{-1} in the manured soils and from 34.8 to 288.9 mL g^{-1} in the non manured soils, whereas for EPM, its K_f values ranged from 16.03 to 113.6 mL g^{-1} in the manured soils and from 12.19 to 78.91 mL g^{-1} in the non manured soils. Correlations tests showed a strong positive correlation between soil TOC and the K_f values of IVM (r = 0.914, p < 0.001) and EPM (r = 0.962, p < 0.001) in the studied soils, regardless of manuring.
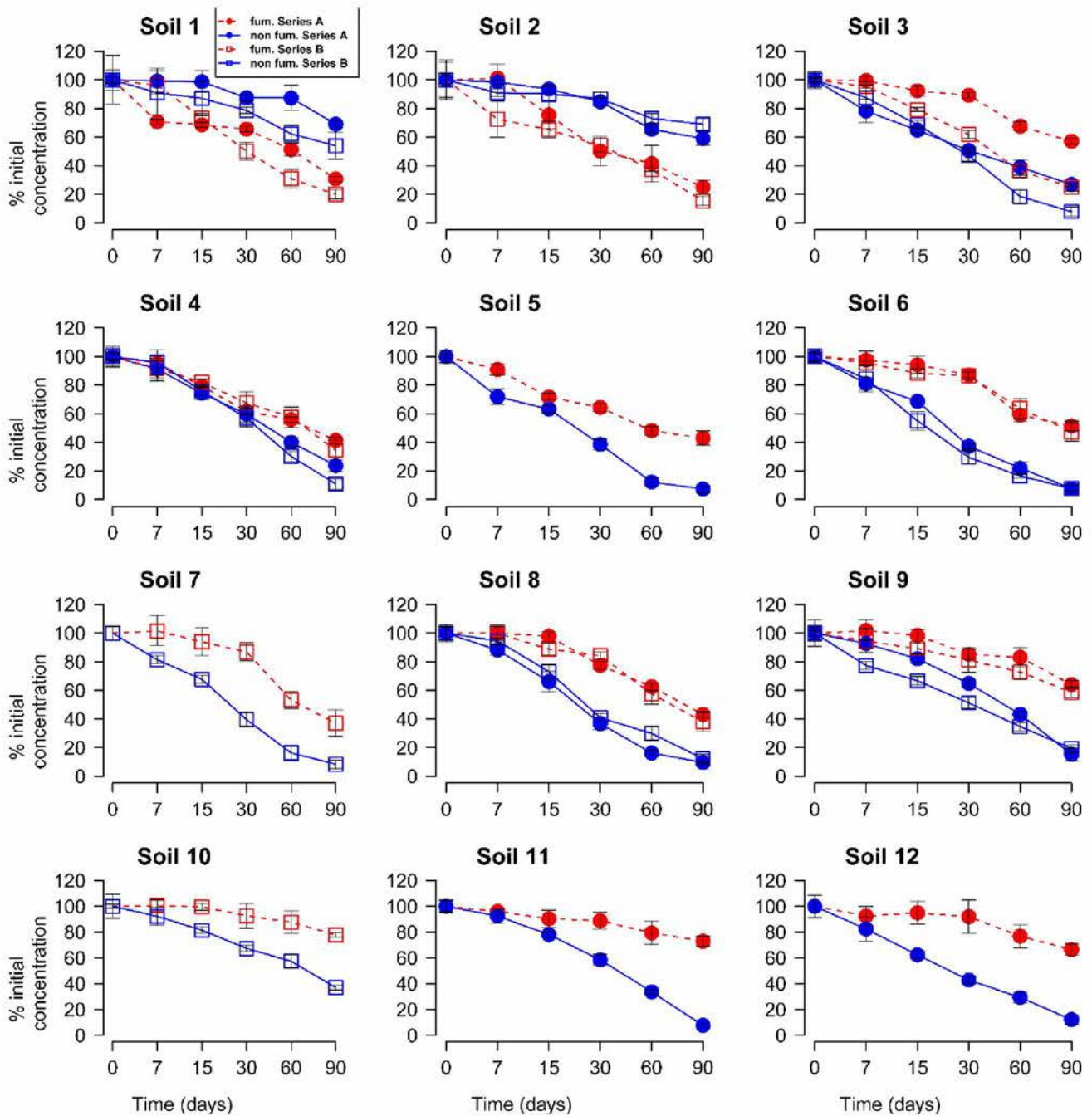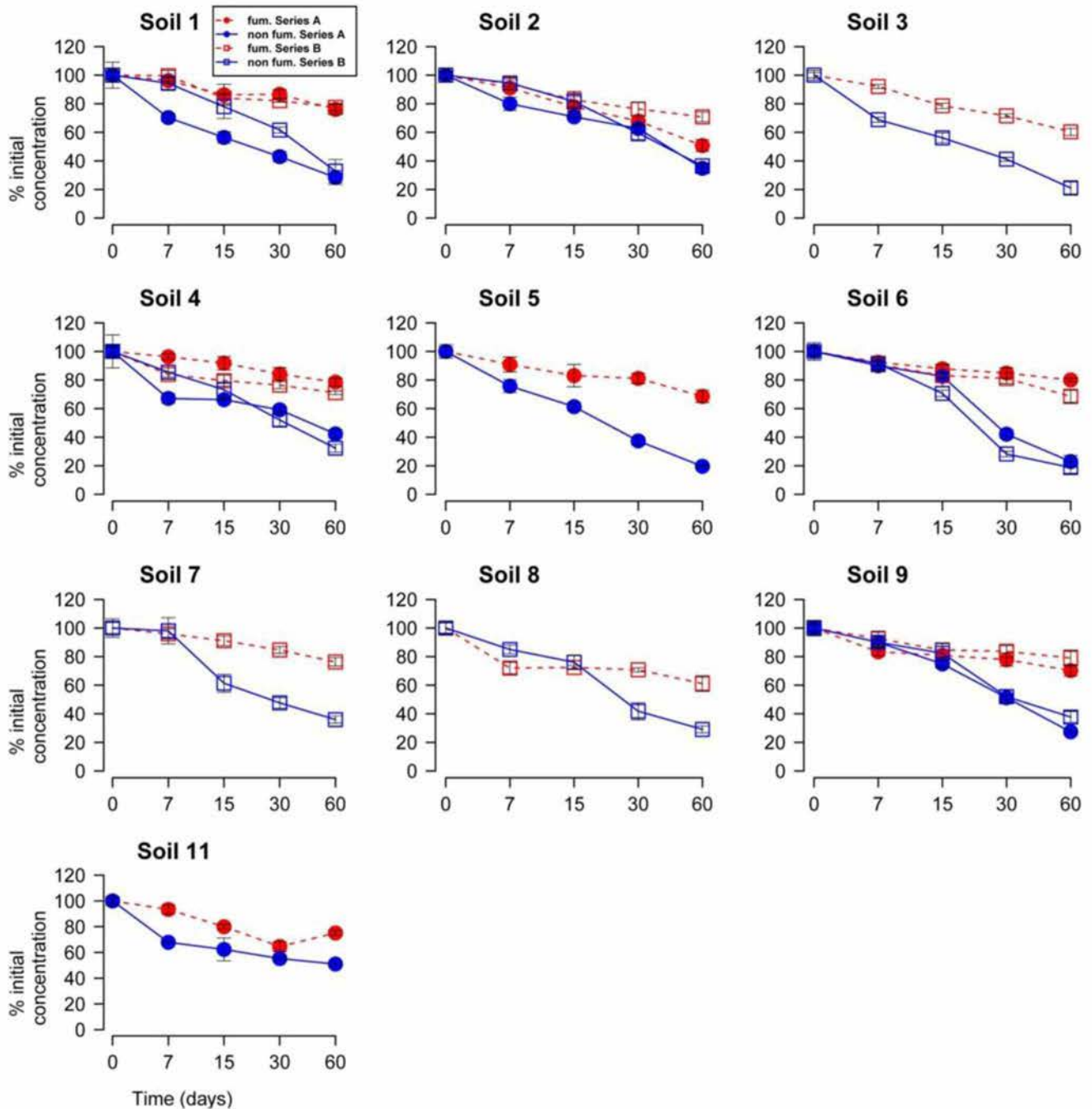
Figure 2.4 The dissipation of eprinomectin (EPM) in fumigated (fum.) and non-fumigated (non fum.) soil samples collected from inside small ruminant barn facilities (series A) and from their associated animal grazing pastures (series B). Each value is the mean of three replicates with error bars representing the standard deviation of the mean.

Table 2.5 Sorption parameters Kf (mL g−1), Kfoc (mL g−1), and n for the anthelmintic albendazole (ABZ), ivermectin (IVM), and eprinomectin (EPM) in six selected soils with variable physicochemical characteristics

Anthelmintics	Soil 1			Soil 2			Soil 3			Soil 4			Soil 5		
	K_f	K_{foc}	n	K_f	K_{foc}	n	K_f	K_{foc}	n	K_f	K_{foc}	n	K_f	K_{foc}	n
Manured															
ABZ	90.59	2236.8	0.82	90.60	1065.8	0.82	43.63	1056.4	0.98	146.5	2861.0	0.59	153.5	10,967	0.59
IVM	97.44	2405.9	0.83	384.6	4524.7	0.64	88.74	2148.7	0.91	90.6	1769.0	0.82	46.1	3293	1.11
EPM	44.74	1104.7	0.83	113.6	1336.5	0.78	39.68	960.8	0.95	43.5	850.0	0.95	16.03	1145	0.99
Non manured															
ABZ	69.6	2023.3	0.72	43.02	612.8	1.20	66.46	1704.1	0.87	131.5	3684.0	0.52	131.7	11,654.0	0.56
IVM	95.9	2786.3	0.75	288.9	4115.4	0.64	77.56	1988.7	0.89	69.3	1941.0	0.84	34.8	3080.0	1.08
EPM	40.5	1177.9	0.82	78.9	1124.1	0.82	31.53	808.5	1.02	39.7	1112.0	0.91	12.2	1079.0	1.10

Figure 2.5 Adsorption isotherms of albendazole (A), ivermectin (B), and eprinomectin (C) in non-manured (black circle, "●") and manured (white diamond, "◊") soil. Data were fitted to the Freundlich equation. Each point is the mean of three replicates ± the standard deviation.

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4. Discussion

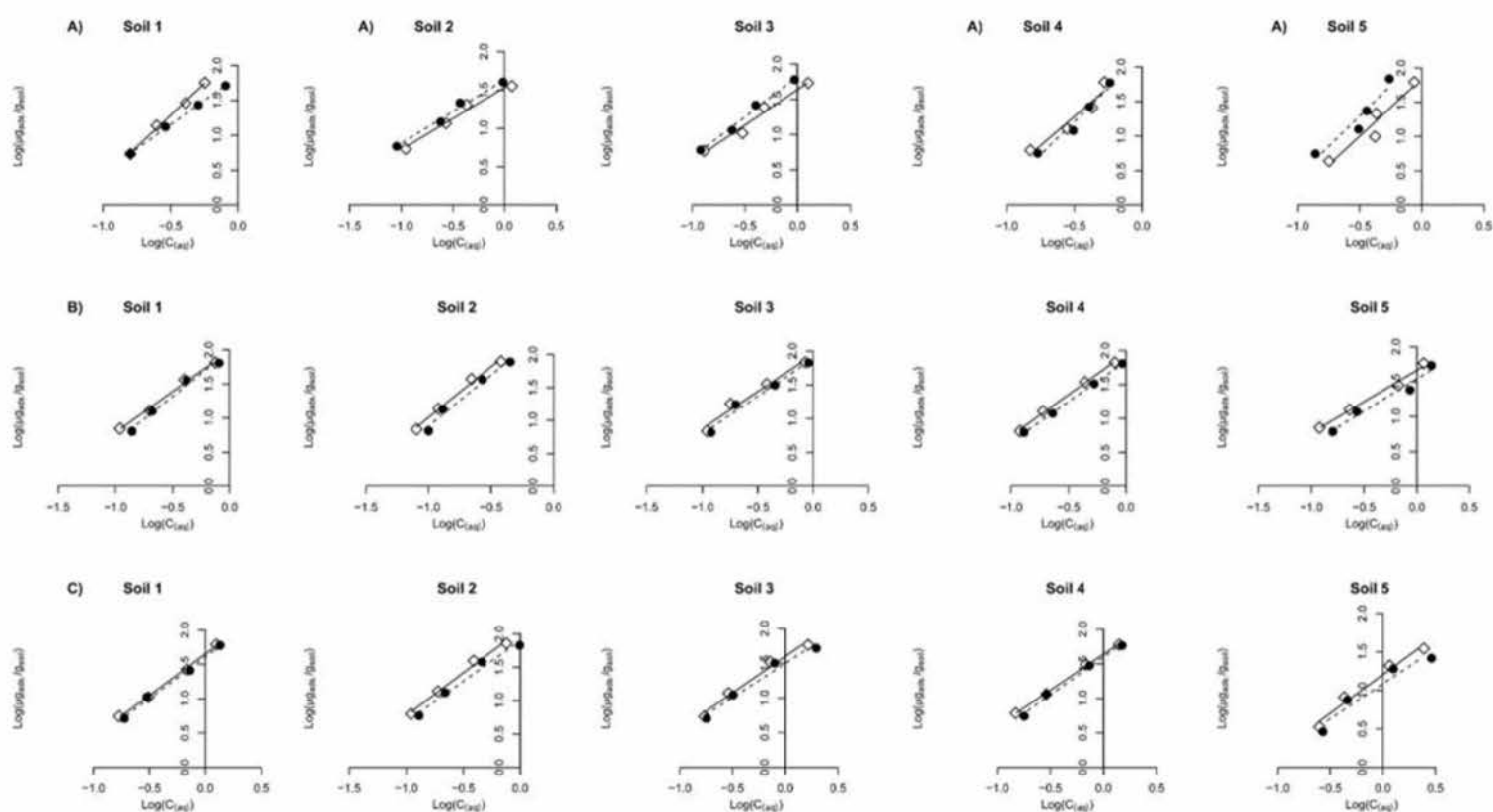Soil constitutes a major environmental sink for AHs, which unintentionally reach soil through direct or indirect routes (Navratilova et al. 2021). Once in the soil, they are expected to interact with biotic and abiotic factors that determine their environmental fate. Still, we know little about the role of these factors and especially of the role of soil microorganisms on the dissipation of AHs. We tested different hypotheses about the role of soil microorganisms (biotic) and soil physicochemical properties (abiotic) and their possible interactions on the dissipation of three of the most widely used AH compounds. We first posed the question "What is the role of microorganisms in the dissipation of AHs in soil?". Comparative studies in fumigated and non-fumigated samples of a range of soils suggested a significant contribution of soil microorganisms in the dissipation of all three tested AHs based on their significantly higher DT_{50} values upon fumigation. ABZ was not persistent with an average DT_{50} value of *ca.* 2 days, which was over-doubled upon fumigation (4.3 days). Still, the rapid dissipation of ABZ even in the fumigated samples might be the result of a multitude of abiotic process being concurrently operative like (i) rapid irreversible adsorption to soil, an assumption supported by Pavlovic et al. (2018) who reported a rapid adsorption of ABZ onto soil colloids (with 30 min), although the levels of bound residues formed were not reported; (ii) oxidation to ABZ-SO and $ABZ-SO_2$ (partially abiotic), being evident in our study; and (iii) transformation to other TPs, beyond its oxidation derivatives, like carbendazim (possessing fungicidal activity) and amino-ABZ (Liou and Chen 2018) which were not determined in our study. In all soils, ABZ was quickly transformed to ABZ-SO and $ABZ-SO_2$ which constituted the major residual component at the end of the incubation. In the only available study looking at the dissipation and transformation of ABZ in soil, Wu and Hu (2013) observed a rapid transformation of ABZ to its two oxidation derivatives and reported DT50 values which were in the range reported in our study (4.95 to 6.3 days). The same transformation pathway of ABZ was also observed in feces (Lagos et al. 2021) and in liquid cultures of fungi belonging to *Aspergillus, Cunninghamella* (Prasad et al. 2008) and *Rhizomucor pusillus* (Prasad et al. 2011). In addition, a *Paenibacillus* bacterial strain isolated from fish was able to rapidly oxidize ABZ to ABZ-SO which was identified as the main transformation product (Jin et al. 2014). These results reinforce the role of the soil microbiota in the transformation of ABZ, mainly through oxidation to ABZ-SO and $ABZ-SO_2$, although other transformation pathways might be operative but not explored in our study. For example, Prasad et al. (2008) detected N-methyl ABZSO formed by the degradation of ABZ by *Cunninghamella* sp.

Considering that ABZ-SO possess anthelmintic activity (Belew et al. 2021) and both ABZ-SO and ABZ-SO_2 constituted the main transformation products of ABZ in our soils but also in animal tissues (Capece et al. 2009) and microbial cultures (Prasad et al. 2008, 2011), we calculated DT_{50} for the sum of the parent and its two oxidation products. Still, the dissipation of the total ABZ residues was significantly retarded by fumigation suggesting that soil microorganisms have an active role in the degradation not only of the parent compound but also of its TPs. It should be stressed that in several of the soils studied, we noted high levels of $ABZ-SO_2$ at the end of the study. $ABZ-SO_2$ is more water-soluble and mobile in the soil profile entailing a possible risk for groundwater through leaching (Porto et al. 2020) and for surface water through surface runoff prevailing in the mountainous regions with slopes above 5% where most livestock units in the studied area are located.

IVM and EPM showed moderate persistence with average DT_{50} values of 34.5 and 30 days, respectively, which are within the range reported in the literature for (i) IVM with DT_{50} values of 16–67 (Krogh et al. 2009) and 32–54 days (de Oliveira Fereira et al., 2019) and (ii) EPM with DT_{50} values of 30–53 days (Litskas et al. 2013). As with ABZ, fumigation extended the DT_{50} values of IVM and EPM by three and four times, respectively, providing the first hard evidence for the major role of the soil microbiota on the dissipation of these ML compounds in agricultural soils. In our study, we did not

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explore the formation of potential TPs of IVM and EPM. Previous soil studies reported the formation of a monosaccharide and an aglycone as transformation products of IVM in soil, although both molecules were considered as minor metabolites (< 10% of the parent) (Krogh et al. 2009), while little is known about the transformation of EPM in soil. We further asked the question whether the repeated exposure of soils to AHs could lead to their accelerated microbial degradation. To address this, we selected soils with different levels of potential prior exposure to the studied AHs that might have triggered the evolution of novel microbial catabolic capacities leading to growth-linked microbial degradation of AHs and accelerated dissipation. Soils with different exposure regimes were defined at two levels: (a) between livestock farms based on their record of the administration of each of the studied AHs and (b) within each livestock farm, whereby soil collection sites adjacent to barns (series A) and associated pastures (series B) were considered as more or less exposed, respectively. ABZ showed lower persistence in the soils with higher presumed exposure to AHs compared to the not previously exposed soils, although the differences observed were not statistically significant. This lack of statistical significance does not rule out the possibility that a soil microbial acclimation towards enhanced biodegradation of ABZ might be at its onset and longer exposure regimes are required for its establishment. IVM and EPM dissipation did not seem to differ between exposed and non-exposed soils. In contrast to AHs tested here and veterinary antibiotics explored in previous studies (Topp et al. 2013), accelerated biodegradation of pesticides in soil is a much more frequent phenomenon that could be triggered even after one or two successive applications (Martin et al. 1990; Houot et al. 2000; Papadopoulou et al. 2016). In contrast to pesticides which are intentionally applied in agricultural soils at high concentration levels (often 2–10 mg kg^{-1}), veterinary drugs are unintentionally released in soils at much lower concentration levels (0.010–2 mg kg^{-1}) (Iglesias et al. 2018; Mesa et al. 2020; Porto et al. 2020). This lower but still regular exposure might require longer times to trigger a microbial catabolic adaptation towards AHs compared to the higher exposure levels of pesticides. Previous studies with biodegradable pesticides like carbofuran have suggested that application of lower dose rates required longer time for enhanced biodegradation to establish in agricultural soils (Karpouzas et al., 2001), although the compounds tested in the current study belong to chemical groups which are not considered highly biodegradable, we do not believe that this constitutes a major reason for the limited acceleration in their degradation. Earlier studies have managed to isolate bacteria able to rapidly degrade BZs and MLs used in agriculture, like TBZ (Perruchon et al. 2017) and abamectin (Ali et al. 2010; Wang et al. 2015), from agricultural soils which had been repeatedly treated with these compounds.

We finally tried to identify soil physicochemical properties that might control the dissipation of AHs. The study of AHs dissipation in a range of soils varying in key physicochemical properties, like pH and TOC, known to affect the dissipation of organic pollutants (Pantelelis et al. 2006; Kah et al. 2007), allowed us to establish solid correlations between chemicals' persistence and soil characteristics. Still, we should note that other soil properties, like texture and cation exchange capacity, not determined in our soils, might also influence to a lower extent the dissipation (and adsorption affinity) of the studied AHs. Soil TOC constituted the most important factor correlating, in contrasting ways, with the persistence of the studied compounds. ABZ DT$_{50}$ values in non-fumigated soils were negatively correlated with TOC, while IVM DT$_{50}$ values were positively correlated with TOC. This contrasting correlation of AHs persistence with soil TOC could be attributed to the different lipophilicity of the studied compounds which might alter the role of soil organic matter in the dissipation of AHs. It is anticipated that for the more water-soluble and less strongly adsorbed in soil ABZ (see adsorption study below), the high microbial biomass associated with soils with high TOC support a faster biodegradation of ABZ and lower DT$_{50}$ values. In contrast, for more lipophilic and strongly adsorbed compounds like IVM, the high soil TOC content provides more adsorption sites for IVM, hence favoring its stronger adsorption and lower bioavailability eventually retarding its degradation in soil. Kravariti

et al. (2010) showed that TOC exerted similar contrasting effect on the degradation of the pesticides chlorpyrifos (less water-soluble) and terbuthylazine (more water-soluble) that differ in their lipophilicity.

Beyond TOC, we noted a clear positive correlation between soil pH and the DT_{50} values of the two MLs. This correlation was valid only in the fumigated samples suggesting its entire abiotic nature. Data regarding the hydrolytic stability of avermectins are scarce. IVM is stable at the pH range 6–7 but becomes hydrolytically unstable at acidic pH 5 and alkaline pH 8 conditions (Fink 1988), whereas nothing is known regarding EPM. For both compounds, their hydrolytic stability in environmental matrices is unknown, while their pKa values suggest that they are neutral at the pH conditions of the soils tested (Table 2.1). Hence, our study is the first to suggest such a purely abiotic significant positive correlation between the persistence of IVM and EPM and soil pH based on measurements obtained from 19 and 15, soils respectively.

Finally, we determined the adsorption affinity of the studied AHs in soils with variable properties that had been either amended or not amended with manure. The selection of soils studied did not intend to directly compare the contribution of degradation and adsorption in the dissipation of AH in selected soils, but to identify soil physicochemical parameters that control AH soil adsorption. Regardless of the addition of manure the adsorption affinity of the studied AHs increased in the order IVM > ABZ > EPM with the mean K_f values (127.4 mL g^{-1}, 96.7 mL g^{-1} and 46.0 mL g^{-1}, respectively) being in the same range with K_f values reported in the literature for IVM (77.7–120 mL g^{-1} (Rath et al. 2016); 34–184 mL g^{-1} (Rath et al. 2019)), ABZ (12.5–30.4 mL g^{-1} (Pavlovic et al. 2018)) and EPM (31–79 mL g^{-1} (Rath et al. 2019); 11.9–39.8 mL g^{-1} (Litskas et al. 2011)). ABZ adsorption did not correlate with the soil TOC content suggesting the involvement of other adsorption mechanisms beyond hydrophobic binding interactions. Furthermore, we did not observe any correlation between pH and ABZ adsorption coefficients which is not surprising considering that in the range of the pH of the studied soils ABZ is expected to occur in its neutral form. In contrast to our study, Pavlovic et al. (2018) observed a significant positive correlation of ABZ with the soil organic matter content and a negative correlation with pH, although their study also included river sediments which exhibit different adsorption behavior compared to soils. In contrast to ABZ, the soil TOC was a major determinant of the adsorption of IVM and EPM in the studied soils. Previous studies have verified the significant role of soil TOC in the adsorption of IVM and EPM (Popova et al. 2013; Rath et al. 2016 and 2019; Litskas et al. 2016), although the contribution of inorganic adsorption sites was also suggested (Krogh et al. 2008; Litskas et al. 2011). The adsorption data for ABZ and IVM are in accord with the correlations observed between persistence and soil TOC. Hence, the limited role of soil TOC in the adsorption of ABZ in the studied soils is conducive with the positive effect of TOC on the dissipation of ABZ. Conversely, the major role of TOC in the adsorption of IVM largely suggests a stronger adsorption of IVM in soils with higher TOC, lower availability, and eventually slower dissipation in line with the negative correlation of TOC with the dissipation of IVM.

5.   Conclusions

Soil constitutes a major sink for synthetic AHs; still, we lack a good understanding of their interactions with the biotic and abiotic soil properties. We showed that soil microorganisms constitute key contributors in the dissipation of ABZ, IVM and EPM. Besides biotic interactions, physicochemical soil parameters like TOC and pH also affect the dissipation of AHs, although their contribution varies according to the compound studied. Despite their primary role in the dissipation of AHs, soil microorganisms did not seem to evolve specialized catabolic capacities for accelerated degradation of AHs under the exposure regimes of our study. We speculate that longer exposure periods might be required for the establishment of growth-linked biodegradation mechanisms for AHs, unintentionally

deposited in soil. Our data provide a comprehensive analysis of the factors determining the soil dissipation of three of the most popular AHs in the global market and set the basis for the further exploration of the role of soil microorganisms in the dissipation of AHs.

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Annex I – Chapter 2 Supplementary Data

Supplementary Table S2.1 The coordinates and record of administration of anthelmintics (AHs) in the livestock farms from where soil samples were collected.

Soil	Map coordinates	Soil sample	Collection point	Record of AHs administration
1	39°16'21.4"N 26°15'55.7"E	1A	Inside barn facilities	Eprinomectin/Albendazole
		1B	Grazing pasture	Eprinomectin/Albendazole
2	39°19'30.7"N 26°21'13.2"E	2A	Inside barn facilities	Eprinomectin/Albendazole
		2B	Grazing pasture	Eprinomectin/Albendazole
3	39°20'42.0"N 26°19'07.4"E	3A	Inside barn facilities	Ivermectin/ Albendazole
		3B	Grazing pasture	Ivermectin/ Albendazole
4	39°20'57.3"N 26°19'45.1"E	4A	Inside barn facilities	Albendazole
		4B	Grazing pasture	Albendazole
5	39°20'57.3"N 26°19'45.1"E	5	Inside barn facilities	Moxidectin
6	39°16'37.8"N 26°12'45.8"E	6A	Inside barn facilities	Albendazole
		6B	Grazing pasture	Albendazole
7	39°10'35.5"N 26°08'11.8"E	7A	Inside barn facilities	Fenbendazole
		7B	Grazing pasture	Fenbendazole
8	39°10'32.2"N 26°08'13.0"E	8A	Inside barn facilities	Ivermectin
		8B	Grazing pasture	Ivermectin
9	39°10'45.0"N 26°08'22.1"E	9A	Inside barn facilities	Ivermectin
		9B	Grazing pasture	Ivermectin
10	39°11'00.7"N 26°07'28.9"E	10A	Inside barn facilities	Ivermectin
		10B	Grazing pasture	Ivermectin
11	39°10'18.9"N 26°08'53.3"E	11	Inside barn facilities & grazing pasture (composite sample)	Ivermectin/ Albendazole
12	39°09'40.5"N 26°08'23.7"E	12	Inside barn facilities & grazing pasture (composite sample)	Albendazole

Supplementary Table S2.2 The physicochemical properties of the studied soils

Soil sample	Total N (%)	Total organic carbon (%)	pH	EC[a] (mmhos cm^{-1})
1A	0.28	6.0	7.84	2.0
1B	0.19	3.5	6.56	526.0
2A	0.54	20.2	6.55	3.7
2B	0.19	8.6	5.85	185.0
3A	2.25	29.7	6.75	1783.0
3B	0.42	17.8	6.30	1010.0
4A	0.04	6.0	6.29	768.0
4B	0.18	6.5	5.79	322.0
5	0.33	5.0	6.17	102.5
6A	0.32	6.3	6.77	551.0
6B	0.19	3.7	6.13	288.0
7A	1.37	16.2	7.38	1216.0
7B	0.31	8.5	5.85	3.8
8A	0.18	11.4	6.00	86.1
8B	0.25	8.9	5.90	848.0
9A	1.13	12.5	7.01	4.8
9B	0.26	3.1	5.80	281.0
10A	2.03	24.9	7.62	885.0
10B	0.6	20.4	6.90	933.0
11	1.07	11.5	7.02	2.1
12	1.42	14.7	7.93	2.9

Supplementary Table S2.3 The pH and total organic carbon content of the soils used in the adsorption study.

Soil No.	pH	Total organic carbon (%)
Manured soils		
Soil 1	7.38	4.1
Soil 2	7.00	8.5
Soil 3	6.98	4.1
Soil 4	6.21	5.1
Soil 5	7.98	1.4
Non manured soils		
Soil 1	6.87	3.4
Soil 2	6.55	7.0
Soil 3	6.22	3.9
Soil 4	4.97	3.6
Soil 5	7.76	1.1

Supplementary Table S2.4. The kinetic parameters describing the dissipation of the total residues of albendazole (sum of albendazole, albendazole sulfoxide and albendazole sulfone). The dissipation data were fitted to models suggested by the FOCUS kinetic models group including the single first order (SFO), the Hockey Stick (HS) and the first order multicompartment (FOMC).

| Soil sample	Treatment	Kinetic Model	DT_{50} (days)	DT_{90} (days)	x^2 (%)
1A	Non-Fumigated	HS	4.5	337.9	11.2
	Fumigated	FOMC	9.4	>365	2.9
1B	Non-Fumigated	HS	4.3	105.4	3.1
	Fumigated	HS	>365	>365	6.2
2A	Non-Fumigated	HS	1.8	>365	15.7
	Fumigated	FOMC	8.6	>365	6.7
2B	Non-Fumigated	HS	3.9	92.3	2.2
	Fumigated	HS	7.2	362.4	5.4
3A	Non-Fumigated	HS	4.5	>365	3.0
	Fumigated	HS	140.3	>365	10.9
3B	Non-Fumigated	HS	2.4	224.3	5.5
	Fumigated	HS	8.8	61.6	14.9
4A	Non-Fumigated	HS	3.6	199.5	5.1
	Fumigated	HS	13.7	>365	5.1
4B	Non-Fumigated	HS	2.1	60.8	6.5
	Fumigated	HS	3.8	78.3	9.1
5	Non-Fumigated	HS	>365	>365	10.5
	Fumigated	SFO	1.48	4.91	8.9
6A	Non-Fumigated	HS	4.4	>365	2.8
	Fumigated	HS	4.9	>365	14.1
6B	Non-Fumigated	HS	1.8	41.5	10.6
	Fumigated	HS	4.3	>365	6.9
7A	Non-Fumigated	HS	4.15	202.7	12.5
	Fumigated	SFO	35.48	117.9	10.2
7B	Non-Fumigated	SFO	1.27	4.24	14.4
	Fumigated	HS	34.13	122.8	12.4
8A	Non-Fumigated	HS	2.19	119.1	7.1
	Fumigated	HS	6.83	397.9	2.6
8B	Non-Fumigated	HS	4.16	>365	13.7
	Fumigated	HS	4.06	>365	13.4
9A	Non-Fumigated	HS	4.06	>365	15.1
	Fumigated	HS	6.32	266	5.1
9B	Non-Fumigated	HS	1.12	148.5	9.6
	Fumigated	SFO	30.81	102.3	8.6
10A	Non-Fumigated	SFO	223.9	>365	14.8
	Fumigated	SFO	>365	>365	14.8
10B	Non-Fumigated	SFO	114.8	>365	12.5
	Fumigated	SFO	33.7	112.0	6.8
11	Non-Fumigated	HS	2.3	>365	3.8
	Fumigated	HS	8.0	>365	3.7
12	Non-Fumigated	HS	2.2	137.4	12.2
	Fumigated	HS	7.9	>365	5.8

Supplementary Figure S2.1 Map of the island of Lesvos where the collection points of soil samples are indicated.
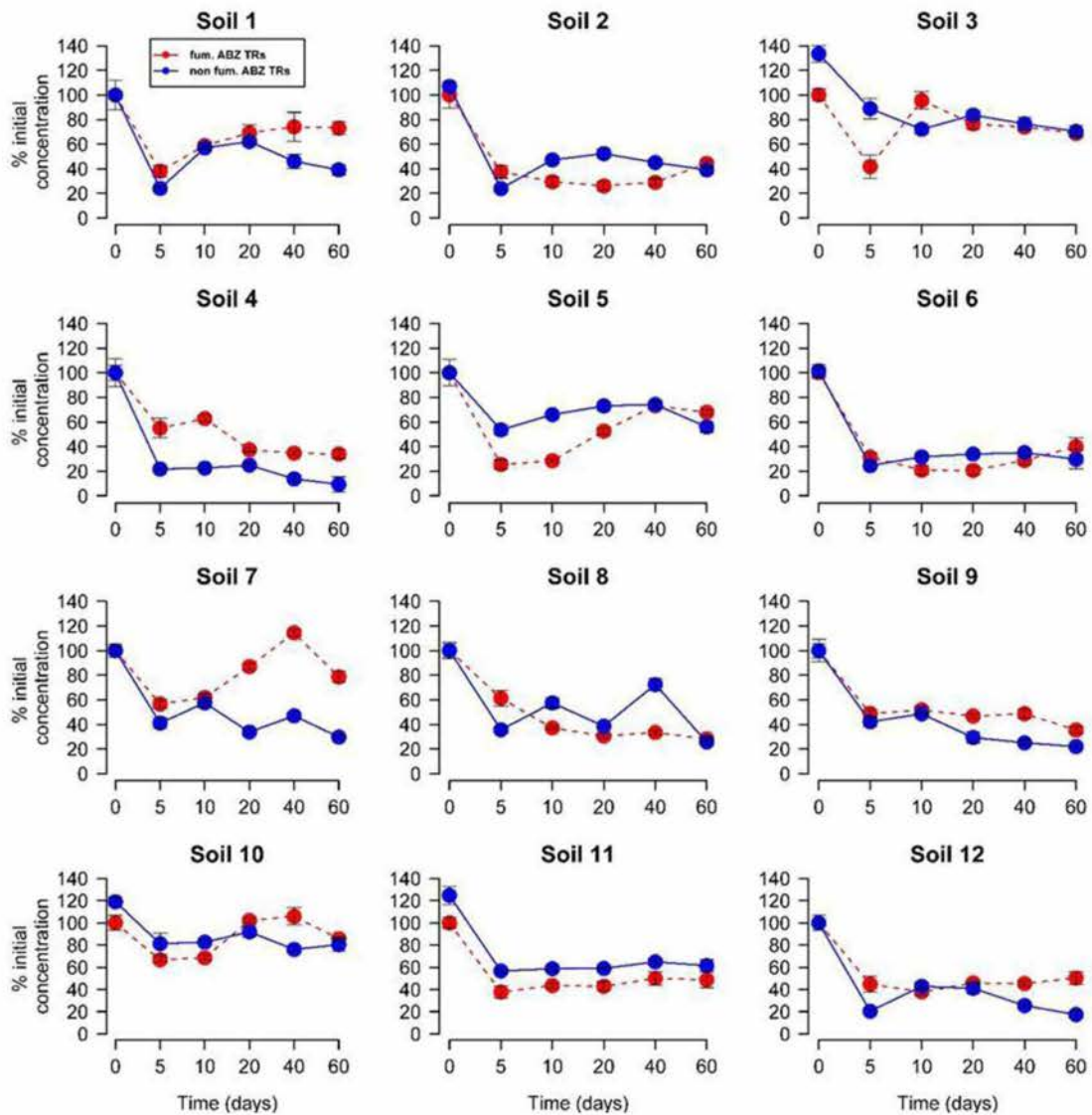
Supplementary Figure S2.2 The dissipation of Albendazole total residues (ABZ TRs) in fumigated (fum.) and non-fumigated (non fum.) samples of soil series A collected from inside barn facilities. Each value is the mean of three replicates with error bars representing the standard deviation of the mean.
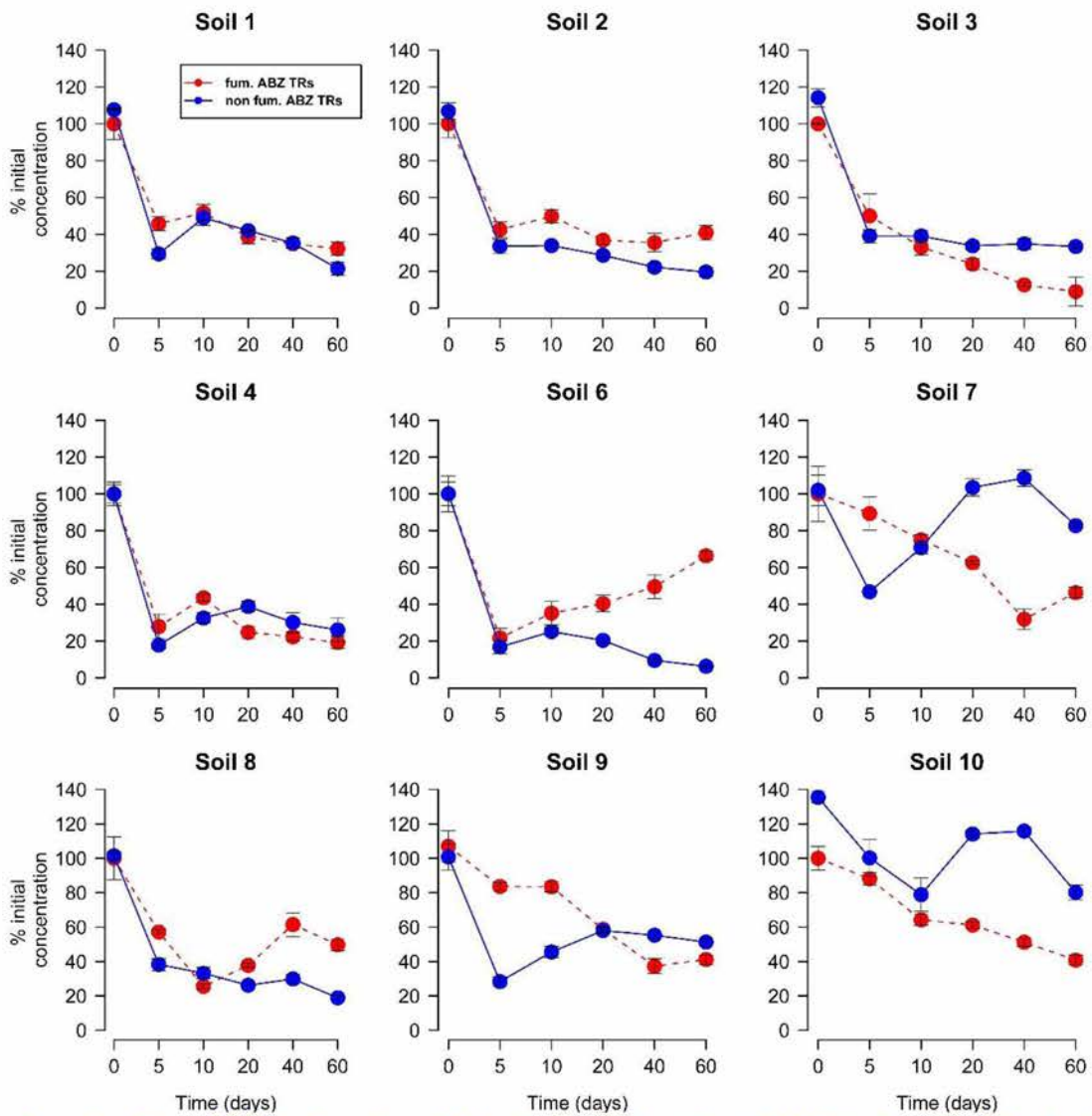
Supplementary Figure S2.3 The dissipation of Albendazole total residues (ABZ TRs) in fumigated (fum.) and non-fumigated (non fum.) samples of soil series B collected from nearby grasslands. Each value is the mean of three replicates with error bars representing the standard deviation of the mean.

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Chapter 3

Interactions of anthelmintic veterinary drugs with the soil microbiota: Toxicity or enhanced biodegradation?

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

Lagos, S. *et al.* (2022) 'Interactions of anthelmintic veterinary drugs with the soil microbiota: Toxicity or enhanced biodegradation?', *Environmental Pollution*, submitted (minor revision).

1. Introduction

Infections by gastrointestinal nematodes (GINs) constitute a major threat for grazing animals worldwide, affecting their welfare and productivity (Kaplan, 2020). For the last 60 years the main strategy for prevention and treatment of GIN infections is the use of anthelminthic (AH) compounds (McKellar and Jackson, 2004; Kaplan, 2020). Benzimidazoles (BZs) and macrocyclic lactones (MLs) are the most widely used classes of AHs (Horvat et al., 2012). The former includes albendazole (ABZ), thiabendazole (TBZ), fenbendazole (FBZ), flubendazole (FLU) and mebendazole (MBZ) (Mckellar and Scott, 1990). MLs (avermectins and milbemycins) are derivatives produced by the soil dwelling actinomycete *Streptomyces avermitilis* (Lasota and Dybas, 1991) with the main AH members being ivermectin (IVM), eprinomectin (EPM) moxidectin and doramectin (Bai and Ogbourne, 2016).

Upon their administration to animals AHs are only partially metabolized by animals and thus excreted through feces and urine. Depending on their administration route, excretion levels range between 60 and 90% of the administrated dose with higher levels expected for AHs administered orally (e.g., ABZ) compared to AHs administered as pour-ons or injectables (e.g., IVM, EPM) (Gottschall et al. 1990; Aksit et al. 2015). In feces AHs can be particularly persistent. ABZ was detected in sheep feces at levels of up to 12.8 mg kg^{-1}, being detectable at 120 h post administration, while lower levels of its transformation products, albendazole sulfoxide (ABZ-SO) and sulfone (ABZ-SO$_2$), were also detected (Prchal et al., 2016; Silveira Porto et al., 2021). IVM and EPM were reported at concentrations of 0.3–3 mg kg^{-1} (Iglesias et al., 2018) and 0.4–3.34 mg kg^{-1} (Halley et al., 2005) in animal feces with a DT$_{50}$ of 89 days. AHs can be also persistent in agricultural soils. Lagos et al (2022) reported in some soils DT$_{50}$ values of total residues of ABZ, ABZ-SO and ABZ-SO$_2$ > 100 days. IVM DT$_{50}$ values ranged from 30 to 66 days (Lagos et al. 2022; Krogh et al. 2009; Dionisio and Rath 2016), while EPM showed DT$_{50}$ values ranging from 20.8 to 57.9 days (Litskas et al. 2013; Lagos et al. 2022).

Once in soil, AHs are expected to interact with soil microorganisms. The exact nature of these interactions and the factors that determine their outcome need to be determined. We hypothesize that the presence of AHs will have a toxic effect on soil microbial communities and ecosystem functioning, or the genetic plasticity of the soil microbiota will facilitate microbial adaptation and thus growth-linked enhanced biodegradation of AHs. Little is known about the toxicity of AHs on the soil microbiota. da Rocha et al. (2020) noted a possible inhibitory effect of abamectin to soil microbial respiration, while Tentu et al. (2017) reported no significant effect of a formulation of abamectin on N mineralization. Ammonia oxidizing microorganisms (AOM), which regulate the first and rate-limiting step of nitrification, the oxidation of ammonia to nitrite (Prosser and Nicol, 2008), have been proposed as ideal bioindicators of the potential toxicity of abiotic stressors on the soil microbiota due to (a) their important ecological role (b) their general sensitivity to biotic and abiotic stressors (c) the availability of sensitive and standardized tools to measure their dynamics and activity (Wessén and Hallin, 2011; Karpouzas, Vryzas and Martin-Laurent, 2022) . Several studies have verified the sensitivity of ammonia oxidizing bacteria (AOB) and archaea (AOA) on other pollutants like pesticides (Feld et al., 2015; Vasileiadis et al., 2018; Karas et al., 2018), while the potential effect of AHs on nitrification remains unknown.

Soil microorganisms have a major role in the degradation of AHs in soil (Boxall et al., 2004). Lagos et al (2022) recently showed that the dissipation of ABZ, IVM and EPM was significantly reduced upon fumigation of soils, while similar results were reported for abamectin (Dionisio and Rath, 2016). In its extreme microbial degradation of organic pollutants in soil could lead to increasing degradation rates driven by the establishment of soil bacteria that carry specialized enzymatic mechanisms for the rapid hydrolysis of organic pollutants and use of their transformation products as energy source. This phenomenon has been called enhanced biodegradation and it is well documented for pesticides

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(Arbeli and Fuentes, 2007; Krutz et al., 2009; Rousidou et al., 2017) and veterinary antibiotics (Topp et al., 2013; 2016) but not for AHs whose sensitivity to this phenomenon is unknown.

We aimed to explore the interactions of widely used AHs, namely ABZ, IVM and EPM, with the soil microbiota. To achieve this goal, we employed mesocosm experiments whereby soil previously known to exhibit fast and slow degradation of the studied AHs were repeatedly treated with these AHs at two dose rates. We tested the hypothesis that repeated application of AHs will lead to accelerated degradation in «fast» soils and accumulation of AHs residues in «slow» soils with reciprocal toxicity on the soil microbiota. The dissipation of AHs was monitored as a measure of the potential enhanced biodegradation of AHs. In parallel we determined the potential effects of AHs on (a) the abundance and diversity of broad phylogenetic groups like total bacteria, fungi and Crenarchaeota via qPCR and amplicon sequencing respectively (b) the activity, abundance, and diversity of AOM, a key functional microbial group, by monitoring potential nitrification, inorganic N pools, the abundance of AOA, AOB and comammox bacteria via qPCR, and their diversity, via amplicon sequencing of the amoA gene.

2. Materials and Methods

2.1. Chemicals

Analytical standards of ABZ (98% purity), ABZ-SO (98% purity) and ABZ-SO$_2$ (97% purity) were purchased from Tokyo Chemical Industry (Zwijndrecht, Belgium), and Santa Cruz Biotech (Heidelberg, Germany) respectively. Analytical standards of IVM (97% purity) and EPM (97% purity) were purchased by Sigma-Aldrich (St Gallen, Switzerland). Stock solutions of IVM and EPM in methanol (1000 mg L^{-1}) and of a mixture of ABZ, ABZ-SO and ABZ-SO$_2$ in acetonitrile (1000 mg L^{-1}) were used for AHs residue analysis. Analytical standards of the AHs were also used for the preparation of methanol solutions that were applied in soils.

2.2. Soils

The soils used were collected in October 2020 from selected sheep farms in Lesvos Island, Greece. The selection of soil samples was based on the results of a previous dissipation study for ABZ, IVM and EPM in soils from 12 sheep farms in Lesvos Island with a history of systematic administration of AHs (Lagos et al., 2022). Based on the DT$_{50}$ values of the three AHs in the soils studied, we selected for each AH one soil which demonstrated a «fast» dissipation pattern and one with a «slow» dissipation pattern. As for IVM and EPM, due to their similar chemical structure, we selected soils where both compounds demonstrated a similar dissipation pattern. Immediately after sampling, soils were placed in portable refrigerators and transferred to the laboratory within 12 h. All soils were partially air-dried overnight and sieved to pass a 3-mm mesh. Measurements of the levels of AHs in randomly selected soil samples showed that their levels were below the limit of detection. The physicochemical properties and the code names of the soil studied are given in Supplementary Table S3.1.

2.3. Experimental setup

A bulk sample of 3500 g from each soil was divided into 9 subsamples. The first three were treated with methanol solutions of ABZ, IVM or EPM aiming to a nominal concentration of 2 mg kg^{-1} for ABZ and 1 mg kg^{-1} for IVM and EPM (designated as low concentration). The second set of triplicate samples were treated with methanol solutions of ABZ, IVM or EPM aiming to a nominal concentration of 20 mg kg^{-1} for ABZ and 10 mg kg^{-1} for IVM and EPM (designated as high concentration). The final subsample (weight 650 g) did not receive any AHs (only the same amount of methanol without AHs) to serve as untreated control. In all cases, the amount of methanolic solution spiked in soils never exceeded the 0.1% v/w (ml g^{-1} soil). The lower concentration level (1 or 2 mg kg^{-1}) was selected as representative of

an AH level expected to be present in manured soil based on (a) measured concentrations of AHs in the feces of animals (Halley et al., 2005; Prchal et al., 2016; Iglesias et al., 2018; Silveira Porto et al., 2021) and (b) our own (unpublished) monitoring data in the feces collected from livestock units where the levels of ABZ and EPM were in the range of 0.13–10.35 and 0.27–3.40 mg kg^{-1}, respectively. The higher concentration (10 or 20 mg kg^{-1}), represented a high exposure scenario not likely to occur in grassland or agricultural soils. The same application regimes were repeated two more times, at 40-day intervals for ABZ and at 60-day intervals for the two MLs. The different application intervals were selected based on the persistence of these compounds in these soils in our previous studies (Lagos et al., 2022). After each AH application, all soil samples were left for 1 h to allow methanol evaporation, and they were thoroughly mixed to ensure a homogeneous distribution of the AHs. Finally, an appropriate amount of water was added to adjust soil moisture levels to 40% of the water holding capacity. Throughout the study, soils were incubated in the dark at 25°C and their moisture content was maintained with regular additions of deionized water.

Immediately after each AH application and at regular intervals thereafter triplicate samples per treatment were used for (i) the determination of AH residues by HPLC, (ii) the measurement of potential nitrification, NO$_3$- and NH$_4$+ levels and (iii) DNA extraction. It should be noted that before the first and third application all soils were pretreated with a 0.5 M solution of (NH4)$_2$SO$_4$ (corresponding to 154 mg N kg^{-1} soil dry weight) aiming to stimulate the growth of all AOM groups.

2.4. Anthelmintic residue analysis

Residues of ABZ, ABZ-SO, ABZ-SO$_2$, IVM and EPM were extracted from soil and analyzed in a Shimatzu HPLC–DAD system equipped with a Grace Smart RP C18 (150 mm × 4.6 mm) as described by Lagos et al. (2022).

2.5. Dissipation kinetics

The kinetic models proposed by the FOCUS working group on pesticide degradation (FOCUS, 2006) were used for calculating dissipation kinetics for the studied AHs. The SFO kinetic model and the biphasic models: HS, FOMC and DFOP model were used. The χ^2 test as well as visual inspection and the distribution of the residuals were used as criteria to assess the agreement between calculated and observed data for a given fit. In all cases, the kinetic model selected to describe the degradation data was the one with the lower χ^2 value and the best fitted residuals to the calculated curve. Kinetic analysis was carried out in the R Studio v4.1.1 (R Core Team 2022, URL https://www.R-project.org/.), utilizing the package mkin, version 1.1.1 (Ranke and Meinecke, 2019).

2.6. Measurement of NO3-, NH4+ levels and Potential Nitrification

NO$_3^-$ and NH$_4^+$ levels in soil were determined spectrophotometrically as described by Doane & Horwáth and Kandeler & Gerber, respectively. Potential nitrification rates were measured according to Kandeler (1996).

2.7. Soil DNA extraction

DNA was extracted from 250 mg of soil with the DNeasy® PowerSoil® Pro Kit from Qiagen® (Hilden, Germany), according to manufacturer's instructions and it was quantified using a Qubit® fluorometer with a Quant-iT™ dsDNA broad range (BR) Assay Kit (Invitrogen®, USA).

2.8. qPCR analysis of the abundance of different soil microbial groups

The abundance of total bacteria and Crenarchaea was determined by targeting the 16S rRNA gene using primers Eub338/Eub518 (Fierer et al., 2005) and 771f/957R (Ochsenreiter et al., 2003) respectively. The abundance of total fungi was determined using primers FR1/FF390 (Chemidlin Prévost-Bouré et al., 2011) that amplify the 18S rRNA gene. The abundance of AOB and AOA was

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determined by amplification of the amoA gene using the primers amoA-1F/amoA-2R (Rotthauwe, Witzel and Liesack, 1997) and Arch-amoAF/Arch-amoAR (Francis et al., 2005), respectively. As for commamox bacteria, we quantified separately the abundance of the amoA gene of clade A and clade B by using an optimized mixture of primers described by Pjevac et al. (2017). A list of the primers, gene targets, and the qPCR thermal cycling conditions used are given in Supplementary Table S3.2. qPCR analyses were performed in a Biorad® CFX Connect Real-Time PCR System. All amplification reactions were carried out in a total volume of 10 μL containing 5 μL of the KAPATaq SYBR Green® PCR master mix (Kapa Biosystems, Massachusetts, USA), 200 ng of BSA, 2 ng of soil DNA (20 ng in the case of Comammox) and 2 μM of each primer. The abundance of each microbial group was determined with the use of standard curves that were produced using serial dilutions of linearized plasmids containing the studied genes. Efficiency in all cases ranged between 84 and 111%, while the R^2 values were > 0.995.

2.9. Amplicon sequencing analysis

Microbial diversity analysis for total bacteria, fungi, protist, AOB, and AOA was performed on samples collected at the end of each application. Analysis for total bacteria, fungi and protist was performed via multiplex amplicon sequencing in a HiSeq 2500 System® - Rapid Mode 2x250 bp paired-end (Illumina Inc., San Diego, USA) in Admera Health (Admera Health, New Jersey, US). The V4 region of the 16S rRNA gene of prokaryotes and the V9 region of the 18S rRNA gene of protists was amplified with primers 515f-806r (Walters et al., 2015; Caporaso et al., 2012) and 1391f-EukBr (Stoeck et al., 2010; Medlin et al., 1988) respectively following the protocol of the Earth Microbiome Project (Caporaso et al., 2018; Amaral-Zettler et al., 2018). The amplification of the ITS2 genomic region, was performed with primers ITS7-ITS4 as described by Ihrmark et al. (2012). Amplicon sequencing analysis of AOB and AOA was performed in a MiSeq System with the v3 reagents 2x300 bp paired-end (Illumina Inc., San Diego, USA) in the Genome Center of the Biomedical Research Foundation-Academia of Athens. For AOB and AOA the *amoA* gene was amplified with primers amoA-1F/amoA-2R (Rotthauwe, Witzel, and Liesack, 1997) and amoA310f - amoA529r (Marusenko et al., 2013) respectively. For all PCR amplifications the Q5® High-Fidelity DNA Polymerase 2x master mix (NEB, Ipswich, Massachusetts, USA) was used. All samples were initially amplified using the primer sets mentioned above, followed by a 2nd PCR using the same primers with indexes (on forward or reverse primer) for meta-barcoding of samples. Primer list and PCR conditions used in amplicon sequencing analysis are presented in Supplementary Table S3.3, while index sequences are presented in Supplementary Tables S3.4 and S3.5.

2.10. Bioinformatic analysis

Sequence pre-analysis consisted of de-multiplexing with Flexbar version 3.1.3 (Dodt et al., 2012). Sequencing quality screening, chimera removal, alignment to reference databases and generation of the amplicon sequence variant (ASV) matrices were performed with the dada2 package of the R version 4.1.1 (Callahan et al., 2016). Silva SSU taxonomic dataset version 138.1 (McLaren, 2020) formatted for dada2 and UNITE general fasta release version 8.2 (Nilsson et al., 2019) were used for the classification of the V4 16S rRNA and ITS2 amplicons respectively. In case of V9 18S rRNA amplicons, the PR2 (Protist Ribosomal Reference) taxonomic database, version 4.14.0 was used (Guillou et al., 2013). For the classification of AOB/AOA amoA amplicons we performed comparison with the alignments of Abell et al. (2012) and Alves et al. (2018), respectively. Microbial diversity coverage was assessed through rarefaction curves prepared with the vegan package, version 2.6-4 (Oksanen, 2007). The microbiome package (Lahti and Shetty, 2019) was used to calculate measures of α-diversity indices like Fisher's diversity index (Fisher et al., 1943), the Shannon index, the inverse Simpson index (Jost, 2006) and ACE (abundance-based coverage estimator) richness estimation (Chao, 1987).

2.11. Statistical analysis

All statistical analysis was performed with the R v4.1.1 software. q-PCR, and relative abundance data, as well as NO_3^-, NH_4^+ levels and potential nitrification, were analyzed with two-way ANOVA and the Tuckey HSD post-hoc analysis or their non-parametric versions, Kruskal-Wallis and the post-hoc Wilcoxon rank sum test., with the agricolae v1.3-3 package (de Mendiburu, 2022). The parametric ANOVA followed by the Tukey's post hoc analysis (or the non-parametric equivalents Kruskal-Wallis and Wilcoxon rank sum test) were implemented to assess α-diversity index differences, while the Kruskal-Wallis test was implemented for differential abundance for ASVs after P-value correction for multiple hypothesis testing with the Benjamini Hochberg algorithm. All hypothesis testing methods were performed using the agricolae package. The β-diversity was evaluated via Nonmetric multidimensional scaling (NMDS) ordination of the reference sites based on the Bray-Curtis similarities (Clarke, 1993). Permutational analysis of variance (Anderson, 2017) that followed β-diversity analyses, was performed with the pairwise Adonis package (Martinez Arbizu, 2020).

3. Results

3.1. Dissipation and transformation patterns of anthelmintics in soils

The dissipation patterns of the AHs are shown in Figure 3.1. The SFO model adequately described the dissipation of AHs in most cases. The dissipation kinetic parameters of AHs are presented in Supplementary Table S6. ABZ was transformed to ABZ-SO and ABZ-SO$_2$, however their formation and decay patterns varied in the two soils. In the «fast» soil, the rapid degradation of ABZ led to the formation of ABZ-SO, which was rapidly and near fully transformed to ABZ-SO$_2$ that accumulated in soil. On the other hand, in the «slow» soil ABZ-SO was also rapidly formed during degradation of ABZ but this time it persisted in soil leading to the formation of low levels of ABZ-SO$_2$. Repeated applications of ABZ in the «fast» soil accelerated its degradation with DT$_{50}$ values decreasing from 3.2 and 4.6 days after the first application to 0.7 and 1.5 days after the third application of the low and high dose rate respectively. This was not the case in the «slow» soil with the DT$_{50}$ values increasing from 3.2 (first application) to 3.4 (third application) days and from 5.3 to 20.5 day at the low and high dose rates, respectively. IVM and EPM showed similar dissipation patterns with DT$_{50}$ values being always > 30 days. No acceleration in the degradation of EPM or IVM was evident in both soils and dose rates. In contrast a gradual increase in the DT$_{50}$ values of both compounds was observed leading to accumulation of their residues in soils. For example, the DT$_{50}$ of the low dose rate of IVM and EPM in the «fast» soil increased from 48.1 (first application) to 79.9 (third application) days and from 55.2 to 74.1 days respectively (Supplementary Table S3.6).
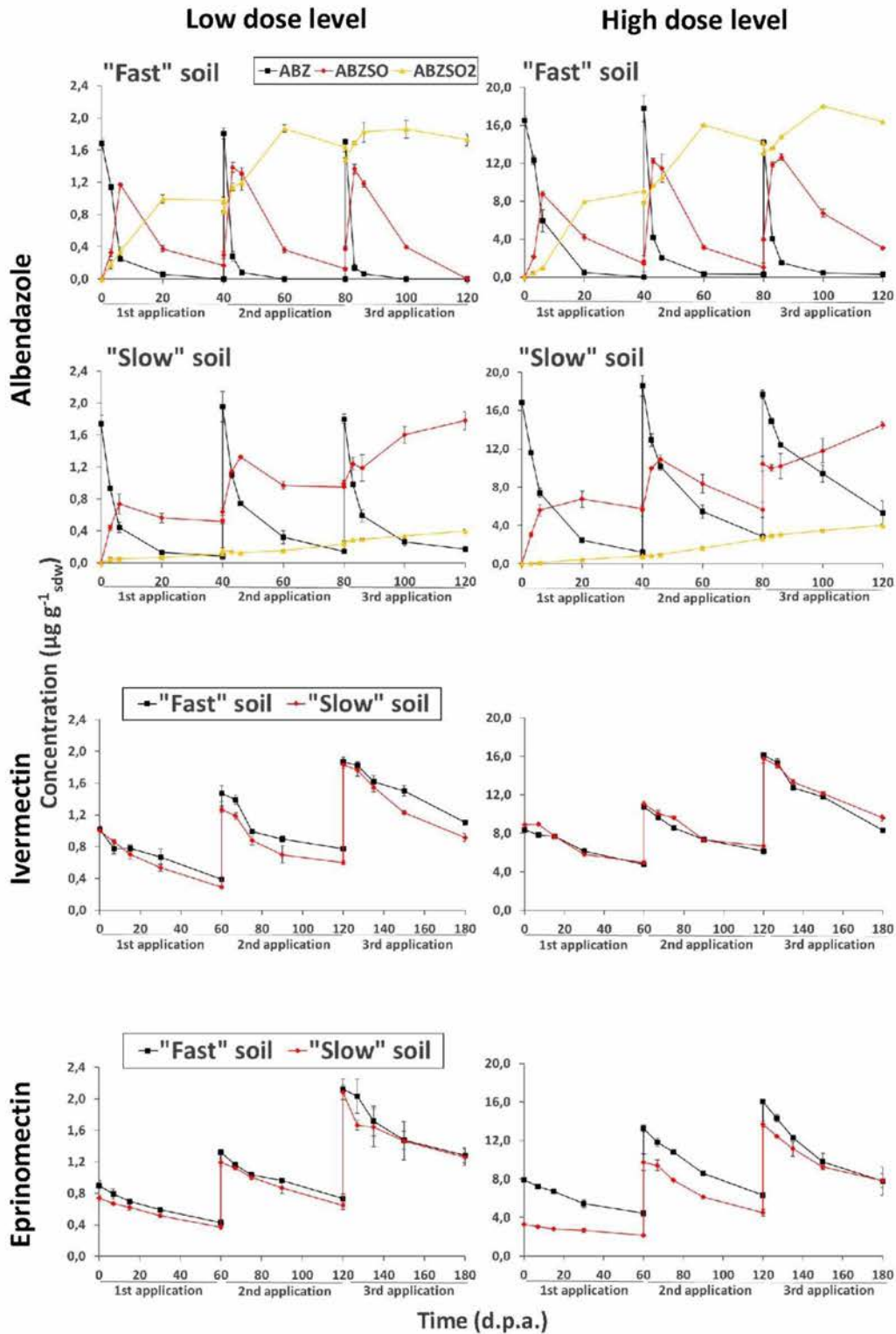
Figure 3.1 The dissipation patterns of the three repeated applications of Albendazole (ABZ), Ivermectin (IVM) and Eprinomectin (EPM) at two dose rates («low» 1 and 2 mg kg^{-1} and «high» 10 and 20 mg kg^{-1}) in «fast» and «slow» soils. The formation and decay patterns of the transformation products of ABZ, Albendazole sulfoxide (ABZ-SO) and Albendazole sulfone (ABZ-SO$_2$) are also presented. Each value is the mean of three replicates with error bars representing the standard deviation of the mean.

3.2. Effects of anthelmintics on the structure and activity of the soil microbial community

3.2.1. Effects on the abundance of total bacteria, fungi and Crenarchaeota

We first explored the possible effects of the three AHs on the abundance of broader soil microbial groups (Supplementary Figure S3.1). The application of AHs induced significant changes in the abundance of the 16S rRNA gene of bacteria which were though limited to specific time points and did not show a temporal pattern. Regarding fungi we noted a significant inhibitory effect in their abundance by all AHs which varied according to AH and soil. ABZ showed the strongest inhibitory effect on fungal abundance which persisted for the whole study duration in the «fast» soil, but only for the first two applications in the «slow» soil. IVM induced a temporary reduction on the abundance of fungi only after the first application in the «slow» soil, while a more consistent inhibitory effect was evident in the «fast» soil and particularly at the highest dose rate. EPM, in both soils, induced a significant but transient reduction in the abundance of fungi at 15 days after each application but recovery was observed thereafter. ABZ significantly reduced the abundance of Crenarchaeota for the whole experimental duration, especially at the «slow» soil. IVM and EPM had similar effects on Crenarchaeota. In the «fast» soil both compounds did not affect the abundance of Crenarchaeota, whereas in the «slow» soil they caused a significant temporary reduction in the abundance of Crenarchaeota at 15 days after the first two application, but recovery was observed thereafter.

3.3. Effects of anthelmintics on the activity and abundance of AOM

3.3.1. Effects on Potential Nitrification, NO_3^-, NH_4^+ levels

The temporal patterns of NO_3^- and NH_4^+ and potential nitrification are shown in Supplementary Figures S3.2 & S3.3 respectively. All three AHs, at both dose rates, induced a significant reduction ($p<0.05$) in potential nitrification compared to the control. ABZ showed the most consistent and persistent inhibitory effect with recovery observed only in the «fast» soil 40 days after the third application. Similar patterns were observed for IVM and EPM, where recovery was evident in most cases at 15 to 60 days after the third application. No clear pattern of effects in NH_4^+ levels was observed. On the other hand, we noted a significant reduction ($p<0.05$) in the levels of NO_3^- in the samples treated with the three AHs compared to the control samples. This reduction becomes visible a few days after the first application of the AHs and persisted for the whole experimental duration.

3.3.2. Effects on the abundance of AOB, AOA and comammox bacteria

The abundance patterns of AOB, AOA and comammox bacteria are presented in Figure 3.2. Regardless of the treatment employed, AOB *amoA* gene copies were more abundant than AOA in all soils. Comammox bacteria, both clade A and clade B, were also detected, although the latter showed the lower abundance of all AOM groups.

AHs significantly reduced ($p<0.05$) the abundance of AOM. ABZ and its TPs, at both rates, imposed the most consistent inhibitory effect on AOB and AOA abundance in both soils, which persisted for the whole experimental duration. Regarding commamox bacteria, a significant reduction in their abundance was observed in both soils and by both dose rates of ABZ only after the first and the second application. IVM and EPM also significantly decreased ($p<0.05$) the abundance of the *amoA* gene of all AOM groups, although the temporal patterns of these effects varied in the different soils. In the «fast» soil IVM induced a significant reduction ($p<0.05$) in the abundance of the *amoA* gene of AOB and AOA only after the first and second application. In contrast in the «slow» soil we observed a significant decrease ($p<0.05$) in the abundance of the *amoA* gene of AOB and AOA from the second application onwards. EPM showed a more persistent inhibitory effect on the abundance of the AOB compared to AOA in the «fast» soil. More specifically, AOA *amoA* gene abundance significantly decreased ($p<0.05$)

upon the first application of EPM but no difference with the control was observed thereafter, while for AOB the reduction in their abundance was evident after all three application of EPM. In the «slow» soil, EPM induced a significant decrease in the abundance of the *amoA* gene from its second application onwards. In line with ABZ, IVM and EPM induced a significant reduction ($p < 0.05$) in the abundance of clade A and clade B comammox bacteria only after the first two applications.

3.4. Effects of anthelmintics on microbial diversity

3.4.1. Effects of anthelmintics on the α-diversity of soil microorganisms

The amplicon sequencing matrix was used for the calculation of α-diversity indices (Supplementary Figures S3.4 and S3.5). AHs did not have a significant effect on the α-diversity of bacteria ($p > 0.05$) (Supplementary Figure S3.4). ABZ induced a significant reduction in all α-diversity indices ($p < 0.05$) for fungi and protists. Whereas IVM and EPM did not significantly affect the α-diversity of both groups,
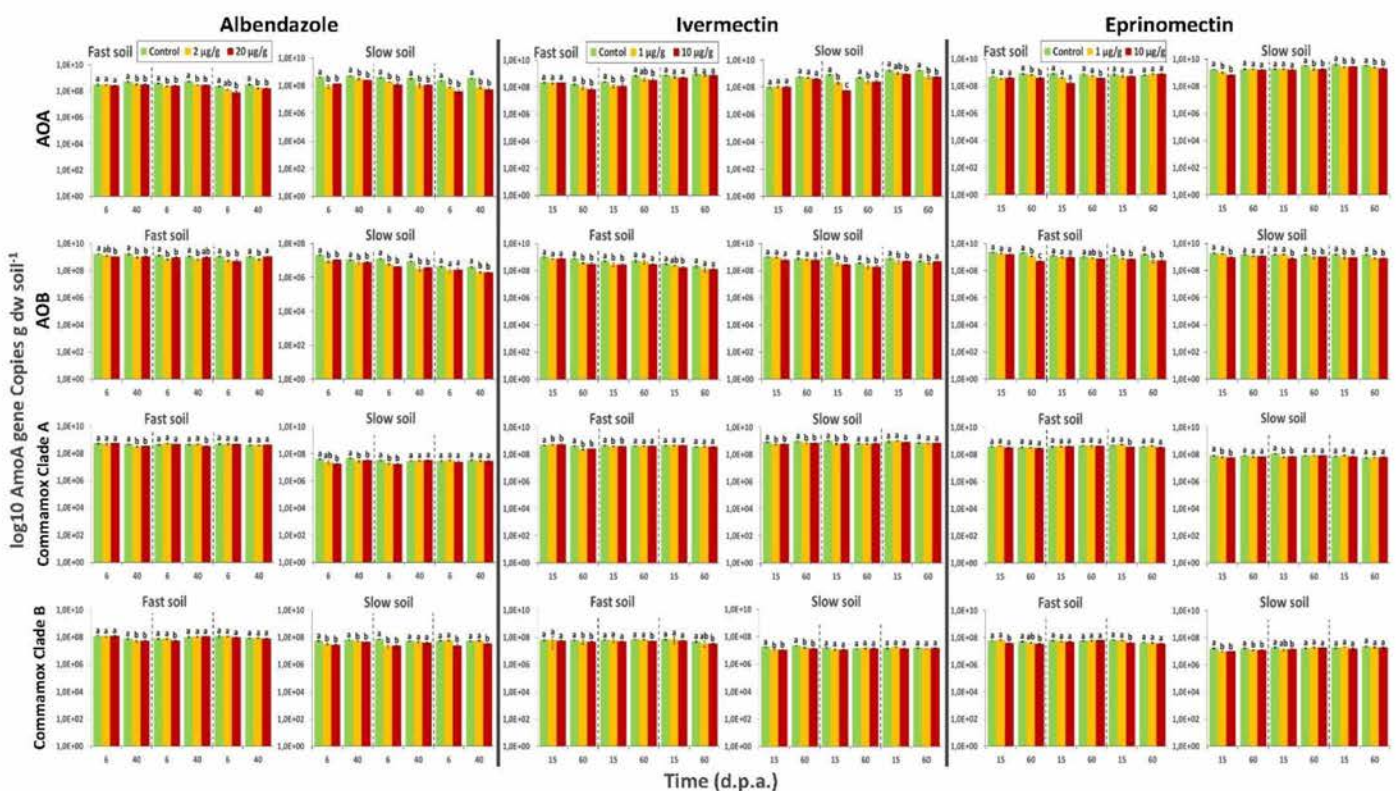

Figure 3.2 Effects of Albendazole (ABZ), Ivermectin (IVM) and Eprinomectin (EPM) on the abundance of the *amoA* gene of AOB, AOA and comammox bacteria (clades A and B) after three repeated applications. The dashed vertical lines indicate the different applications. Each value is the mean from three replicates ± standard deviation. Abundance data are presented on a logarithmic scale (log base = 10). Bars designated by the same letter within each time group are not significantly different at the 0.05 level.

with notable exceptions, a reduction ($p < 0.05$) in the Fisher's α index of fungi by the high dose rate of EPM in the «slow» soil and of Shannon, Inverse Simpson and Fisher's α of protists by IVM in the «fast» soil (Supplementary Figure S3.4). We did not observe any significant effects of AHs on the α-diversity of AOA, whereas ABZ and EPM showed contrasting effects on the α-diversity AOB. ABZ decreased the α-diversity of AOB in the «fast» soil and increased the α-diversity of AOB in the «slow» soil. EPM increased the α-diversity indices in the «fast» soil ($p < 0.05$) and decreased only in Fisher's α and ACE indices in the «slow» soil (Supplementary Figure S3.5).

3.4.2. Effects of anthelmintics on the β-diversity of soil microorganisms

We further determined the effects of AHs on the composition of the bacterial, fungal, protistan and AOM communities. Regardless of AH treatment the bacterial community in the studied soils were

dominated by Proteobacteria, Acinetobacteriota, Planctomycecota, Chloroflexi, Firmicutes and Bacteroidota which together accounted for > 90% of the total bacterial community (Supplementary Figure S3.6). In all soils the fungal community was dominated by *Sporormiella* and *Trichoderma* ASVs (Supplementary Figure S3.7). Protists of the Divisions *Lobosa, Pseudofungi, Cercozoa* and *Ampicomplexa* prevailed in the studied soils (Supplementary Figures S3.8).

We looked in the effects of AHs on the β-diversity of the different microbial groups. As expected, soil was the main structuring parameter of all microbial communities (p < 0.001) masking the effects of other factors (Supplementary Figure S3.9). Hence, the effects of AHs were studied separately in each soil. Pairwise-PERMANOVA analysis between the different treatments showed that ABZ, at both dose rates, significantly altered (p<0.05) the composition of the bacterial community (Figure 3.3). The effect of IVM and EPM varied in the two soils. In the «fast» soil, IVM showed a significant effect on the bacterial community only at the high dose rate, whereas EPM showed no significant effect. In the «fast» soil both AHs at both dose rates induced significant changes on the composition of the bacterial community. Regarding fungi, ABZ was again the sole AH which had a significant effect (p<0.05) on the composition of the fungal community at both soils, unlike IVM and EPM whose effects (p<0.05) were limited only to the «fast» soil (Figure 3.4). AHs did not have a significant effect (p>0.05) on the community of protists, with the sole exception of the high dose rate of ABZ in the «slow» soil (p<0.01) (Figure 3.5). ABZ showed a consistent and significant effect on the community of AOB and AOA in both soils, unlike IVM which showed no significant effect (Supplementary Figures S3.11 and S3.12). EPM significantly affected (p<0.05) the composition of the AOB community in both soils and dose rates, whereas this effect was restricted only to the «slow» soil at the high dose rate for AOA.

To further identify microorganisms that are associated with specific AH treatments, we performed a differential abundance analysis for the 500 most abundant ASVs in each microbial group (Supplementary Figures S3.13, S3.14 and S3.15). Amongst bacteria, the abundance of ASVs belonging to *Chitinophagaceae* (both soils), *Agromyces, Lautropia* and *Pirellula* showed a significant positive association with ABZ, whereas ASVs of *Shingomonas* (both soils), *Streptomyces* and *Promicromonosporaceae* (fast soil), *Planococcaceae, Rubrobacter, Beijenrinckiaceae* (slow soil) were negatively affected by ABZ. For IVM we identified ASVs belonging to *Gemmata, Thermomonas*, and *Haliangium* (slow soil) that showed significant negative association with IVM-treated samples while ASVs belonging to *Conexibacter* where significantly more abundant in presence of IVM in slow soil only.
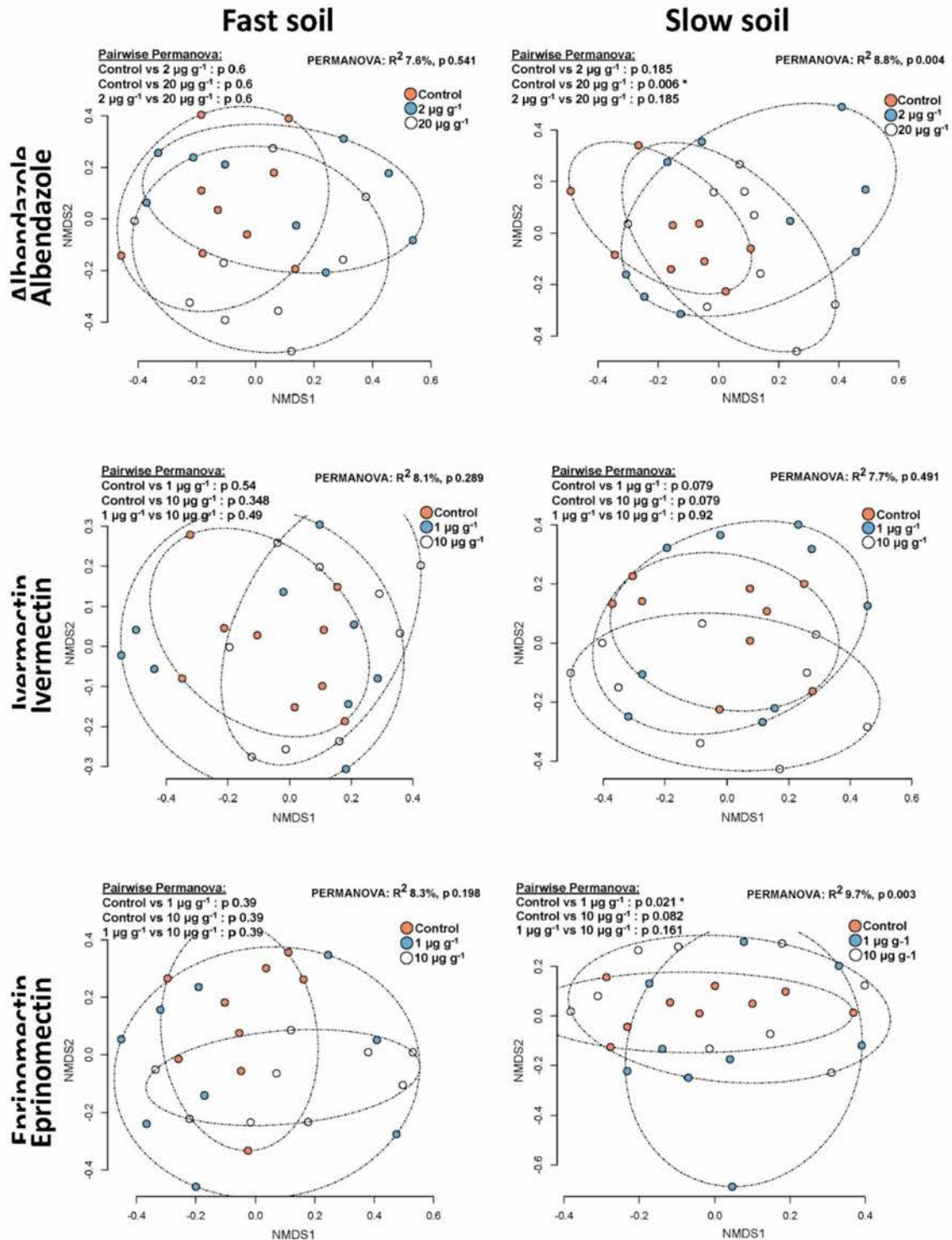
Figure 3.4 Non-metric multidimensional scaling (nMDS) ordination plot illustrating the effects of albendazole (ABZ), ivermectin (IVM), and eprinomectin (EPM) on the structure of the fungal community in the «fast» and «slow» soils. Samples were ordinated according to the dose rate (control, 1 or 2 mg kg⁻¹, 10 or 20 mg kg⁻¹) of the AHs. Pairwise comparisons of the soil communities between different treatments are also presented. Significant difference (p<0.05) between groups is indicated with an asterisk (*).

Finally, we identified ASVs from both studied soils belonging to Conexibacter, Gemmata, Planctomicrobium, Chloroflexi (Caldilineaceae, Ardenticatenales) whose abundance was positively associated with EPM-treated samples. Regarding fungi all responsive ASVs belonged to Sporormiela and Trichoderma and hence no clear phylogenetically patterns between AHs and fungal ASVs were observed. Amongst protists we noted the most distinct associations between AHs treatments and specific ASVs. Pirsonia (clade Stramenopiles) ASVs were the most common responders to AH

exposure. Their abundance, and of other ASVs belonging to the same clade (Leukarachnion, Andriamonas, Aurantiochytrium, Bumilleriopsis, Oomycota) showed a significant positive correlation with ABZ.
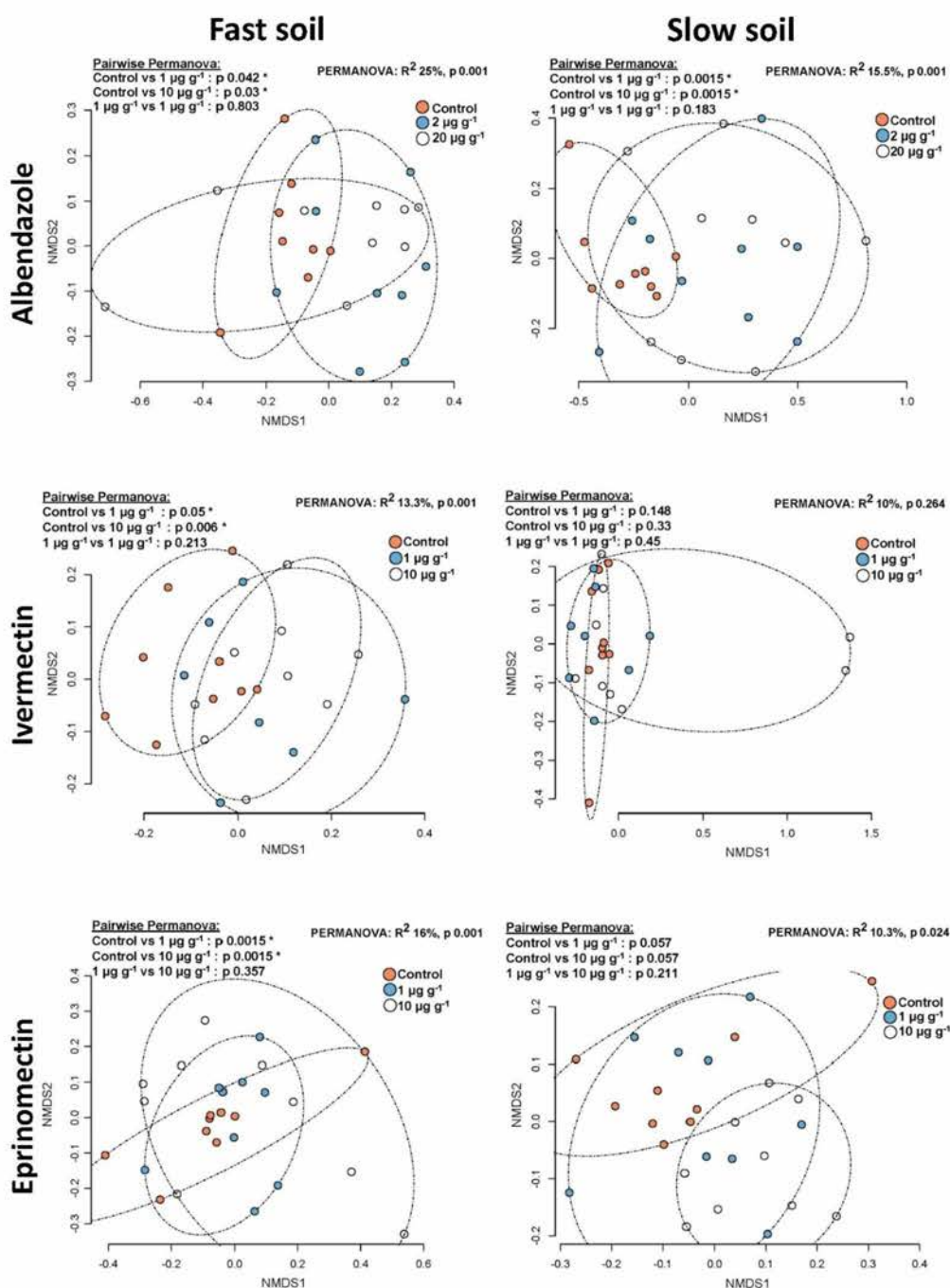

Figure 3.5 Non-metric multidimensional scaling (nMDS) ordination plot illustrating the effects of albendazole (ABZ), ivermectin (IVM), and eprinomectin (EPM) on the structure of the protist community in the «fast» and «slow» soils. Samples were ordinated according to the dose rate (control, 1 or 2 mg kg^{-1}, 10 or 20 mg kg^{-1}) of the AHs. Pairwise comparisons of the soil communities between different treatments are also presented. Significant difference ($p<0.05$) between groups is indicated with an asterisk (*).

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4. Discussion

In this study we disentangled the type of interactions expected to occur between soil microbiota and AHs that reach soil via direct and indirect means. We asked the question whether the repeated exposure of soils to AHs will lead to microbial adaptation and eventually enhanced biodegradation of AHs or will result in accumulation of their residues and toxicity to the soil microbiota.

First, we followed the dissipation of AHs in soils to determine potential acceleration upon three repeated applications and at the same time define the levels and the duration of the exposure of the soil microbiota to the AHs. Only ABZ showed an acceleration in its dissipation and only in the soil previously characterized by Lagos et al. (2022) as «fast». The dissipation of ABZ in the two soils led to the formation of the same two oxidation products, ABZ-SO and ABZ-SO$_2$, in line with previous studies (Lagos et al., 2022; Navrátilová et al., 2021). However, their formation and decay kinetics varied in the two soils as described previously (Section 3.1). We speculate that the slow transformation and eventual accumulation of ABZ-SO, a bioactive AH molecule instead of the inactive ABZ-SO$_2$ (Belew et al., 2021), in the «slow» soil inhibited the potential establishment of an active microbial community capable of rapidly degrading the parent compound. Previous studies with pesticides like chlorpyrifos and chlorothalonil have shown that formation of biologically active intermediates could inhibit the microbial degradation of their parent compound (Motonaga et al., 1998; Robertson et al., 1998). On the other hand, IVM and EPM were moderately persistent in the two soils, in line with previous studies (Lagos et al., 2022; Krogh et al., 2009; Litskas et al., 2013; Iglesias et al., 2018), and did not show signs of accelerated biodegradation.

In the absence of enhanced biodegradation for ABZ in the «slow» soil and of IVM, EPM in both soils, we explored the potential toxicity of the accumulating residues of AHs on the soil microbiota. First, we investigated potential effects on the abundance and diversity of broad phylogenetic groups across the soil food web like bacteria (and Crenarchaeota), fungi and protists. The studied AHs did not affect the abundance of total bacteria. On the other hand, we noticed clear effects of AHs on the abundance of fungi and Crenarchaeota. ABZ had the most notable inhibitory effect on the abundance of fungi and Crenarchaeota, whereas the effects of IVM and EPM were soil-specific and transient. The higher toxicity of ABZ on fungal abundance is not surprising considering that several benzimidazole compounds, structurally similar to ABZ, like TBZ and carbendazim, are used as fungicides.

We expanded our research and looked for potential effects of AHs on the diversity of bacteria, fungi and protists, their main predators in soil ecosystems (Geisen et al., 2018). None of the studied AHs had an effect on the α-diversity of bacteria, whereas ABZ had a strong negative effect on the α-diversity of fungi and protists even at the lower dose rate. ABZ was again the AH with the stronger effect on the community of bacteria and fungi in both soils, unlike IVM and EPM whose effects were limited either to one of the two soils or were significant only at the high dose rate. Unlike their prey, the community of protists was not affected by the AHs. There is lack of available studies regarding AHs effects on the diversity and abundance of the soil microbiota. Previous studies with other BZ compounds showed contrasting results. For example, Papadopoulou et al. (2016) and Papadopoulou et al. (2018) showed that TBZ would not affect the composition of the soil microbial community at concentration levels equivalent or even higher than the ones used in the current study. Whereas carbendazim reduced the α-diversity of bacteria and fungi in soil (Wang et al., 2009; Ma et al., 2021). Similarly, no studies have explored the potential effects of IVM and EPM on the soil microbial diversity. In the only study available with another ML, abamectin, applied in soil at concentrations of 0.1 and 1 mg kg^{-1}, induced temporary effects on microbial diversity as determined by shotgun metagenomics (Qiu et al., 2022).

Finally, we tried to identify specific ASVs with differential abundance between the control and AH-spiked soil samples. *Chitinophagaceae* and *Sphingomonas* were negatively and positively associated

respectively with ABZ in both soils. *Chitinophagaceae* are responsible for the degradation of chitin and cellulose (Rosenberg, 2014; Bailey et al., 2013), while *Sphingomonas* are known as versatile degraders of organic pollutants (Li et al., 2007). *Conexibacter* were positively associated with soils treated with the MLs. They were suggested to have a role in nitrification in oxygen-limited condition and others showed their role as saccharolytic bacteria (Monciardini et al., 2003; Seki et al., 2012). The fungal community in all soils was dominated by *Sporormiella* which is not surprising if we consider that (i) *Sporormiella* are obligate coprophilous fungi dwelling in animal dung (Mungai et al., 2012; Gill et al., 2013), and have been used as a proxy for the presence of large herbivores on the landscape in the past (Rozas-Davila et al., 2016), or to estimate livestock densities and grazing consequences (Baker et al., 2016) (ii) the soils studied were collected from nearby grasslands or the interior part of livestock units. The presence of AHs in soil led to clear positive or negative responses by protists with members of the genus *Pirsonia* being positively affected by ABZ along with other members of the supergroup *Stramenopiles*. *Pirsonia* are considered marine protists acting mostly as diatom parasites, although recent findings propose that phagoheterotrophic members could exist in soil (Cho et al., 2022; Cavalier-Smith and Chao, 2006). Overall, the presence of AHs, and mostly of ABZ was shown to affect several microorganisms involved in key ecosystem functions like nutrient cycling, plant growth promotion, predation and population control with unexpected consequences for soil ecosystem functioning.

Based on the effects of AHs on the abundance and diversity of broad microbial groups, we further focused on the potential effects of AHs on N cycling and especially on ammonia oxidation. The temporal patterns of potential nitrification, NO_3^- and the abundance of AOB and AOA suggested a clear inhibitory effect on the nitrification process by all three AHs, although ABZ was the compound with the stronger effect. We also detected commamox bacteria of both clade A and clade B in the studied soils. Their response to AHs varied between compounds and seemed to coincide with the level and the duration of the exposure. Hence the non-persistent ABZ induced significant reductions in their abundance at the first two application cycles. Whereas the more persistent and accumulating IVM and EPM reduced the abundance of commamox bacteria only after the second and third applications.

Despite its short persistence, ABZ was consistently the most toxic to broad phylogenetic groups and functional microbial groups like AOM. We speculate that the persistent inhibitory effect of ABZ on soil microbes at concentration levels expected to be encountered in soil is supported, besides the parent compound, by its transformation products ABZ-SO, which carries anthelminthic activity, but probably also ABZ-SO$_2$ since its accumulation in the «fast» soil, instead of ABZ-SO, did not alter the inhibitory effects on AOM. Similar observations have been reported for pesticides where transformation products were equally or more toxic on AOM compared to the parent compound (Vasileiadis et al., 2018). Further *in vitro* toxicity tests with ABZ and its two transformation products against selected AOM strains and in vivo in soil will further verify our hypothesis. Overall, little is known on the effects of AHs on AOM. In the few studies available, Papadopoulou et al. (2016) reported no inhibitory effects of TBZ (used mostly as a fungicide) on the abundance and activity of AOB and AOA. Whereas Konopka et al. (2015) reported that IVM applied in soil as a mixture with the antibiotics monensin, and zinc bacitracin at a concentration of 10 mg kg^{-1} suppressed the abundance of AOB but not of AOA. We also investigated potential effects of AHs on the diversity of AOA and AOB. We noted only subtle effects with ABZ being the only compound which induced significant changes in the composition of the AOB and AOA communities in both soils but only at the high dose rate. The lack of concurrence between activity/abundance data and diversity data for AOM has been reported before for other pollutants (Vasileiadis et al., 2018) and implies that AHs do not eliminate or drastically alter the composition of the AOM community but instead they affect the activity of the members of the established AOM community.

5. Conclusions

In soil AHs interact with the soil microbiota with the underlying mechanisms determining the outcome remaining unknown. We showed that the exposure of soils to AHs at concentration levels expected to be encountered in agricultural and grassland settings could have beneficial or detrimental effects on the soil microbiota. A classic example was ABZ which, on the one hand, exhibited enhanced degradation upon repeated application in one of the soils tested, while at the same time it imposed a negative effect on the abundance, activity and diversity of most phylogenetically and functionally distinct microbial groups studied. MLs like IVM and EPM did not show signs of accelerated degradation and accumulated in soils triggering inhibitory effects on most microbial endpoints measured, although their effects were always less persistent and acute compared to ABZ. We speculate that the formation and persistence of the transformation products of ABZ, ABZ-SO and ABZ-SO$_2$, in soil along with its inherently, due to its mode of action, higher microbial toxicity explain the long-term inhibitory effects of ABZ to the soil microbiota. Overall, our findings are expected to benchmark future studies on the toxicity of AHs on the soil microbiota and will pave the way for potential reconsideration of soil microbes as protection goals in the environmental risk analysis of AHs.

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Annex II – Chapter 3 Supplementary Data

Supplementary Table S3.1 The physicochemical properties of the studied soils

Soil code names	Total N (%)	Total organic carbon (%)	pH
1A (ABZ «fast» soil)	0.28	6.0	7.84
7B (ABZ «slow» soil)	0.31	8.5	5.85
6A (IVM & EPM «fast» soil)	0.32	6.3	6.77
11A (IVM & EPM «slow» soil)	1.07	11.5	7.02

Supplementary Table S3.2 The primers, sequences and thermocycling conditions used for qPCR analysis of the abundance in studied soils of total bacteria, total fungi, Crenarchaeota, AOB, AOA and Commamox bacteria (Clade A & b).

Primer	Thermocycling Conditions		Sequence (5' – 3')	Target gene	Reference
	initial denaturation (1 cycle)	Amplification stage (n cycles)			
Eub 338 - Eub 518	95 °C for 3 min	95 °C for 15 sec - 60 °C for 20 sec - 72 °C for 10 sec n = 35	ACT CCT ACG GGA GGC AGC AG ATT ACC GCG GCT GCT GG	16S rRNA (bacteria)	Fierer et al., 2005
FR1 - FF 390	95 °C for 3 min	95 °C for 15 sec - 50 °C for 35 sec - 72 °C for 10 sec n = 40	AIC CAT TCA ATC GGT AIT CGA TAA CGA ACG AGA CCT	18S rRNA (fungi)	Chemidlin et al., 2011
771F - 957R	95 °C for 3 min	95 °C for 3 sec - 55 °C for 30 sec - 72 °C for 55 sec n = 35	ACG GTG AGG GAT GAA AGC CGG CGT TGA CTC CAA TTG	16S rRNA (archaea)	Ochsenreiter et al., 2003
amoA-1F/ amoA-2R	95 °C for 3 min	95 °C for 5 sec - 57 °C for 10 sec - 72 °C for 30 sec n = 40	GGG GTT TCT ACT GGT GGT CCC CTC KGS AAA GCC TTC TTC	amoA (bacteria)	Rotthauwe et al., 1997
Arch-amoAF/ Arch-amoAR	95 °C for 3 min	95 °C for 15 sec - 60 °C for 20 sec - 72 °C for 10 sec n = 45	STA ATG GTC TGG CTT AGA CG GCG GCC ATC CAT CTG TAT GT	amoA (archaea)	Francis et al., 2005
comaA-244f_(a, b, c)/ comaA-659r_(c, d, e, f)	95 °C for 3 min	95 °C for 3 sec - 52 °C for 20 sec - 72 °C for 2 sec n = 40	TAYAAYTGGGTSAAYTA ARATCATSGTGCTRTG	amoA (Commamox bacteria – Clade A)	Pjevac et al., 2017
comaB-244f_(a, b, c)/ comaB-659r_(a, c, d)	95 °C for 3 min	95 °C for 3 sec - 52 °C for 20 sec - 72 °C for 2 sec n = 40	TAYTTCTGGACRTTYTA ARATCCARACDGTGTG	amoA (Commamox bacteria – Clade B)	Pjevac et al., 2017

Supplementary Table S3.3 The primers, sequences and thermocycling conditions used for amplicon sequencing analysis of the bacterial, fungal protist, AOB and AOA communities.

Primer	Thermocycling Conditions	Sequence (5' – 3')	Target	Reference
515f – 806r	98°C for 10 s, 50°C for 30 s, 72°C for 30 s (25 + 7 cycles) [b]; 72°C for 10 min	NNNNNNNNN**GT**GTGYCAGCMGCCGCGGT AA[a] GGACTACNVGGGTWTCTAAT	Bacterial V4 region of the 16S rRNA gene	Walters et al.,2015
fITS7 – ITS4	98°C for 10 s, 55°C for 30 s, 72°C for 30 s (25 + 7 cycles) [b]; 72oC for 10 min	GTGARTCATCGAATCTTTG NNNNNNNNN**GA**TCCTCCGCTTATTGATATG C[a]	Fungal ITS2 genomic region	Ihrmark et al.2012
1391f-EukBr	98°C for 10 s, 57°C for 30 s, 72°C for 30 s (30 + 10 cycles) [b]; 72°C for 10 min	GTACACACCGCCCGTC NNNNNNNNN**CA**TGATCCTTCTGCAGGTTC ACCTAC	V9 region of the 18S rRNA gene	Stoeck et al., 2010
amoA-1F/ amoA-2R	98°C for 10 s, 54°C for 30 s, 72°C for 30 s (30 + 7 cycles) [b]; 72°C for 10 min	NNNNNNNNN**AA**GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	amoA gene	Rotthauwe et al., 1997
amoA310f - amoA529r	98°C for 10 s, 54°C for 30 s, 72°C for 30 s (30 + 7 cycles) [b]; 72°C for 10 min	NNNNNNNNN**GG**TGGATACCBTCWGCAATG GCAACMGGACTATTGTAGAA	amoA gene	(Marusenko et al., 2013)

[a] the sample index (consecutive Ns) and linker (bold letters) prior to the extension bases in the forward or reverse primer are indicated. Indexed sequences are listed in Supplementary Tables S3 & S4

[b] the first number in parentheses indicates the number of cycles performed in the first PCR where the unindexed primers were used, while the second number indicates the additional cycles performed in the sample indexing PCR.

Supplementary Table S3.4 Sample code names and the forward (f) or reverse (r) primer index sequences (5' - 3') associated with them regarding the first amplicon library.

Library	Sample ID	515f primer index	ITS4r primer index	EukBr primer index	amoA-1F primer index	amoA310f primer index
1st Library	19A _ABZ	TTCTTCTTC	TTATTACCG	TTATTACCG	TTATTACGC	TTATTAGGC
	20A _ABZ	TTCTTCAAG	TTATTAGGC	TTATTCTCC	TTATTCGTC	TTATACTCC
	21A _ABZ	TTCTTGTCA	TTATTCTCC	TTATTCAGG	TTATATGGC	TTATACAGG
	22A _ABZ	TTCTTGAGT	TTATTCGTG	TTATTGCGT	TTATCTCTC	TTATAGCTG
	23A _ABZ	TTCTTGGAC	TTATTGCGA	TTATTGGAC	TTATCCTAC	TTATAGGAC
	24A _ABZ	TTCTATAGG	TTATACTGG	TTATATCGC	TTATCGAGC	TTATGTTGG
	25A _ABZ	TTCTATCTC	TTATACCTC	TTATAAGCC	TTATGGTTC	TTATGTCTC
	26A _ABZ	TTCTATGCA	TTATACGCA	TTATACCAG	TTAATCTGC	TTATGAACC
	27A _ABZ	TTCTAACAG	TTATAGACC	TTATACGGT	TTAATGCTC	TTATGACGT
	28A _ABZ	TTCTAGTTG	TTATGTTCG	TTATAGTGG	TTAAGCATC	TTATGAGAG
	29A _ABZ	TTCTCTTGT	TTATGTGAC	TTATCTTCG	TTACTTGAC	TTATGGTCA
	30A _ABZ	TTCTCTAAC	TTATGAAGG	TTATCCATC	TTACAATGC	TTAATCTCG
	31A _ABZ	TTCTCAATG	TTATGAGCT	TTATCCGCA	TTACGTCGC	TTAATCAGC
	32A _ABZ	TTCTCAGAA	TTATGCCAT	TTATCGACT	TTCTATCCT	TTAATGGTG
	33A _ABZ	TTCATATGG	TTATGGTGT	TTATGTCCT	TTCTACTTC	TTAACTACC
	34A _ABZ	TTCATAGTC	TTAATTCGC	TTATGTGTC	TTCTACGAT	TTAACTCTG
	35A _ABZ	TTCATCACA	TTAATCCAG	TTATGATGC	TTCTCTTGC	TTAACTGGA
	36A _ABZ	TTCATCGAT	TTAATCGGT	TTATGAGAG	TTCTCTGTT	TTAACAAGG
	19B _ABZ	TTCAATCGT	TTAATGTGG	TTATGCCGA	TTCTCATCT	TTAACACAC
	20B _ABZ	TTCAATGAC	TTAATGCCT	TTAATTCGG	TTCTCGCAT	TTAACCGTT
	21B _ABZ	TTCAACTAG	TTAATGGAC	TTAATTGCC	TTCTGTGAC	TTAACGTAG
	22B _ABZ	TTCAACATC	TTAACTTCC	TTAATCCTC	TTCTGACTC	TTAACGCCT
	23B _ABZ	TTCAAGAGA	TTAACTAGG	TTAATCGAG	TTCTGGATT	TTAAGTCCA
	24B _ABZ	TTCAAGGTT	TTAACAGTC	TTAACTAGC	TTCATTCGC	TTAAGTGAC
	25B _ABZ	TTCAGTTCA	TTAACCTTG	TTAACTCCA	TTCATCGTT	TTAAGAGCT
	26B _ABZ	TTCAGTAAG	TTAACCGAA	TTAACATGG	TTCATGTCT	TTAAGCATG
	27B _ABZ	TTCAGACTT	TTAACGACA	TTAACACAC	TTCATGGAC	TTAAGGAGT
	28B _ABZ	TTCAGAGGA	TTACTTACG	TTAACGTTC	TTCAAGCTT	TTACTTACG
	29B _ABZ	TTCAGCAGT	TTACTTGTC	TTAACGGCG	TTCCGTTAT	TTACTTGTC
	30B _ABZ	TTCAGCCAA	TTACTAGAG	TTAAGAGCT	TTCCGAGCT	TTACTATGG
	31B _ABZ	TTCAGGTAT	TTACTCTGA	TTAAGCTAC	TTCCGCTGC	TTACTAGCA
	32B _ABZ	TTCGTTCTA	TTACTCCTT	TTACTTACG	TTCCGCCTT	TTACTCCTT

33B _ABZ	TTCGTTGGT	TTACTGGCA	TTACTTGGT	TTCGTTACT	TTACTCGAG
34B _ABZ	TTCGTAGAG	TTACATTGC	TTACTAGTC	TTCGTTGTC	TTACATCAC
35B _ABZ	TTCGTGATC	TTACAGTAG	TTACTCTGA	TTCGTCTAT	TTACAACCT
36B _ABZ	TTCGATGTG	TTACAGGTT	TTACTGAGC	TTCGTGTGC	TTACAAGTG
19C _ABZ	TTCGAATCA	TTACCTAAC	TTACATTCC	TTCGACATT	TTACACTGT
20C _ABZ	TTCGACAAT	TTACCTCTA	TTACAACGT	TTCGACGGC	TTACACATC
21C _ABZ	TTCGAGCAC	TTACCTGGT	TTACACGAC	TTCGCATAC	TTACAGTCG
22C _ABZ	TTGTTCAGA	TTACCATCG	TTACAGCTC	TTCGCCGCT	TTACAGAGA
23C _ABZ	TTGTTCGTT	TTACCGTTC	TTACAGGCA	TTCGCGATC	TTACCTAGT
24C _ABZ	TTGTTGTAG	TTACGTCAG	TTACCAACA	TTGTTCTCT	TTACCACTA
25C _ABZ	TTGTATCGA	TTACGATAC	TTACCGTAT	TTGTTGCTT	TTACCAGAT
26C _ABZ	TTGTAATGG	TTACGACCA	TTACCGCGA	TTGTCTACT	TTACCGAAC
27C _ABZ	TTGTAAGTC	TTACGCCGC	TTACGTTAG	TTGTGTCAT	TTACCGCGG
28C _ABZ	TTGTAGAAC	TTACGCGTA	TTACGACAA	TTGTGCGTT	TTACGATAC
29C _ABZ	TTGTCTTCA	TTAGTTCTG	TTACGCATT	TTGATTCCT	TTACGCTTA
30C _ABZ	TTGTCTCTT	TTAGTTGGA	TTACGGCCG	TTGACTGAT	TTACGCAAT
31C _ABZ	TTGTCAGGT	TTAGTAACC	TTAGTTCAC	TTGAGCTAT	TTACGCCGC
32C _ABZ	TTGTCGATA	TTAGTACGT	TTAGTCGTT	TTGAGGACT	TTAGTACGA
33C _ABZ	TTGTGTATC	TTAGATCCT	TTAGTGCTA	TTGCTTGTT	TTAGTCTAC
34C _ABZ	TTGTGTGAA	TTAGATGAG	TTAGATGAG	TTGCTACAT	TTAGTCGCT
35C _ABZ	TTGTGACTA	TTAGACTAC	TTAGACTTC	TTGCATTCT	TTAGTGTGT
36C _ABZ	TTGTGCAAT	TTAGACATG	TTAGACACT	TTGCCGATT	TTAGTGATC
19A _IVM	TTGTGGTGT	TTAGAGTCA	TTAGAGCAT	TTGGTGGAT	TTAGTGGAA
20A _IVM	TTGATAGCA	TTAGCAGAT	TTAGCTTGT	TTGGATCTT	TTAGATAGC
21A _IVM	TTGATCTTG	TTAGCCTGT	TTAGCATCC	TTGGCGTCT	TTAGAACTC
22A _IVM	TTGATCAAC	TTAGGTACA	TTAGCCTAA	TATTAAGGC	TTAGAAGGT
23A _IVM	TTGATGAGG	TTAGGCGCC	TTAGCCGGC	TATTCTCCT	TTAGACTTG
24A _IVM	TTGAACTCA	TTCTTATGG	TTAGCGAGG	TATTCTGAC	TTAGACCAT
25A _IVM	TTGAAGTTC	TTCTTACTC	TTCTTATGG	TATTCCTTC	TTAGAGAAG
26A _IVM	TTGAAGGAA	TTCTTAGCA	TTCTTACTC	TATTCGCGC	TTAGCTCAA
27A _IVM	TTGACTATG	TTCTTCAGT	TTCTTAGCA	TATTCGGTT	TTAGCATCT
28A _IVM	TTGACGTGA	TTCTTCGAC	TTCTTCCAT	TATTGTTGC	TTAGCCTGA
29A _IVM	TTGACGAAT	TTCTTGAAG	TTCTTCGGC	TATTGAGCT	TTAGCCACG
30A _IVM	TTGAGTTGG	TTCTTGGTT	TTCTTGTCT	TATTGGATC	TTAGCGGCC
31A _IVM	TTGAGTCAT	TTCTATTCC	TTCTTGAGA	TATATCCTC	TTAGGCGTC
32A _IVM	TTGAGAGTG	TTCTATAGG	TTCTATACC	TATAATCGC	TTGTTAAGG

33A _IVM	TTGAGCCTC	TTCTAACAG	TTCTAACGA	TATAAGCCT	TTGTTACTC
34A _IVM	TTGAGGACA	TTCTACCGA	TTCTACGCG	TATAAGGTC	TTGTTGTTG
35A _IVM	TTGCATAAG	TTCTAGTTG	TTCTAGTTC	TATACATGC	TTGTTGAAC
36A _IVM	TTGCATGTT	TTCTAGCCT	TTCTCTTAC	TATCTTGTC	TTGTTGGCT
19B _IVM	TTGCAACAA	TTCTAGGAA	TTCTCTAGT	TATCTAAGC	TTGTAATCG
20B _IVM	TTGCAGTAT	TTCTCTTAG	TTCTCACAG	TATCTCGCT	TTGTACTGA
21B _IVM	TTGCCTAGT	TTCTCTACA	TTCTCCGTT	TATCCGTCT	TTGTACACT
22B _IVM	TTGCCTCAC	TTCTCTCTT	TTCTCGCTA	TATCGATTC	TTGTACCAG
23B _IVM	TTGCCAATC	TTCTCTGGC	TTCTGTTGA	TATCGCGAC	TTGTACGTC
24B _IVM	TTGCCAGAG	TTCTCCATC	TTCTGTAAG	TATCGGCTT	TTGTAGCGC
25B _IVM	TTGGTTGTC	TTCTCCGCT	TTCTGGCAC	TATGTCTGC	TTGTCTTAC
26B _IVM	TTGGTATGA	TTCTCGTGA	TTCAATTGG	TATGTGACT	TTGTCTATG
27B _IVM	TTGGTCTAT	TTCTGTGTA	TTCAATCTC	TATGATGCT	TTGTCTCCT
28B _IVM	TTGGTGCCA	TTCTGAACC	TTCAACAAG	TATGACATC	TTGTCAACA
29B _IVM	TTGGAACTT	TTCTGACGT	TTCAACGGA	TATGGACGC	TTGTCAGTT
30B _IVM	TTGGACATA	TTCTGCTCA	TTCAAGTCA	TATGGCTCT	TTGTCCGAA
31B _IVM	TTGGACGAC	TTCATTGTG	TTCAAGAGT	TAATTGTGC	TTGTCGTGT
32B _IVM	TATATCAGG	TTCATCTTC	TTCACTTCT	TAATTGCCT	TTGTGTAGT
33B _IVM	TATATCGTC	TTCATGTCA	TTCACAATC	TAATACGAC	TTGTGTCAA
34B _IVM	TATATGCAC	TTCAGTTAC	TTCACACGT	TAATCCGCT	TTGTGAGGA
35B _IVM	TATAACGAG	TTCAGTCCT	TTCACCACA	TAATGTGTC	TTGTGGATA
36B _IVM	TATAAGTGG	TTCAGATTG	TTCAGTCCG	TAAGCTTAC	TTGTGGCCG
19C _IVM	TATAAGCCA	TTCAGAAGA	TTCAGTGTT	TAAGCAAGC	TTGATAACC
20C _IVM	TATAGTCTC	TTCAGCTGT	TTCAGCTTA	TAAGCCGTC	TTGATAGAG
21C _IVM	TATAGACAG	TTCAGGCTA	TTCAGCGCC	TAAGCGCAT	TTGATCTGT
NTC_1	TATAGAGGT	TTCCTTCAT	TTCAGGCGA	TAAGGTCCT	TTGATCGCA
NTC_2	TATAGCAAC	TTCCTAATG	TTCCTTATC	TAAGGCAAT	TTGAATACG
NTC_3	TATAGGATG	TTCCTACGA	TTCCTTCAA	TACTATCTC	TTGAATGGC

Library	Sample ID	515f primer index	ITS4r primer index	EukBr primer index	amoA-1F primer index	amoA310f primer index
	22C _IVM	TTCTTCTTC	TTATTACCG	TTATTACCG	TTATTACGC	TTATTAGGC
	23C _IVM	TTCTTCAAG	TTATTAGGC	TTATTCTCC	TTATTCGTC	TTATACTCC
	24C _IVM	TTCTTGTCA	TTATTCTCC	TTATTCAGG	TTATATGGC	TTATACAGG
	25C _IVM	TTCTTGAGT	TTATTCGTG	TTATTGCGT	TTATCTCTC	TTATAGCTG
	26C _IVM	TTCTTGGAC	TTATTGCGA	TTATTGGAC	TTATCCTAC	TTATAGGAC
	27C _IVM	TTCTATAGG	TTATACTGG	TTATATCGC	TTATCGAGC	TTATGTTGG
	28C _IVM	TTCTATCTC	TTATACCTC	TTATAAGCC	TTATGGTTC	TTATGTCTC
	29C _IVM	TTCTATGCA	TTATACGCA	TTATACCAG	TTAATCTGC	TTATGAACC
	30C _IVM	TTCTAACAG	TTATAGACC	TTATACGGT	TTAATGCTC	TTATGACGT
	31C _IVM	TTCTAGTTG	TTATGTTCG	TTATAGTGG	TTAAGCATC	TTATGAGAG
	32C _IVM	TTCTCTTGT	TTATGTGAC	TTATCTTCG	TTACTTGAC	TTATGGTCA
	33C _IVM	TTCTCTAAC	TTATGAAGG	TTATCCATC	TTACAATGC	TTAATCTCG
	34C _IVM	TTCTCAATG	TTATGAGCT	TTATCCGCA	TTACGTCGC	TTAATCAGC
	35C _IVM	TTCTCAGAA	TTATGCCAT	TTATCGACT	TTCTATCCT	TTAATGGTG
	36C _IVM	TTCATATGG	TTATGGTGT	TTATGTCCT	TTCTACTTC	TTAACTACC
	19A _EPM	TTCATAGTC	TTAATTCGC	TTATGTGTC	TTCTACGAT	TTAACTCTG
	20A _EPM	TTCATCACA	TTAATCCAG	TTATGATGC	TTCTCTTGC	TTAACTGGA
	21A _EPM	TTCATCGAT	TTAATCGGT	TTATGAGAG	TTCTCTGTT	TTAACAAGG
	22A _EPM	TTCAATCGT	TTAATGTGG	TTATGCCGA	TTCTCATCT	TTAACACAC
	23A _EPM	TTCAATGAC	TTAATGCCT	TTAATTCGG	TTCTCGCAT	TTAACCGTT
	24A _EPM	TTCAACTAG	TTAATGGAC	TTAATTGCC	TTCTGTGAC	TTAACGTAG
	25A _EPM	TTCAACATC	TTAACTTCC	TTAATCCTC	TTCTGACTC	TTAACGCCT
	26A _EPM	TTCAAGAGA	TTAACTAGG	TTAATCGAG	TTCTGGATT	TTAAGTCCA
	27A _EPM	TTCAAGGTT	TTAACAGTC	TTAACTAGC	TTCATTCGC	TTAAGTGAC
	28A _EPM	TTCAGTTCA	TTAACCTTG	TTAACTCCA	TTCATCGTT	TTAAGAGCT
	29A _EPM	TTCAGTAAG	TTAACCGAA	TTAACATGG	TTCATGTCT	TTAAGCATG
	30A _EPM	TTCAGACTT	TTAACGACA	TTAACACAC	TTCATGGAC	TTAAGGAGT
2ⁿᵈ Library	31A _EPM	TTCAGAGGA	TTACTTACG	TTAACGTTC	TTCAAGCTT	TTACTTACG
	32A _EPM	TTCAGCAGT	TTACTTGTC	TTAACGGCG	TTCCGTTAT	TTACTTGTC
	33A _EPM	TTCAGCCAA	TTACTAGAG	TTAAGAGCT	TTCCGAGCT	TTACTATGG
	34A _EPM	TTCAGGTAT	TTACTCTGA	TTAAGCTAC	TTCCGCTGC	TTACTAGCA
	35A _EPM	TTCGTTCTA	TTACTCCTT	TTACTTACG	TTCCGCCTT	TTACTCCTT
	36A _EPM	TTCGTTGGT	TTACTGGCA	TTACTTGGT	TTCGTTACT	TTACTCGAG
	19B _EPM	TTCGTAGAG	TTACATTGC	TTACTAGTC	TTCGTTGTC	TTACATCAC
	20B _EPM	TTCGTGATC	TTACAGTAG	TTACTCTGA	TTCGTCTAT	TTACAACCT
	21B _EPM	TTCGATGTG	TTACAGGTT	TTACTGAGC	TTCGTGTGC	TTACAAGTG
	22B _EPM	TTCGAATCA	TTACCTAAC	TTACATTCC	TTCGACATT	TTACACTGT
	23B _EPM	TTCGACAAT	TTACCTCTA	TTACAACGT	TTCGACGGC	TTACACATC
	24B _EPM	TTCGAGCAC	TTACCTGGT	TTACACGAC	TTCGCATAC	TTACAGTCG
	25B _EPM	TTGTTCAGA	TTACCATCG	TTACAGCTC	TTCGCCGCT	TTACAGAGA
	26B _EPM	TTGTTCGTT	TTACCGTTC	TTACAGGCA	TTCGCGATC	TTACCTAGT
	27B _EPM	TTGTTGTAG	TTACGTCAG	TTACCAACA	TTGTTCTCT	TTACCACTA
	28B _EPM	TTGTATCGA	TTACGATAC	TTACCGTAT	TTGTTGCTT	TTACCAGAT
	29B _EPM	TTGTAATGG	TTACGACCA	TTACCGCGA	TTGTCTACT	TTACCGAAC
	30B _EPM	TTGTAAGTC	TTACGCCGC	TTACGTTAG	TTGTGTCAT	TTACCGCGG
	31B _EPM	TTGTAGAAC	TTACGCGTA	TTACGACAA	TTGTGCGTT	TTACGATAC
	32B _EPM	TTGTCTTCA	TTAGTTCTG	TTACGCATT	TTGATTCCT	TTACGCTTA
	33B _EPM	TTGTCTCTT	TTAGTTGGA	TTACGGCCG	TTGACTGAT	TTACGCAAT
	34B _EPM	TTGTCAGGT	TTAGTAACC	TTAGTTCAC	TTGAGCTAT	TTACGCCGC
	35B _EPM	TTGTCGATA	TTAGTACGT	TTAGTCGTT	TTGAGGACT	TTAGTACGA
	36B _EPM	TTGTGTATC	TTAGATCCT	TTAGTGCTA	TTGCTTGTT	TTAGTCTAC
	19C _EPM	TTGTGTGAA	TTAGATGAG	TTAGATGAG	TTGCTACAT	TTAGTCGCT
	20C _EPM	TTGTGACTA	TTAGACTAC	TTAGACTTC	TTGCATTCT	TTAGTGTGT
	21C _EPM	TTGTGCAAT	TTAGACATG	TTAGACACT	TTGCCGATT	TTAGTGATC
	22C _EPM	TTGTGGTGT	TTAGAGTCA	TTAGAGCAT	TTGGTGGAT	TTAGTGGAA
	23C _EPM	TTGATAGCA	TTAGCAGAT	TTAGCTTGT	TTGGATCTT	TTAGATAGC
	24C _EPM	TTGATCTTG	TTAGCCTGT	TTAGCATCC	TTGGCGTCT	TTAGAACTC

25C _EPM	TTGATCAAC	TTAGGTACA	TTAGCCTAA	TATTAAGGC	TTAGAAGGT
26C _EPM	TTGATGAGG	TTAGGCGCC	TTAGCCGGC	TATTCTCCT	TTAGACTTG
27C _EPM	TTGAACTCA	TTCTTATGG	TTAGCGAGG	TATTCTGAC	TTAGACCAT
28C _EPM	TTGAAGTTC	TTCTTACTC	TTCTTATGG	TATTCCTTC	TTAGAGAAG
29C _EPM	TTGAAGGAA	TTCTTAGCA	TTCTTACTC	TATTCGCGC	TTAGCTCAA
30C _EPM	TTGACTATG	TTCTTCAGT	TTCTTAGCA	TATTCGGTT	TTAGCATCT
31C _EPM	TTGACGTGA	TTCTTCGAC	TTCTTCCAT	TATTGTTGC	TTAGCCTGA
32C _EPM	TTGACGAAT	TTCTTGAAG	TTCTTCGGC	TATTGAGCT	TTAGCCACG
33C _EPM	TTGAGTTGG	TTCTTGGTT	TTCTTGTCT	TATTGGATC	TTAGCGGCC
34C _EPM	TTGAGTCAT	TTCTATTCC	TTCTTGAGA	TATATCCTC	TTAGGCGTC
35C _EPM	TTGAGAGTG	TTCTATAGG	TTCTATACC	TATAATCGC	TTGTTAAGG
36C _EPM	TTGAGCCTC	TTCTAACAG	TTCTAACGA	TATAAGCCT	TTGTTACTC
NTC_4	TTGAGGACA	TTCTACCGA	TTCTACGCG	TATAAGGTC	TTGTTGTTG
NTC_5	TTGCATAAG	TTCTAGTTG	TTCTAGTTC	TATACATGC	TTGTTGAAC
NTC_6	TTGCATGTT	TTCTAGCCT	TTCTCTTAC	TATCTTGTC	TTGTTGGCT

Soil/treatment	Circle of application	DT$_{50}$ (days)	χ^2 (%)
Albendazole			
Fast soil 2 μg g^{-1}		**3.2**	9.0
Fast soil 20 μg g^{-1}	1st	**4.6**	9.5
Slow soil 2 μg g^{-1}	application	3.2	6.8
Slow soil 20 μg g^{-1}		5.2	3.6
Fast soil 2 μg g^{-1}		1.14	2.97
Fast soil 20 μg g^{-1}	2nd	2.54	7.33
Slow soil 2 μg g^{-1}	application	3.93	1.48
Slow soil 20 μg g^{-1}		7.28	1.68
Fast soil 2 μg g^{-1}		**0.7**	4.1
Fast soil 20 μg g^{-1}	3rd	**1.5**	4.9
Slow soil 2 μg g^{-1}	application	3.4	1.1
Slow soil 20 μg g^{-1}		20.4	3.3
Ivermectin			
Fast soil 1 μg g^{-1}		48.1	6.2
Fast soil 10 μg g^{-1}	1st	72.1	2.2
Slow soil 1 μg g^{-1}	application	33.1	1.3
Slow soil 10 μg g^{-1}		61.3	4.5
Fast soil 1 μg g^{-1}		54.9	8.2
Fast soil 10 μg g^{-1}	2nd	70.4	3.2
Slow soil 1 μg g^{-1}	application	50.7	8.5
Slow soil 10 μg g^{-1}		76.6	4.3
Fast soil 1 μg g^{-1}		79.9	1.8
Fast soil 10 μg g^{-1}	3rd	65.3	2.8
Slow soil 1 μg g^{-1}	application	56.1	2.1
Slow soil 10 μg g^{-1}		82.2	1.6
Eprinomectin			
Fast soil 1 μg g^{-1}		55.2	2.1
Fast soil 10 μg g^{-1}	1st	67.7	2.1
Slow soil 1 μg g^{-1}	application	60.5	0.6
Slow soil 10 μg g^{-1}		69.7	1.4
Fast soil 1 μg g^{-1}		71.9	3.1
Fast soil 10 μg g^{-1}	2nd	54.4	1.5
Slow soil 1 μg g^{-1}	application	66.5	0.8
Slow soil 10 μg g^{-1}		59.4	3.1
Fast soil 1 μg g^{-1}		74.1	3.9
Fast soil 10 μg g^{-1}	3rd	59.5	3.6
Slow soil 1 μg g^{-1}	application	77.1	5.3
Slow soil 10 μg g^{-1}		79.7	3.1

Supplementary Figure S3.1 Effects of Albendazole (ABZ), Ivermectin (IVM) and Eprinomectin (EPM) on the abundance of the 16S rRNA gene of bacteria, 18S rRNA gene of fungi and 16S rRNA gene of Crenarchaeota in the «fast» and «slow» soils after three repeated applications. The dashed vertical lines indicate the different applications. Each value is the mean from three replicates ± standard deviation. The abundance data are presented on a logarithmic scale (log base = 10). Bars designated by the same letter within each time group are not significantly different at the 0.05 level.

Supplementary Figure S3.2 The levels ammonium and nitrate in the «fast» and the «slow» soils either untreated (control) or repeatedly treated with two different dose rates of Albendazole (ABZ), Ivermectin (IVM) and Eprinomectin (EPM). Each value is the mean from three replicates ± standard deviation. At each time point bars designated by the same letter are not significantly different at the 0.05 level.

Albendazole
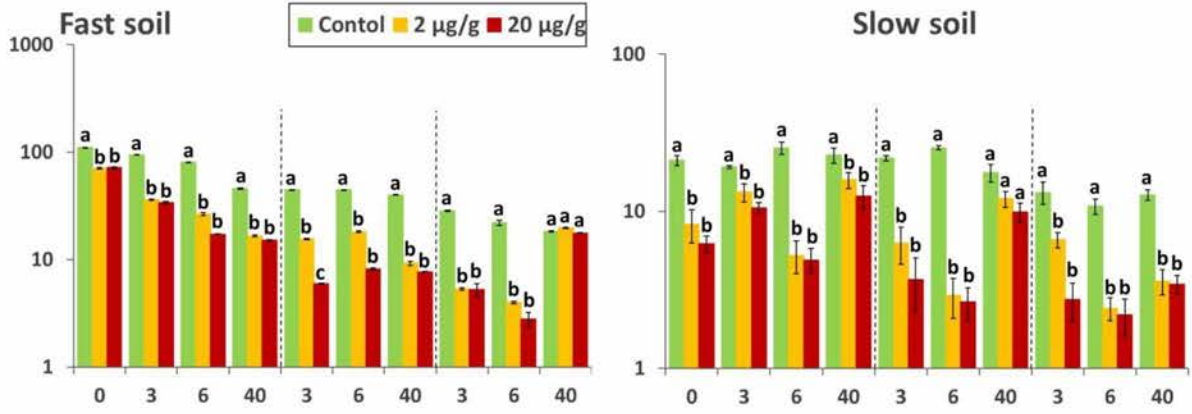
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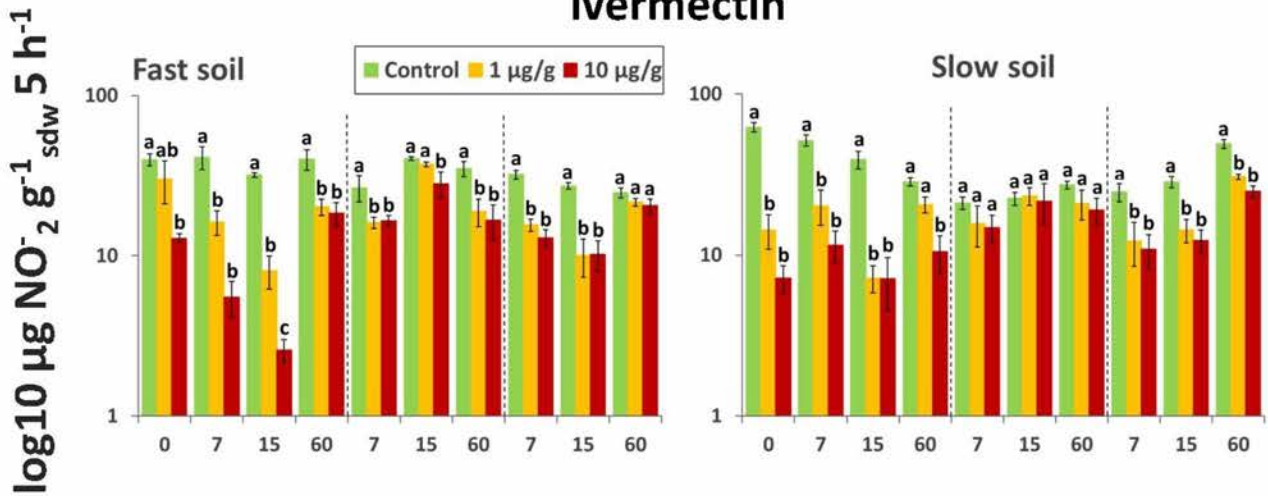
Eprinomectin
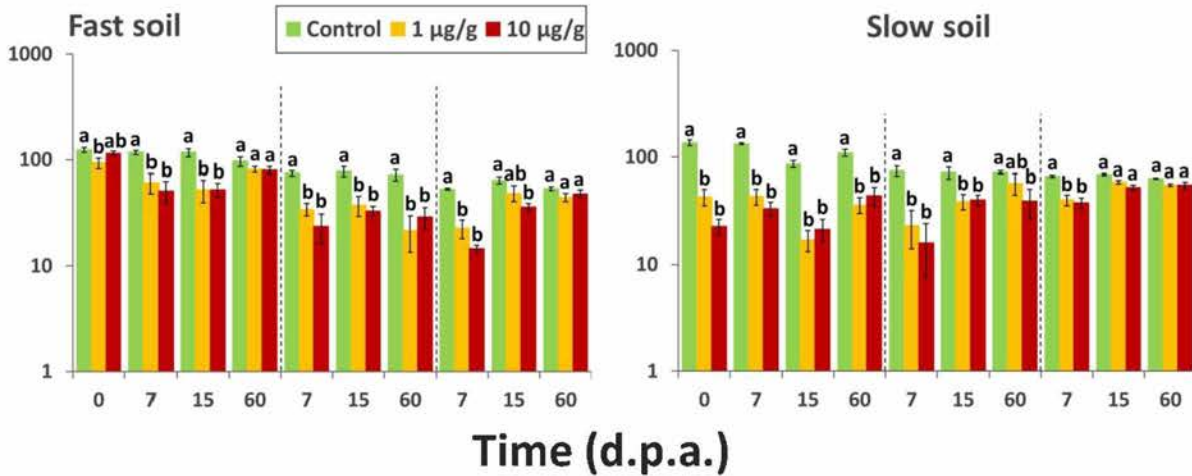
Supplementary Figure S3.3 The potential nitrification rates in the «fast» and the «slow» soils either untreated (control) or repeatedly treated with two different dose rates of Albendazole (ABZ), Ivermectin (IVM) and Eprinomectin (EPM) . Each value is the mean from three replicates ± standard deviation. At each time point bars designated by the same letter are not significantly different at the the 0.05 level.
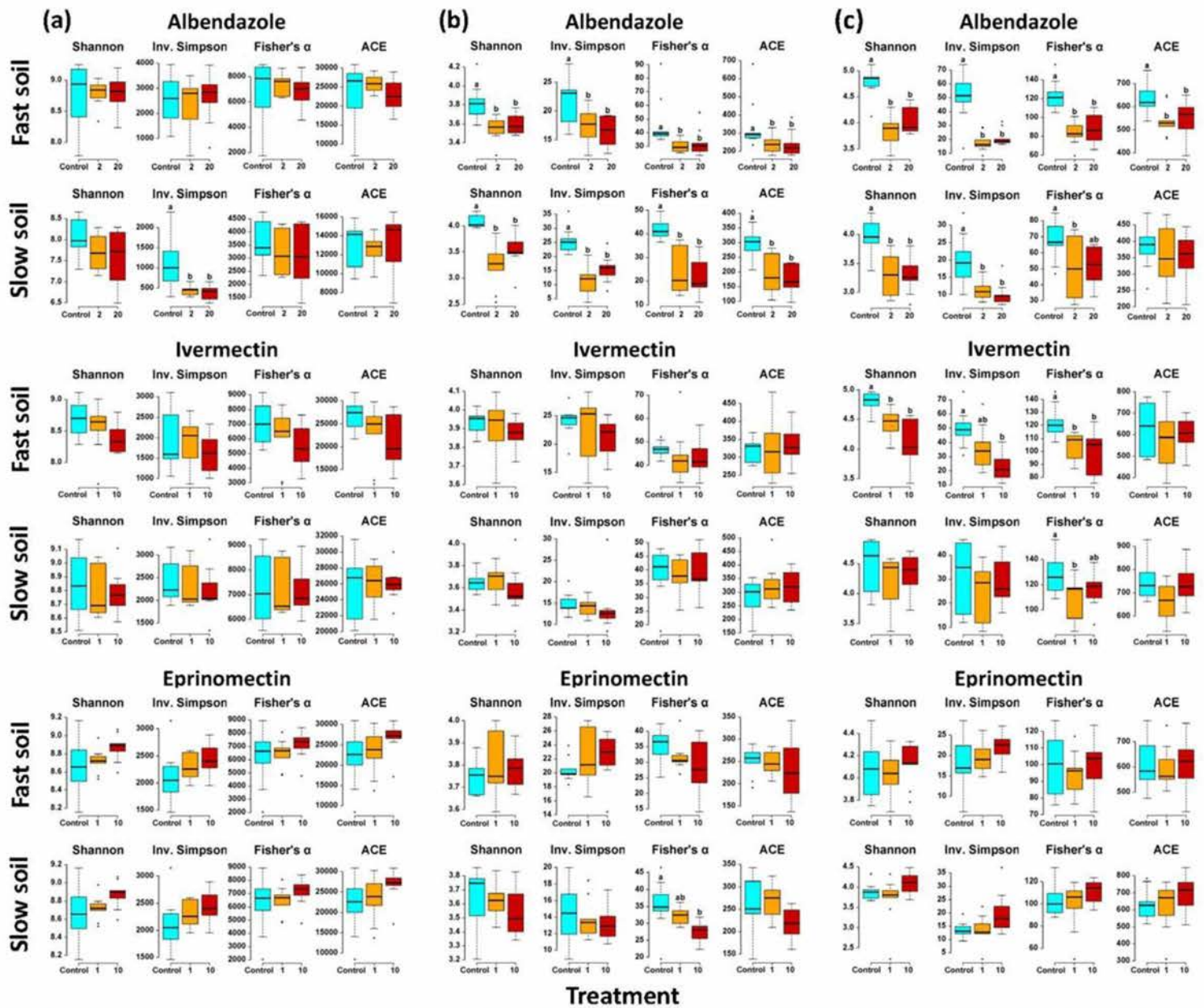
Supplementary Figure S3.4 The α-diversity indices of (a) bacteria (b) fungi (c) protists in the «fast» and the «slow» soils which were not treated (control) or treated with 1 (2) and 10 (20) mg Kg^{-1} of Albendazole (ABZ), Ivermectin (IVM) or Eprinomectin (EPM). Each value is the mean from three replicates ± standard deviation. Bars designated by different letter within each index are significantly different at the 0.05 level.
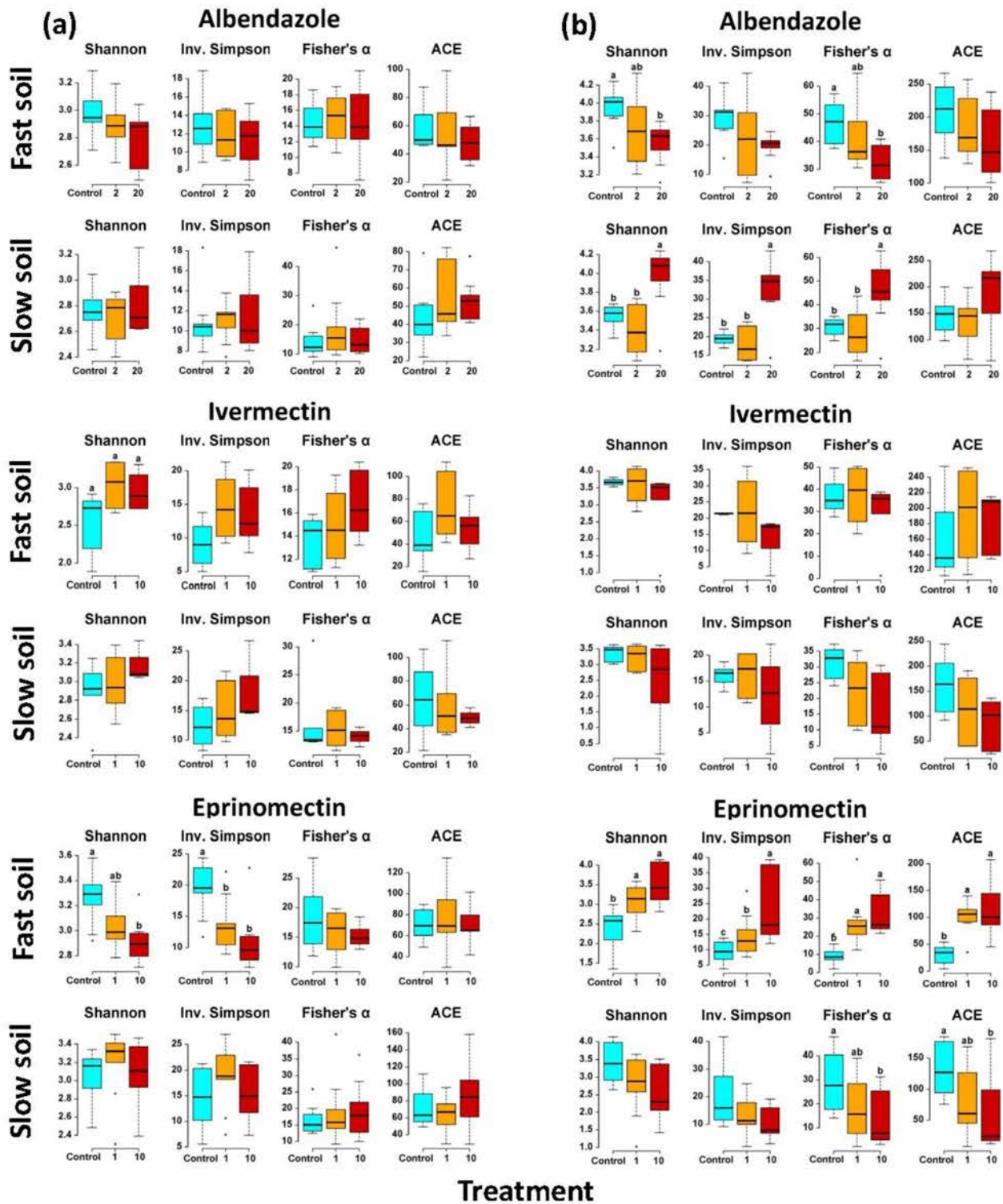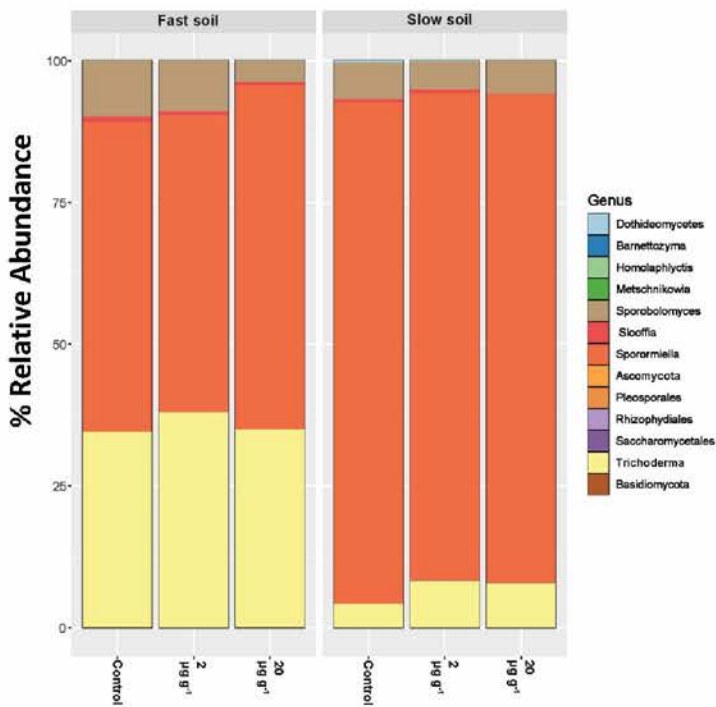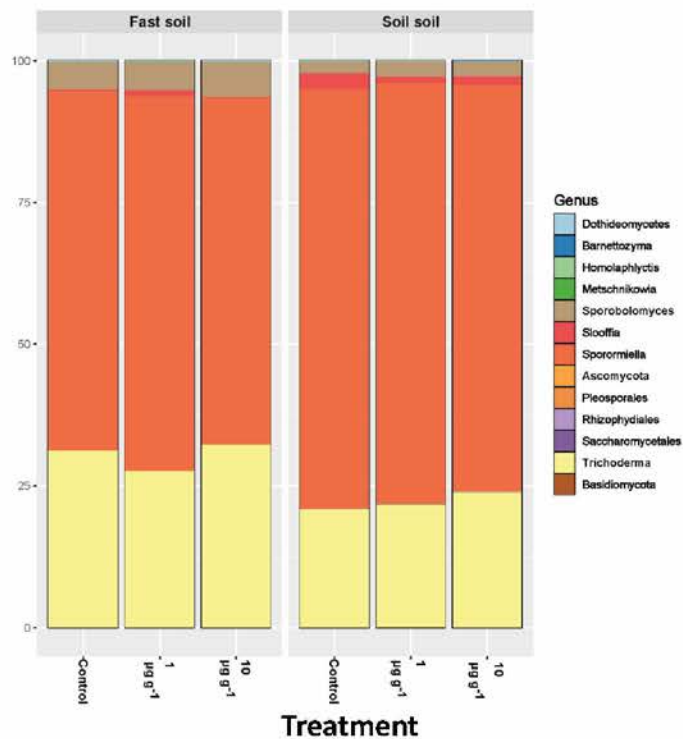
Supplementary Figure S3.5 The α-diversity indices of (a) AOA and (b) AOB in the «fast» and the «slow» soils which were not treated (control) or treated with 1 (2) and 10 (20) mg Kg^{-1} of Albendazole (ABZ), Ivermectin (IVM) or Eprinomectin (EPM). Each value is the mean from three replicates ± standard deviation. Bars designated by different letter within each index are significantly different at the 0.05 level.
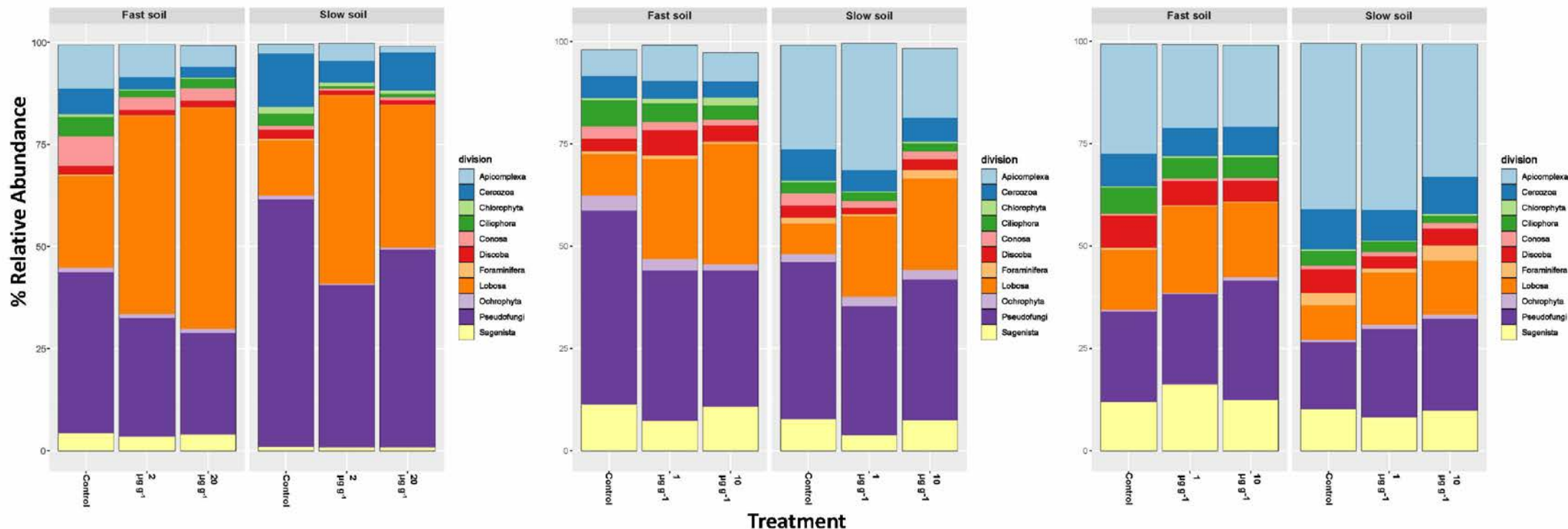
Supplementary Figure S3.6 The composition (in terms of relative abundance) of the bacterial community (phyla which represent >1% of the total community are presented) in the «fast» and the «slow» soils which were either untreated or repeatedly treated with 1 (2) and 10 (20) mg Kg^{-1}, of Albendazole, Ivermectin and Eprinomectin.
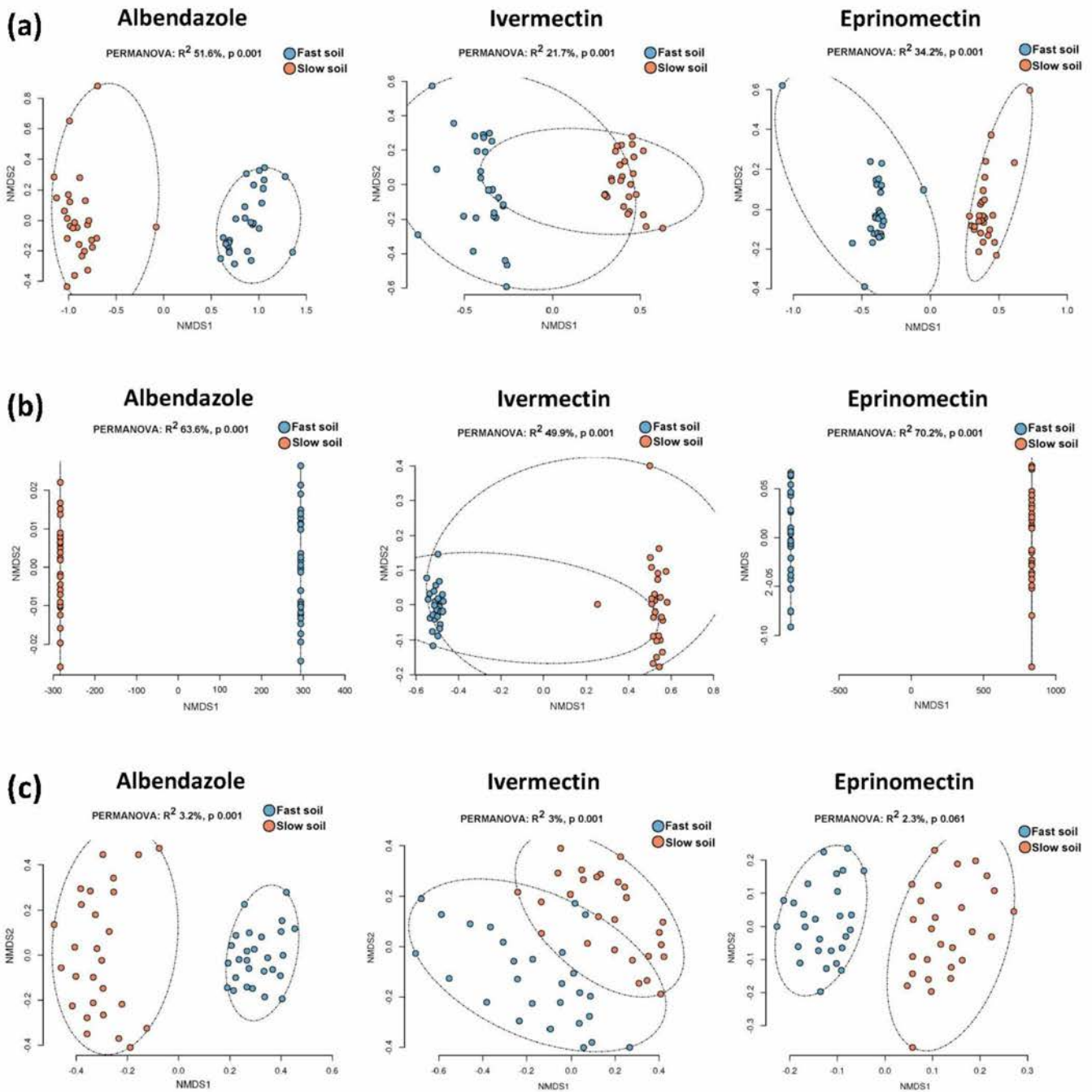
Supplementary Figure S3.7 The composition (in terms of relative abundance) of the fungal community (phyla which represent >1% of the total community are presented) in the «fast» and the «slow» soils which were either untreated or repeatedly treated with 1 (2) and 10 (20) mg Kg^{-1}, of Albendazole, Ivermectin and Eprinomectin.
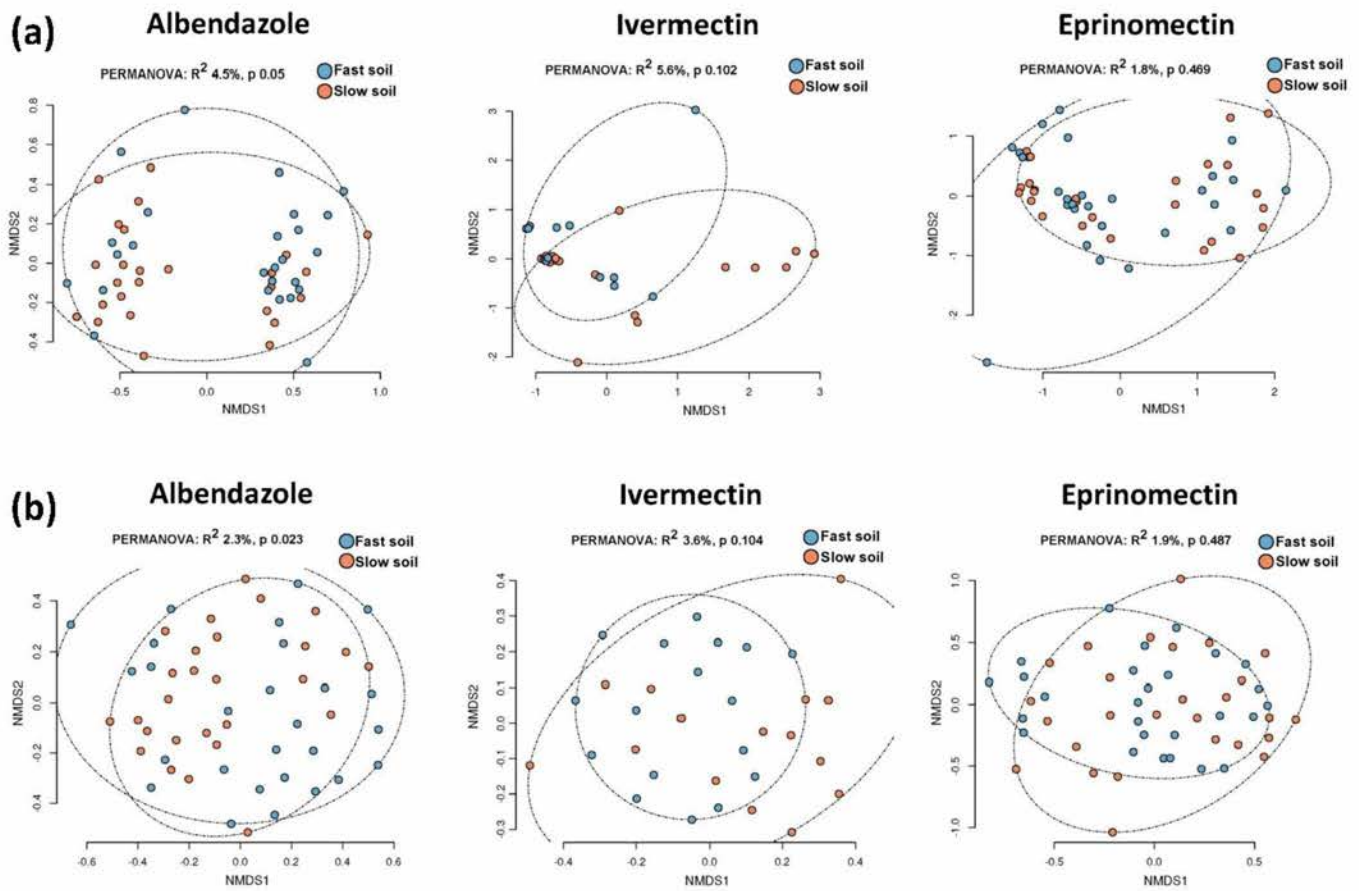
Supplementary Figure S3.8 The composition (in terms of relative abundance) of the protists community (phyla which represent >1% of the total community are presented) in the «fast» and the «slow» soils which were either untreated or repeatedly treated with 1 (2) and 10 (20) mg Kg^{-1}, of Albendazole, Ivermectin and Eprinomectin.
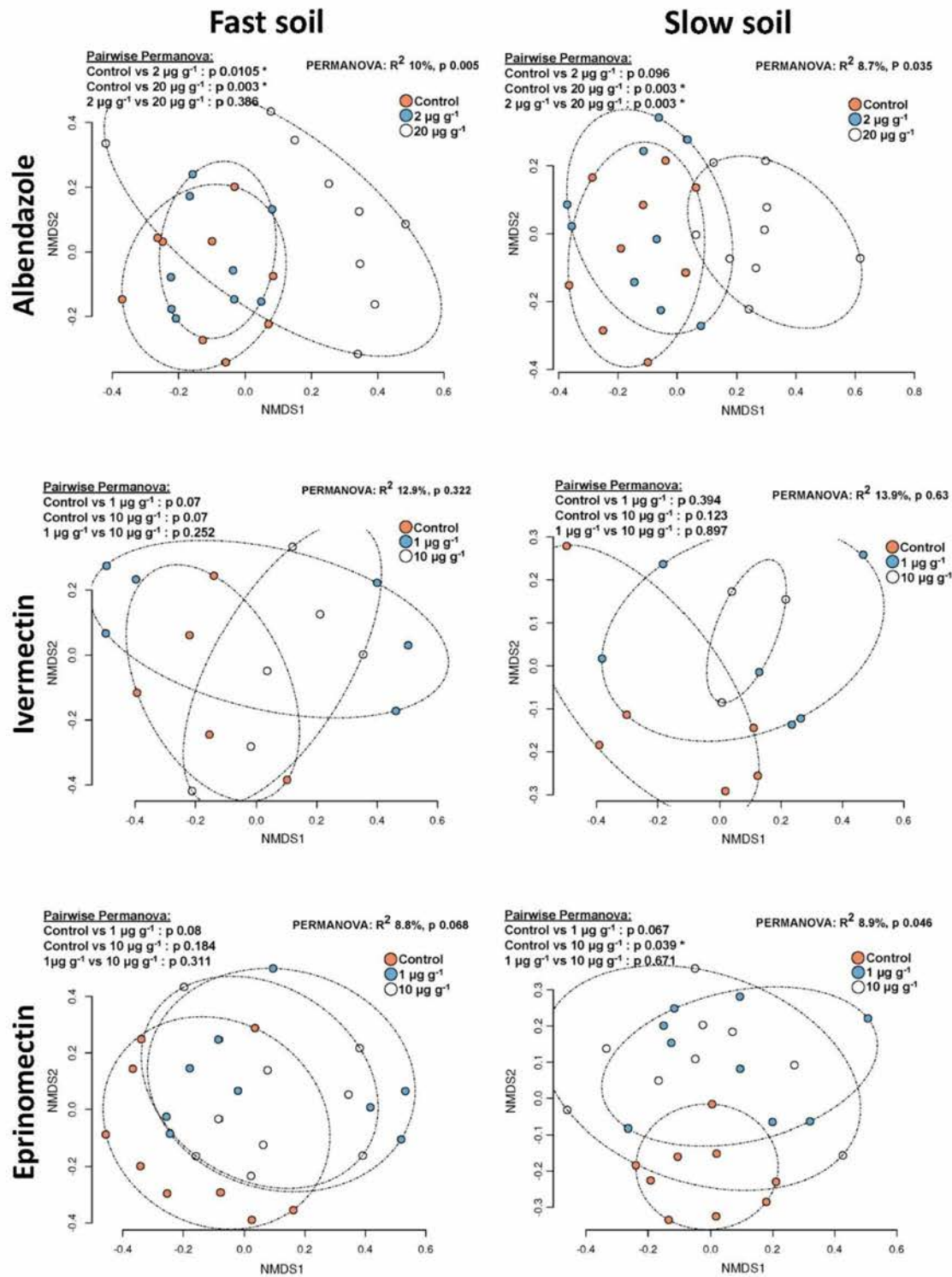
Supplementary Figure S3.9 Non-metric multidimensional scaling (NMDS) ordination plot illustrating differences in the composition of the (a) bacterial, (b) fungal and (c) protists community between the different soil samples. Samples are ordinated according to different soil in which AHs were applied.

Supplementary Figure S3.10 Non-metric multidimensional scaling (NMDS) ordination plot illustrating differences in the composition of the (a) AOB and (b) AOA community between the different soil samples. Samples are ordinated according to different soil in which AHs were applied.
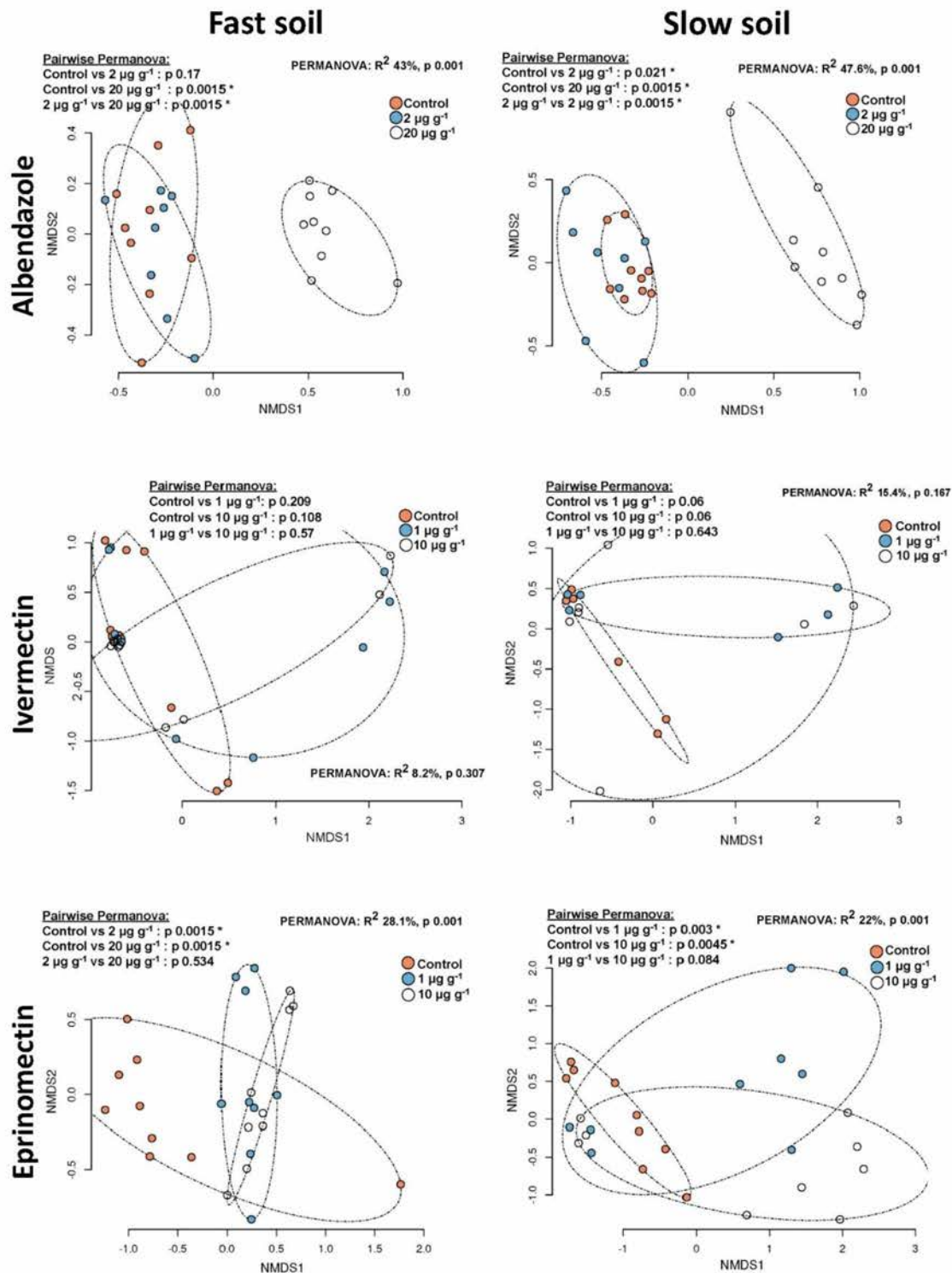
Supplementary Figure S3.11 Non-metric multidimensional scaling (NMDS) ordination plot illustrating the effects of albendazole (ABZ) (a, b), ivermectin (IVM) (c, d), and eprinomectin (EPM) (e, f) on the structure of the community of ammonia-oxidizing bacteria (AOB) in the «fast» (a, c, e) and the «slow» (b, d, f) soil. Samples were ordinated according to the dose rate of the AHs (control, 1 (2) and 10 (20) mg Kg^{-1}. Pairwise comparisons between different treatments are also presented. Significant difference ($p<0.05$) between groups is indicated with an asterisk (*).

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Supplementary Figure S3.12 Non-metric multidimensional scaling (NMDS) ordination plot illustrating the effects of albendazole (ABZ) (a, b), ivermectin (IVM) (c, d), and eprinomectin (EPM) (e, f) on the structure of the community of ammonia-oxidizing archaea (AOA) in the «fast» (a, c, e) and the «slow» (b, d, f) soil. Samples were ordinated according to the dose rate of the AHs (control, 1 (2) and 10 (20) mg Kg^{-1}. Pairwise comparisons between different treatments are also presented. Significant difference (p<0.05) between groups is indicated with an asterisk (*).

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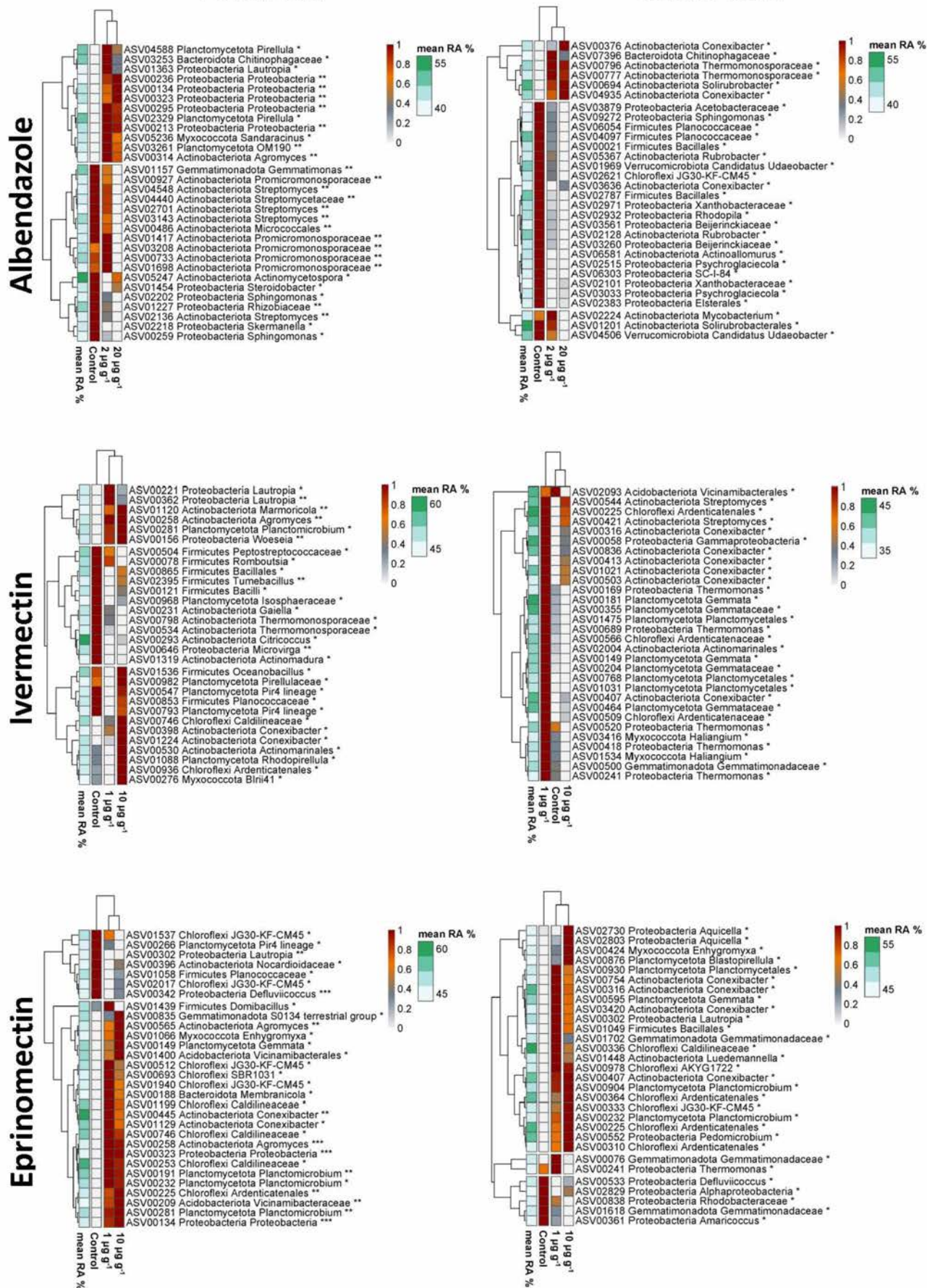
Supplementary Figure S3.13 Heatmaps showing bacterial ASVs that showed significant differential abundance in the different AH treatments (control, 1 (2) and 10 (20) mg Kg⁻¹). Relative abundance (RA) values are presented «ranked» (with values from 0 to 1).
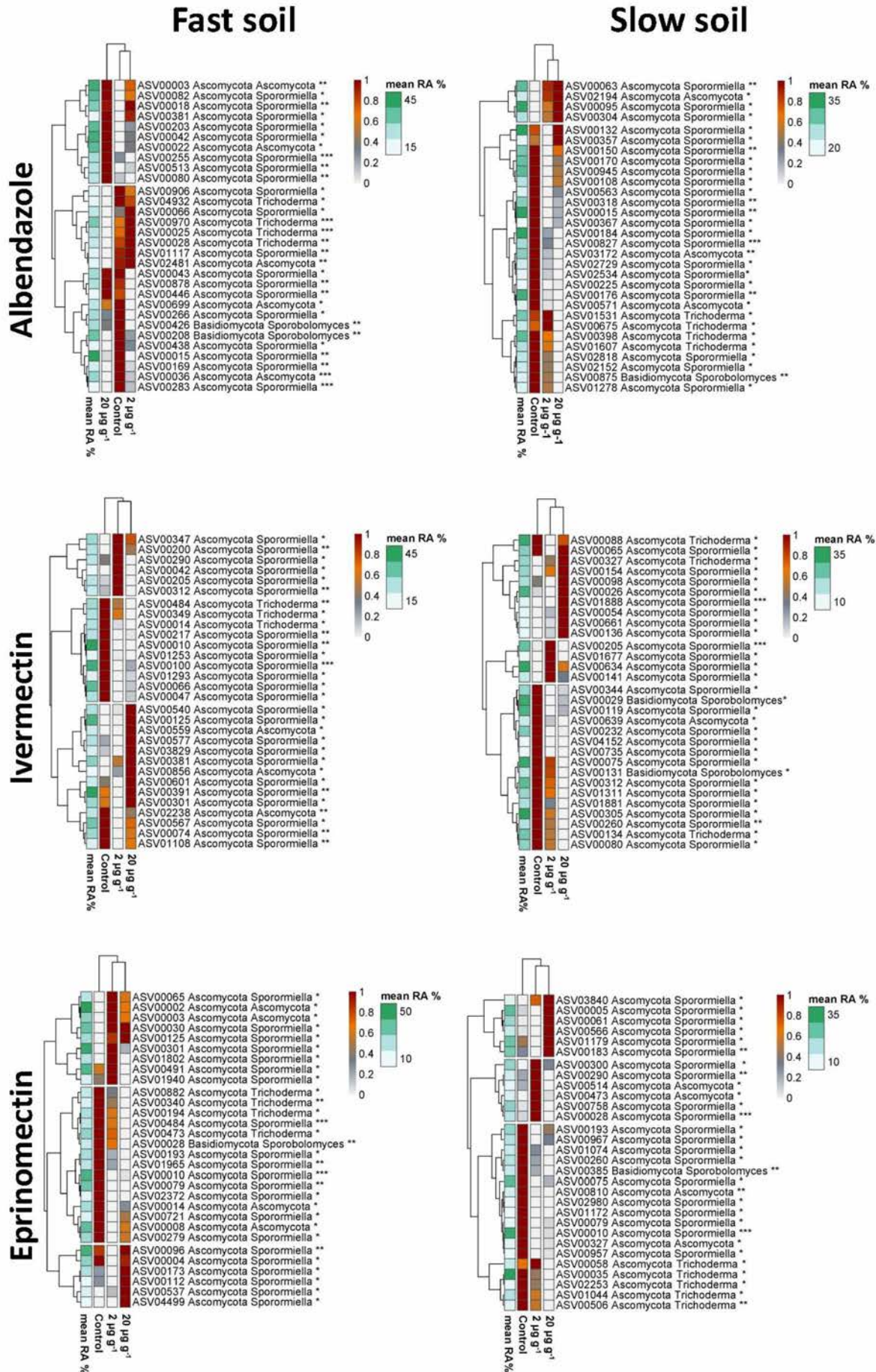
Supplementary Figure S3.14 Heatmaps showing fungal ASVs that showed significant differential abundance in the different AH treatments (control, 1 (2) and 10 (20) mg Kg⁻¹). Relative abundance (RA) values are presented «ranked» (with values from 0 to 1).

Supplementary Figure S3.15 Heatmaps showing protists ASVs that showed significant differential abundance in the different AH treatments (control, 1 (2) and 10 (20) mg Kg^{-1}). Relative abundance (RA) values are presented «ranked» (with values from 0 to 1).

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Chapter 4

Bioaugmentation of animal feces as a mean to mitigate environmental contamination with anthelmintic benzimidazoles.

1. Introduction

Infections by gastrointestinal nematodes (GINs) affect both the health and the welfare of grazing ruminants, causing reduced productivity, ailing health and necessitating heavy chemical drug use since their control relies on repeated dosing with anthelmintic (AH) drugs (Kaplan et al., 2020). Benzimidazoles (BZs) constitute one of the most important groups of anthelmintic compounds including albendazole (ABZ), fenbendazole (FBZ), flubendazole (FLU), ricobendazole, mebendazole (MBZ) and thiabendazole (TBZ) (McKellar and Scott, 1990). Upon their application BZs are excreted (i) intact in feces (75% of the dose) and urine (14% of the dose), which was the case for TBZ (Tocco et al., 1964) and FBZ (McKellar et al., 2002) (ii) oxidized to bioactive sulfoxides and inactive sulfones or hydrolyzed to inactive derivatives, like ABZ does (Gyurik et al., 1981). Recently Porto et al. (2020) reported that administration of ABZ and FBZ to sheep resulted in the detection of concentrations of 12.8 and 10 mg kg^{-1} in feces, respectively, while active (sulfoxide) and inactive (sulfones) derivatives were also detected in feces at levels below 0.5 mg kg^{-1} until 120 h after administration. Similar studies with ABZ in lamp provided analogous results with the parent compound (7.7 mg kg^{-1}) and its sulfoxide (2.8 mg kg^{-1}) and sulfone (2.4 mg kg^{-1}) being the main residual components in animal feces until 72 h (Prchal et al., 2016). Other monitoring studies have reported residues of BZs in feces exceeding 1000 mg kg^{-1} (Gyurik et al., 1981).

When in feces BZs are particularly persistent with more than 72% and 80% of FLU and FBZ respectively, remaining in pig fecal material after 102 days (Kreuzig et al., 2007). The persistence of BZs in fecal material could lead to accumulation of their residues in piles in the floor of livestock farms. These constitute a previously not considered point source for the contamination of natural water resources via surface runoff or leaching. In addition, the potential use of this pilled fecal material as manure in agricultural fields facilitates the dispersal of benzimidazole residues in agricultural soils with reciprocal risk for further translocation to other environmental compartments. Monitoring studies have detected residues of MBZ (14 ng L^{-1}) in surface water systems in Brazil (Sodre et al., 2018), of TBZ (1.43–1.46 ng L^{-1}), FBZ (3.9–6.4 ng L^{-1}) and FBZ sulfoxide (3.7–5.1 ng L^{-1}) in marine waters and sediments in Korea (Kim et al., 2017), and of ABZ in riverine water (11 ng L^{-1}), seawater (8 ng L^{-1}) and drinking water (2.8 ng L^{-1}) (Sim et al., 2013; Petrovic et al., 2014). Considering (i) the high toxicity of BZs to aquatic (Wagil et al., 2015) and terrestrial organisms (Wang et al., 2009) and (ii) their demonstrated uptake and translocation to upper plant parts (Raisova et al., 2017), their environmental dispersal constitutes a potential threat for natural resources and for human health.

Little is known about the environmental fate of BZ AHs in soils. ABZ is rapidly oxidized to sulfoxide (ABZ-SO), which possesses anthelmintic properties (used as ricobendazole in veterinary practice), and sulfone (ABZ-SO$_2$). The DT$_{50}$ values of ABZ in lab and field studies ranged from 0.1 to 4.2 days (Lagos et al., 2019) and from 4.95 to 6.3 days (Wu and Hu, 2013) respectively. Regarding FBZ and FLU, Kreuzig et al. (2007) reported DT$_{50}$ values of 54 and 174 days respectively. The oxidized derivatives of ABZ (ABZ-SO, ABZ-SO$_2$) and FBZ (FBZ-SO) are more polar (see Table 1, water solubilities) and prone to leaching to groundwater, as shown by Porto et al. (2020). Regarding TBZ, it is considered moderately to highly persistent in soil with DT$_{50}$ values ranging from 43.3 to over 365 days (European Commission (EC), 2001; Omirou et al., 2012; Karas et al., 2015; Papazlatani et al., 2019).

Previous studies have suggested that soil microorganisms have a role in the degradation of BZs in soil (Lagos et al., 2019). However, microorganisms able to degrade AH BZ compounds are yet to be

isolated. The sole exception is a bacterial consortium able to degrade TBZ and use it as a C source. It was recently isolated from a soil collected from a disposal site receiving TBZ-contaminated wastewaters from a fruit-packaging industry (Perruchon et al., 2017a). It was characterized by a stable composition of 18 main members, as determined by shotgun metagenomic analysis, most of them belonging to α- and γ-Proteobacteria (Vasileiadis et al., 2020). Stable isotope probing, amplicon sequencing, metatranscriptomic and metaproteomic analysis revealed that a *Sphingomonas* sp. is responsible for the degradation of TBZ, while other members like a *Hydrogenophaga* support the main degrader through the supply of B12 to counterbalance the auxotrophy of *Sphingomonas* (Vasileiadis et al., 2020). The consortium has been previously used successfully for the bioremediation of soils from wastewater disposal sites contaminated with up to 400 mg kg-1 of TBZ (Papadopoulou et al., 2018). This comes as no surprise since it is now well documented that biodegradation of organic pollutants in the environment is performed by microbial consortia rather than single organism (Billet et al., 2019). The collaboration between members of microbial consortia, at nutritional and pollutant transformation levels, ensures higher tolerance to biotic and abiotic stress encountering in the environment (Festa et al., 2016) and hence higher bioaugmentation performance compared to single organism inoculation (Jacques et al., 2008).

Different biobased methods have been proposed to mitigate the environmental dispersal of veterinary drugs. These have focused on the treatment of feces prior to their soil application to reduce their veterinary drug load. Composting, anaerobic digestion or storage in lagoons have been widely applied in swine fecal material for the removal of veterinary antibiotics with variable results (Selvam et al., 2013; Spiel- meyer et al., 2015; Widyasari-Mehta et al., 2016; Berendsen et al., 2018), while their efficiency to remove AHs remains unknown. Bioaugmentation of fecal material with tailored-made inocula able to degrade and detoxify veterinary drugs pose an interesting biotechnological approach to avert environmental contamination by these compounds. This approach has been used solely for removing veterinary antibiotics from soils treated with contaminated manures and showed encouraging results (Hirth et al., 2016; Hong et al., 2020). However, its efficacy for removing persistent AH BZs from fecal material remains unknown and should be explored as a preventive measure to minimize environmental dispersal of persistent AHs like BZs.

Our study aimed to investigate the potential of bioaugmentation for the decontamination of fecal material from BZ AH compounds. In the absence of tailored-made bacterial inocula able to degrade the different BZ AH compounds, we hypothesized that the TBZ-degrading bacterial consortium, previously isolated by our group, would be also able to achieve appreciable degradation of other AHs of the same chemical group. To verify this hypothesis, we first examined the degrading capacity of the bacterial consortium against all BZ AH compounds in a minimal growth media. Based on the outcome of this first *in vitro* screening we employed a further experiment to (i) explore the bioaugmentation potential of the bacterial consortium in sheep fecal material contaminated with FBZ, ABZ and TBZ and (ii) define the role of the indigenous fecal microbiota as antagonists of the exogenous bacterial inoculum and/or as contributors on the degradation of the AH compounds. Our study provides first evidence for the potential of bioaugmentation as a mean to alleviate environmental dispersal of AH BZs.

2. Materials and methods

2.1. Bacterial consortium

The bacterial consortium used was isolated from the soil of a wastewater disposal site in Cyprus as described by Perruchon et al. (2017a). The consortium was routinely cultivated in minimal medium supplemented with nitrogen (MSMN) and TBZ (50 mg L^{-1}) as a sole C source, in a shaking incubator at 25°C.

2.2. Chemicals and reagents

Analytical standards of FBZ (98% purity), fenbendazole sulfone (FBZ-SO) (99% purity), FLU (98% purity), MBZ (98% purity) and ABZ (98% purity) were purchased by Tokyo Chemical Industry, while ricobendazole (ABZ-SO) (97% purity) and TBZ (> 98% purity) were purchased by Santa Cruz Biotech (chemical structures in Fig. 4.1). Dense solutions of each of the above BZ compounds in DMSO (11–17 mg ml^{-1}), prepared with the analytical standards, were used for the preparation of (i) growth media amended with the AH compounds and (ii) methanolic solutions (1000 mg L^{-1}) which were used for analytical purposes. Tween® 20 for molecular biology (Sigma-Aldrich, USA) was used in liquid culture studies.
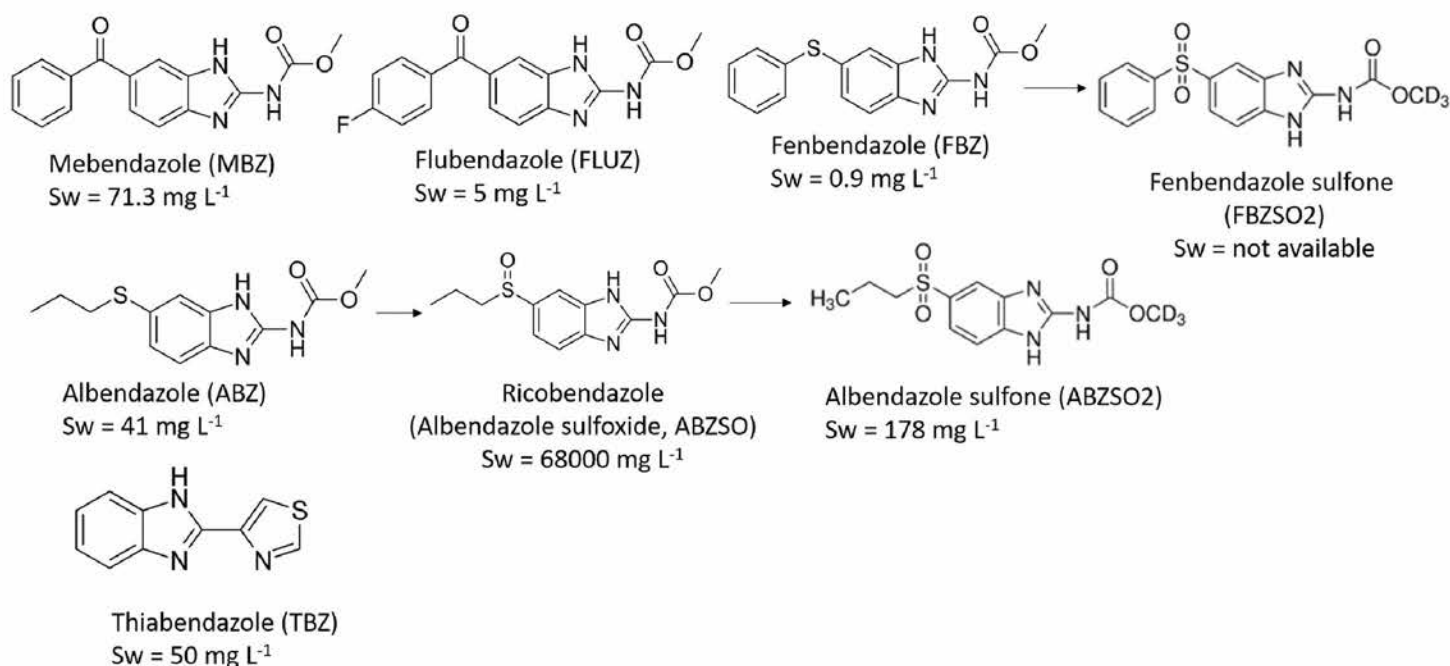

Figure 4.1 The chemical structures and water solubilities (derived from the PubChem https://pubchem.ncbi.nlm.nih.gov and DrugBank https://go.drugbank.com databases) of the anthelmintic benzimidazole compounds and their transformation products.

2.3. Inoculum preparation

An actively growing culture of the bacterial consortium in MSMN + TBZ (50 mg L^{-1}) was used as inoculum. Inoculation of liquid cultures was performed as described by Perruchon et al. (2017a). Briefly, bacterial cells from a fresh culture of the consortium were harvested at the mid-log phase, washed three times with sterile ddH$_2$O and adjusted to OD_{600} = 0.1 before inoculation aiming to an initial inoculum level of 106 cells ml^{-1}. The inoculation of feces was performed as described by Papado-

poulou et al. (2018) for soil. Briefly, a fresh culture (250 ml) of the consortium was harvested at 72 h, when the consortium and the TBZ-degrading bacterium *Sphingomonas* were at the mid-log phase of growth, as determined by q-PCR analysis of total bacteria and the *Sphingomonas* (Perruchon et al., 2017a). Cells were washed three times in sterile ddH$_2$O before resuspended to appropriate volumes of ddH$_2$O which were used for the inoculation of soil samples aiming to a final inoculum density of 6.5 × 10^6 cells g^{-1} soil (on a dry weight basis).

2.4. Biodegradation of benzimidazole anthelmintic compounds in liquid cultures

The degradation capacity of the bacterial consortium towards all BZs was initially screened in liquid culture assays. For each benzimidazole compound six flasks containing 10 ml of MSMN + AH (15 mg L^{-1}) + Tween 20 (0.1%) were prepared. Tween 20, a non-ionic surfactant was added to facilitate the initial dissolution of AH compounds. The first three flasks were inoculated with an appropriate volume of a fresh culture of the bacterial consortium grown in MSMN + TBZ (50 mg L-1) as described above. The remaining three flasks received the same amount of MSMN without bacterial cells to act as abiotic controls. All samples were placed in a shaking incubator at 200 rpm and 25◦C. The degradation of BZs was determined via HPLC analysis of their residues in samples of the liquid culture (0.5 ml) collected immediately after the commencement of the experiment and at regular intervals thereafter. In the case of ABZ the formation of its oxidized derivatives ABZ-SO and ABZ-SO$_2$ was also monitored.

Based on the results of this first screening, a second liquid culture experiment was employed, this time focusing only on the compounds that were more efficiently degraded by the bacterial consortium (TBZ, ABZ and FBZ). The same experimental plan as described in the first liquid culture experiment was followed. The only exception was that this time all cultures were amended with Tween 20 (0.1%) at each sampling time point during the degradation study. The regular addition of Tween 20 was deemed necessary in order to ensure maximum and continuous dissolution of the non-polar BZ compounds tested (e.g. ALB, FBZ). This was expected to maximize bioavailability of BZ compounds to the inoculum and optimum biodegradation performance.

2.5. Bioaugmentation of fecal material

Sheep feces were collected from commercial livestock farms located in Giannitsa, Northern Greece. The animals in the farm had not received any treatment with any of the BZ compounds tested. For each AH compound eight lots of fecal material, 320 g each, were prepared. The first four were fumigated with chloroform as follows. Fecal samples were contained in a polyester fine mesh net, and they were fumigated under vacuum with chloroform for a week at 30◦C in a vacuum desiccator. After that, the samples were incubated moist at room temperature (23–25◦C) for a week to allow the proliferation of any microbial propagules that escaped fumigation so as to be vulnerable to a second fumigation treatment which followed. In contrast, the other four samples were maintained without fumigation. Fumigated and non-fumigated samples were treated with 1 ml aliquots of methanolic solutions of each anthelmintic compound resulting in concentrations of 5 and 50 mg kg^{-1}. Samples were left for an hour to allow methanol to evaporate. Subsequently, half of the samples were bioaugmented with the bacterial suspension as described above, while the rest received the same amount of water without bacterial cells to serve as non- bioaugmented controls. All samples were then mixed by hand to ensure uniform distribution of the inoculum and the chemicals. Samples were divided into subsamples of 15 g which were placed in aerated plastic bags and incubated in the dark at 25◦C. The degradation of BZs was determined via HPLC analysis of triplicate samples from each

treatment collected immediately upon preparation and at 3, 6, 10, 20 and 40 days. This experimental setup resulted in 8 treatments: (i) 5 mg kg^{-1} of anthelmintic, bioaugmented (ii) 5 mg kg^{-1} of anthelmintic, non-bioaugmented (iii) 50 mg kg^{-1} of anthelmintic, bioaugmented, (iv) 50 mg kg^{-1} of anthelmintic, non-bioaugmented, (v) 5 mg kg^{-1} of anthelmintic, bioaugmented, fumigated (vi) 5 mg kg^{-1} of anthelmintic, non-bioaugmented, fumigated (vii) 50 mg kg^{-1} of anthelmintic, bioaugmented, fumigated (viii) 50 mg kg^{-1} of anthelmintic, non-bioaugmented, fumigated.

2.6. Determination of the residues of anthelminthic compounds in liquid cultures and fecal material

2.6.1. Extraction of anthelmintics from liquid cultures and the fecal material

Extraction of AHs from the liquid medium was achieved by mixing 0.5 ml of the culture with 1 ml of methanol. The mixture was vortexed for 30 s, centrifuged at max speed for 1 min and then used for HPLC analysis. Tests at three concentration levels (0.05, 1 and 15 mg L-1) showed recoveries for all AHs exceeding 85%. ABZ, ABZSO2, FBZ and FBZ-SO were extracted from feces using the Quechers method. Briefly, 5 g of feces were mixed with 5 ml of ddH$_2$O and 10 ml of acetonitrile and vortexed

for 1 min. A mixture of salts composed of 4 g MgSO$_4$, 1 g NaCl and 1.5 g C$_6$H$_5$Na$_3$O$_7$·2H$_2$O were added. The mixture was then vortexed for a minute and centrifuged at 7500 rpm for 5 min. An aliquot of the clear supernatant (1.5 ml) was collected and mixed with 0.225 g MgSO^4 and 0.0375 g PSA. The mixture was vortexed for 30 sec, centrifuged for 1 min at 4400 rpm and the clear supernatant was filtered through a 0.45 μm syringe before analyzed by HPLC. ABZ-SO was extracted from 5 g of feces with 10 ml of acetonitrile. The mixture was agitated for 1 h at 300 rpm in a shaking platform in flasks covered with aluminum foil. The supernatant was collected, and the fecal material was re-extracted with another 10 ml of acetonitrile. Extracts were pooled, centrifuged at 7500 rpm for 5 min and the clear supernatant was filtered through 0.45 μm syringe filters before analyzed by HPLC. Extraction of TBZ from fecal material was as described in Perruchon et al. (2017b). Tests for ABZ, FBZ and their oxidative derivatives in fecal samples at three fortification levels (0.05, 0.5 and 5 mg kg^{-1}) resulted in recoveries higher than 90% in all cases.

2.6.2. HPLC analysis

The concentrations of BZ compounds were determined in a Shimadzu HPLC-PDA system equipped with CNH Athena RP C18 150 mm (CNW Technologies, Dusseldorf, Germany). AHs were eluted at a flow rate of 1 ml min^{-1} using mobile phases of different strengths. FLU, MBZ and FBZ, FBZ-SO were eluted using a mobile phase composed of 50:50 acetonitrile:water (v/v) + 0.1% H$_3$PO$_4$ and detected at 245 and 210 nm respectively. ABZ, ABZ-SO$_2$ and ABZ-SO (or ricobendazole) were eluted using a gradient mobile phase of 30:70 acetonitrile:water (v/v) + 0.1% H$_3$PO$_4$ and they were detected at 227 nm. Finally, TBZ was eluted using a mobile phase composed of 39:60.5:0.5 acetonitrile:water:NH$_3$ (v/v) and detected at 254 nm. In all cases the concentrations of the anthelmintic compounds were determined using an external calibration curve.

2.7. Data analysis

The single first order (SFO) and three biphasic kinetic models (Hockey Stick (HS), first order multi-compartment and double first order in parallel) were used (FOCUS, 2006) to calculate degradation kinetics for all anthelmintic compounds in liquid cultures and in feces. The goodness of fit was assessed using the χ^2 test (> 15%, for α of 0.05), visual inspection and the distribution of residuals. Significant

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differences (level of significance 5%) in compounds degradation rates (k_{deg} and k_1) were determined with the student's t- test. The confidence intervals of the degradation rates obtained by fitting the kinetic models to the degradation data were converted to standard deviations using the formula:

$$SD = \sqrt{N} \times (Upper\ limit - Lower\ limit)/3.92$$

where N = sample size and 3.92 is the standard error for a 95% confidence interval.

3. Results

3.1. Degradation of benzimidazole anthelmintics by the bacterial consortium in liquid cultures

3.1.1. Experiment I

The degradation and metabolism of the different BZ AH compounds are shown in Fig. 2. As expected, the bacterial consortium degraded TBZ rapidly (DT50 = 2.9 days). Moreover, it significantly (p < 0.05) accelerated the degradation of ABZ, ricobendazole (or ABZ-SO) and FBZ with DT_{50} values of 67.6, 69.7 and 224.5 (extrapolated) days respectively, whereas it failed to degrade FLU and MBZ with DT_{50} values > 365 days (extrapolated) (Table 4.1). No abiotic degradation of any of the compounds tested was observed in the time frame of our study (DT_{50} values > 365 days, extrapolated). In the presence of the bacterial consortium ABZ was oxidized to ABZ-SO which accumulated in the liquid culture at the end of the study, while trace amounts of $ABZ-SO_2$ were also detected (Fig. 4.2f). The high variability in the biodegradation pattern of ABZ was due to the higher degradation of the compound in one of the three inoculated replicates as shown in the inserted graph in Fig. 4.2f.

3.1.2. Experiment II

Based on the results of Experiment I, we further focused on the degradation of ABZ and FBZ, which were the two compounds other than TBZ showing some level of biodegradation. In addition, cultures were amended with Tween 20 at each sampling time point, unlike experiment I where cultures had been amended with Tween 20 only at the start of the study. The degradation and transformation patterns of the tested anthelmintic compounds are presented in Fig. 4.3. The bacterial consortium imposed a clear and significant acceleration in the degradation of TBZ, ABZ and FBZ with DT_{50} values of 3.1, 6.7 and 28.3 days respectively, compared to over 365 days (extrapolated) in the non-inoculated controls (Table 4.1). As in Experiment I, ABZ was oxidized to ABZ-SO which was not degraded further (Fig. 4.3c).

Table 4.1 The degradation kinetic parameters (DT_{50}, degradation rates k_{deg} or k_1/k_2) for the different anthelmintics in MSMN inoculated or not inoculated (control) with the bacterial consortium in Experiments I and II.

| Anthelmintic	Treatment	Kinetic model	χ^2 (%)	K_{deg} (d^{-1})	K_1 (d^{-1})	K_2 (d^{-1})	DT_{50} (d)
			Experiment I				
Albendazole (ABZ)	Control	SFO	4.67	< 0.0001			> 365
	Inoculated	HS	2.66		0.1354	0.0062	67.6
Fenbendazole (FBZ)	Control	SFO	10.02	< 0.0001			> 365
	Inoculated	HS	13.27		0.3345	< 0.0001	224.5
Flubendazole (FLU)	Control	SFO	8.55	< 0.0001			> 365
	Inoculated	HS	19.87		0.333	< 0.0001	> 365
Mebendazole (MBZ)	Control	SFO	4.35	< 0.0001			> 365
	Inoculated	SFO	10.30	< 0.0001			> 365
Ricobendazole	Control	SFO	6.50	< 0.0001			> 365
	Inoculated	HS	2.41		0.1008	0.0053	69.7
Thiabendazole (TBZ)	Control	SFO	4.79	< 0.0001			> 365
	Inoculated	SFO	24.49	0.2384			2.9
			Experiment II				
Thiabendazole (TBZ)	Control	SFO	6.54	0.0168			> 365
	Inoculated	SFO	24.14	0.2264			3.07
Albendazole (ABZ)	Control	SFO	8.26	0.0018			> 365
	Inoculated	SFO	17.42	0.1033			6.71
Fenbendazole (FBZ)	Control	SFO	3.68	< 0.0001			> 365
	Inoculated	HS	5.81		0.1624	0.0019	28.3
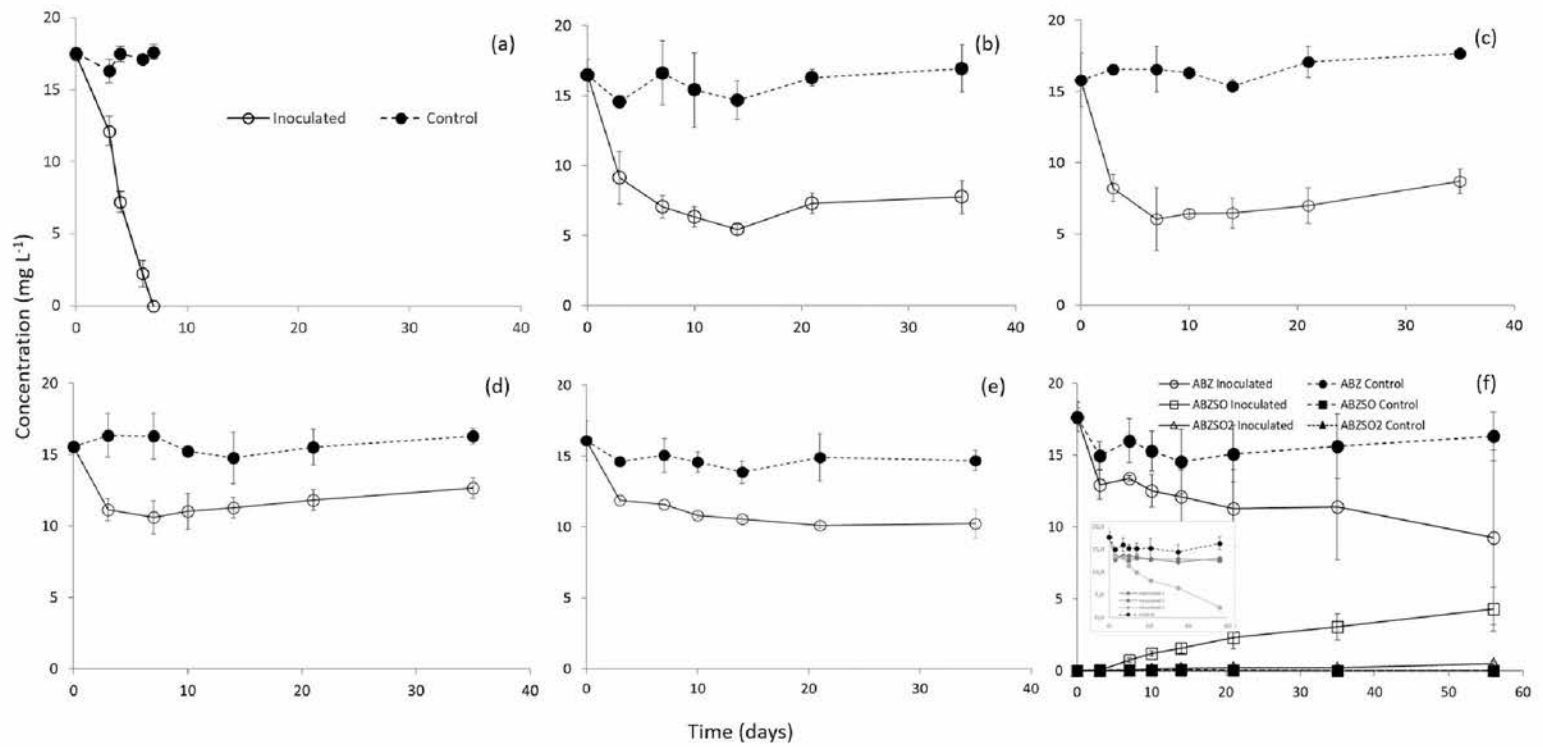
Figure 4.2 The degradation of thiabendazole (TBZ) (a), fenbendazole (FBZ) (b), flubendazole (FLUZ) (c), mebendazole (MBZ) (d), ricobendazole (or albendazole sulfoxide, ABZSO) (e), and albendazole (ABZ) (f) in MSMN liquid cultures inoculated (○) or not inoculated (●) with the bacterial consortium. The formation patterns of the oxidation products of ABZ, ABZSO and albendazole sulfone (ABZSO2) are also presented. Each value is the mean of three replicates + the standard deviation of the mean. The inserted graph in (f) shows the degradation patterns of ABZ in the three individual inoculated replicates to demonstrate the high variability be- tween replicates.

3.2.   Bioaugmentation of fecal material

We further explored the bioaugmentation capacity of the bacterial consortium against TBZ, FBZ, and ABZ in fortified fecal material. We also included a set of sterilized-fumigated samples per treatment to evidence the role of the indigenous microbial community of feces either as degraders of BZs and/or as competitors of the introduced exogenous inoculum. Inoculation of the fecal material with the bacterial consortium significantly accelerated ($p < 0.05$) the degradation of TBZ at both concentration levels (5 and 50 mg kg^{-1}), with DT_{50} values of 88.5 and 35.7 days respectively, compared to 115 and 77.8 days in the corresponding non-inoculated samples (Table 4.2, Fig. 4.4a and c). The beneficial effect of inoculation was magnified in fumigated samples where significantly lower DT_{50} values ($p < 0.01$) were observed (31.6 and 33.8 days) compared to the non-inoculated fumigated samples (125 and 201.7 days) (Table 4.2).

Bioaugmentation accelerated the degradation of FBZ in the non-fumigated fecal material (DT_{50} 24.2 days at the 5 mg kg^{-1} level and 25.4 days at the 50 mg kg^{-1} level), compared to the corresponding non-inoculated samples (DT_{50} 35.5 and 39.9 days, respectively), although the effects were not significant ($p > 0.05$) (Table 4.2, Fig. 4.4e and g). Conversely, in fumigated feces bioaugmentation significantly increased ($p < 0.01$) the degradation of FBZ at both concentration levels (DT_{50} =16.3 and 19.5 days)

Figure 4.3 The degradation of thiabendazole (TBZ) (a) and fenbendazole (FBZ) (b) in MSMN inoculated (○) or not inoculated (●) with the bacterial consortium. The degradation of albendazole (ALB) and the formation of its oxidation products albendazole sulfoxide (ABZSO) and albendazole sulfone (ABZSO2) in MSMN inoculated or not inoculated with the bacterial consortium (c). Each value is the mean of three replicates + the standard deviation of the mean.

compared to their corresponding fumigated non-inoculated samples (DT_{50} = 74.4 and 57.8 days) (Table 4.2, Fig. 4.4f and h). Regardless of inoculation or fumigation of fecal samples at both concentration levels, FBZ was transformed to FBZ-SO which was not further degraded and accumulated at low amounts (Fig. 4.4e–h). Similarly to FBZ, bioaugmentation of non-fumigated feces accelerated the degradation of ABZ, although this effect was not statistically significant ($p > 0.05$), with
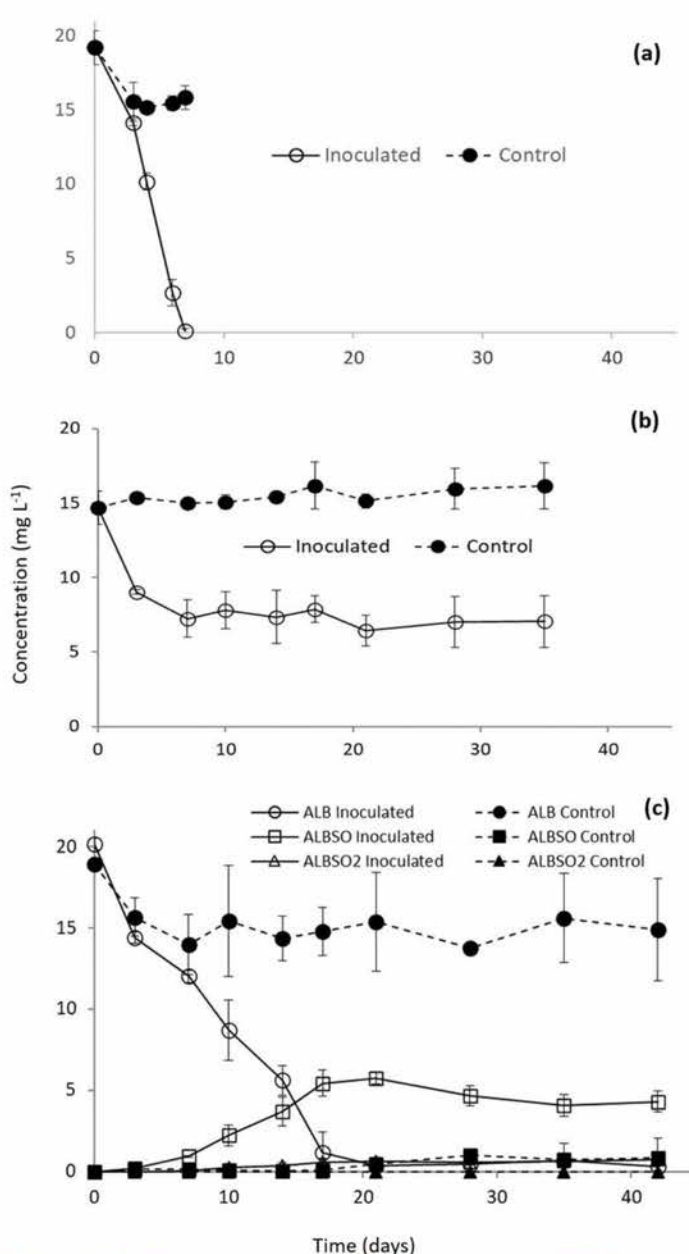
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DT$_{50}$ values of 5.3 and 6.3 days (5 and 50 mg kg^{-1} treatment respectively) in bioaugmented compared to the corresponding non-bioaugmented samples (5.7 and 6.8 days) (Table 4.2, Fig. 4.4i and k). This was not consistent in fumigated feces where bioaugmentation significantly accelerated ($p < 0.05$) the degradation of ABZ (DT$_{50}$ = 3.4 and 6.4 days) compared to the corresponding non-inoculated samples (DT$_{50}$ = 17.6 and 16.1 days) (Table 4.2, Fig. 4.4j and l). In all fecal samples ABZ was oxidized to ABZ-SO whose concentration peaked at 3–6 days post application. ABZ-SO was further oxidized to ABZ-SO$_2$ which accumulated in all fecal samples at the end of the study (Fig. 4.4i–l). When the sum of ABZ and its oxidation derivatives was used for the calculation of DT$_{50}$ a similar pattern was observed whereby bioaugmentation resulted in a non-significant ($p > 0.05$) reduction in DT$_{50}$ values from 13.0 (5 mg kg-1 level) and 9.96 (50 mg kg-1) days to 7.4 and 9.65 days respectively (Table 2). Conversely, in fumigated samples bioaugmentation induced a significant acceleration ($p < 0.05$) in the degradation of total ABZ residues with DT$_{50}$ values of 11.2 (5 mg kg^{-1} level) and 9.1 days (10 mg kg^{-1}) compared to 33.6 and 16.6 days in the corresponding non-bioaugmented controls (Table 4.2).

When the degradation rates of TBZ, FBZ and ABZ in corresponding fumigated and non-fumigated control samples were compared it was evident that fumigation significantly reduced ($p < 0.05$) the degradation rates of all three BZs. The same pattern was also evident in the corresponding inoculated samples (fumigated vs non-fumigated), although significant differences ($p < 0.05$) were only observed at the low fortification rate (5 mg kg^{-1}).

4. Discussion

The removal of veterinary drugs from feces pilled inside livestock farms or destined for use as manures in agricultural soils could be an efficient means to mitigate environmental dispersal of these pollutants. Unlike antibiotics, little is known about the efficiency of different methods to detoxify feces and manures contaminated with AH compounds. We tested the potential of bioaugmentation for the removal of BZ AHs from fecal material as a means to minimize their environmental dispersal.

We hypothesized that a bacterial consortium able to degrade the anthelmintic BZ TBZ, would be able to effectively degrade other AH compounds of the same chemical group. Initial tests in liquid cultures showed that, beyond TBZ, the bacterial consortium was able to accelerate the degradation of ABZ, of its oxidation analog ricobendazole (or ABZ-SO) and to a lower extent of FBZ. However, it failed in degrading the other BZ compounds tested like FLU and MBZ. The different degradation activity of the consortium against the tested BZ is probably a reflection of the differences in their chemical structure and primarily in the size of the substituents of the benzoyl moiety of their benzimidazole ring. ABZ and ricobendazole are characterized by low molecular weight thioalkyl substituents compared to the thio- or oxo-phenyl substituents in FBZ, FLU and MBZ (Fig. 4.1). Previous studies by Perruchon et al. (2017a) also suggested that the substituents of the benzimidazole moiety strongly influence the capacity of the bacterial consortium to degrade BZ derivatives.

Table 4.2 The degradation kinetics of thiabendazole (TBZ), fenbendazole (FBZ), albendazole (ABZ) and albendazole + ABZ-SO + ABZ-SO$_2$ in fecal material, fortified with different levels of anthelmintics, which were bioaugmented (inoculated) or not (control) with the bacterial consortium – Experiment II.

| Anthelmintic	Treatment	Kinetic model[a]	χ^2 (%)	K_{deg} (d^{-1})	K_1 (d^{-1})	K_2 (d^{-1})	DT$_{50}$ (d)
Thiabendazole (TBZ)	5 mg kg^{-1} Control	SFO	5.05	0.0060			115.0
	5 mg kg^{-1} Inoculated	SFO	3.86	0.0078			88.5
	50 mg kg^{-1} Control	HS	3.13		0.0618	0.0057	77.8
	50 mg kg^{-1} Inoculated	HS	2.09		0.0499	0.0128	35.7
	5 mg kg^{-1} Control – Fumigated	SFO	5.64	0.0055			125.0
	5 mg kg^{-1} Inoculated – Fumigated	SFO	1.93	0.0219			31.6
	50 mg kg^{-1} Control – Fumigated	SFO	2.23	0.0034			201.7
	50 mg kg^{-1} Inoculated – Fumigated	SFO	4.17	0.0205			33.8
Fenbendazole (FBZ)	5 mg kg^{-1} Control	SFO	4.78	0.0195			35.5
	5 mg kg^{-1} Inoculated	SFO	4.07	0.0287			24.2
	50 mg kg^{-1} Control	SFO	4.14	0.0174			39.9
	50 mg kg^{-1} Inoculated	SFO	5.77	0.0273			25.4
	5 mg kg^{-1} Control – Fumigated	SFO	6.73	0.0093			74.4
	5 mg kg^{-1} Inoculated – Fumigated	SFO	2.15	0.0426			16.3
	50 mg kg^{-1} Control – Fumigated	SFO	2.55	0.0120			57.8
	50 mg kg^{-1} Inoculated – Fumigated	SFO	5.95	0.0355			19.5
Albendazole (ABZ)	5 mg kg^{-1} Control	HS	1.81		0.1210	0.0162	5.72
	5 mg kg^{-1} Inoculated	HS	5.11		0.1614	0.0288	5.31
	50 mg kg^{-1} Control	HS	7.41		0.1018	0.0406	6.81
	50 mg kg^{-1} Inoculated	HS	5.35		0.1094	0.0295	6.33
	5 mg kg^{-1} Control – Fumigated	SFO	6.64	0.0394			17.6
	5 mg kg^{-1} Inoculated – Fumigated	SFO	10.48	0.2027			3.42
	50 mg kg^{-1} Control – Fumigated	SFO	6.77	0.0431			16.1
	50 mg kg^{-1} Inoculated – Fumigated	SFO	10.12	0.1079			6.42
Albendazole + ABZSO + ABZSO2	5 mg kg^{-1} Control	HS	1.26		0.0532	0.0139	13.0
	5 mg kg^{-1} Inoculated	HS	1.61		0.0936	0.0245	7.4
	50 mg kg^{-1} Control	HS	7.34		0.0696	0.0207	9.96
	50 mg kg^{-1} Inoculated	HS	9.37		0.0718	0.0159	9.65
	5 mg kg^{-1} Control – Fumigated	SFO	2.47	0.0206			33.6
	5 mg kg^{-1} Inoculated – Fumigated	HS	15.1		0.0622	0.0059	11.2
	50 mg kg^{-1} Control – Fumigated	HS	5.04		0.0417	0.0067	16.6
	50 mg kg^{-1} Inoculated – Fumigated	HS	6.29		0.0686	8.0E-8	9.1
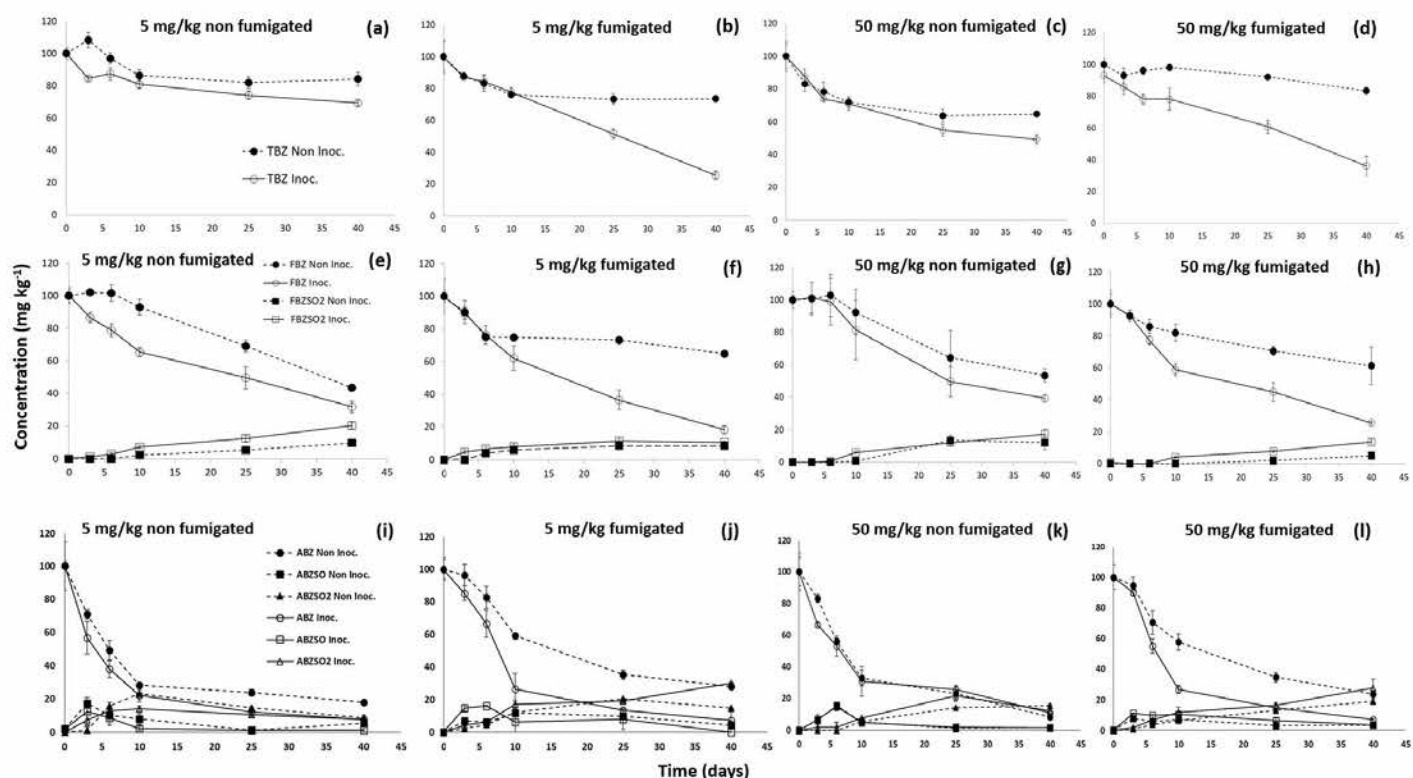
Figure 4.4 The degradation of 5 mg kg^{-1} (a, b) and 50 mg kg^{-1} (c, d) of thiabendazole (TBZ) in fumigated (b, d) or not fumigated (a, c) feces inoculated or not inoculated with the bacterial consortium. The degradation of fenbendazole (FBZ) and the formation of its oxidation product fenbendazole sulfone (FBZ-SO) in fumigated (f, h) or not fumigated (e, g) feces fortified with 5 mg kg^{-1} (e, f) and 50 mg kg^{-1} (g, h) of FBZ and inoculated or not inoculated with the bacterial consortium. The degradation of albendazole and the formation of its oxidation products albendazole sulfoxide (ABZ-SO) and albendazole sulfone (ABZ-SO$_2$) in fumigated (j, l) or not fumigated (l, k) feces fortified with 5 mg kg^{-1} (i, j) and 50 mg kg^{-1} (k, l) of ABZ and inoculated or not inoculated with the bacterial consortium. Each value is the mean of three replicates + the standard deviation of the mean.

The selectivity of bacteria in degrading organic pollutants is the result of the structural affinity of the pollutants to the active site of the specialized enzymes controlling the first steps of the transformation process (Theriot and Grunden, 2011; Jiang et al., 2021). In this frame, Vasileiadis et al. (2020) showed, via meta-transcriptomic and -proteomic analysis, that a multicomponent carbazole dioxygenase-like enzyme, found in the metagenome assembled genome of a *Sphingomonas*, the main degrader of TBZ in the bacterial consortium, is responsible for the transformation of TBZ. It was further demonstrated that the active site pocket of the terminal oxygenase component of this carbazole dioxygenase had the highest affinity for carbazole (ΔG = -7.5 kcal mol^{-1}), and a slightly lower affinity for TBZ (ΔG = -6.8 kcal mol^{-1}). We speculate that the bulky substituents of the benzyl moiety of the BZ ring of MBZ, FLU and FBZ do not facilitate optimum fitting in the active site pocket of the carbazole-dioxygenase enzyme of *Sphingomonas* which shows high affinity for carbazole and TBZ, both lacking such substituents.

We further aimed to optimize the degradation capacity of the bacterial consortium against ABZ and FBZ, for which a slight acceleration in its biodegradation was observed in the first test. Considering the limited water solubility of these molecules (41 and 0.9 mg L^{-1} respectively), we assumed that a regular addition of a non-ionic surfactant like Tween 20, with known capacity to increase the dissolution of such molecules (Woertz and Kinney, 2004), will increase their bioavailability and thus their degradation by the bacterial consortium. Indeed, the regular addition of Tween 20 drastically

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enhanced the biodegradation of (i) ABZ (from DT_{50} = 67.2 days in the first experiment to 6.7 days in the second experiment) and (ii) FBZ (from DT_{50} = 224.5 days to 28.3 days).

Several previous studies have also showed that addition of Tween 20, Tween 80 or other non-ionic surfactants accelerated the biodegradation of lipophilic compounds like pyrene (Zhang et al., 2013), fluoranthene, phenanthrene (Luning Prak and Pritchard, 2002) and crude oil (Xu et al., 2018) by bacteria and fungi. This has been mostly attributed to the increased bioavailability of pollutants on the cell surface of microbial degraders (Zhang et al., 2013), although the use of surfactants by the bacteria as co-substrates has been also proposed (Wang et al., 2018). In our study the degradation of Tween 20 was not followed, hence its role as co-substrate for the members of the consortium cannot be ruled out. Based on the successful biodegradation of TBZ, ABZ and FBZ by the bacterial consortium in liquid cultures, we further assessed its bioaugmentation capacity in feces at two concentration levels representing a lower, realistic level (5 mg kg^{-1}) and a higher, less frequently encountered level (50 mg kg^{-1}) of BZs (Thiele-Bruhn, 2003). Bioaugmentation of non-fumigated feces resulted in a moderate enhancement of the degradation of TBZ, FBZ, ABZ and its oxidation derivatives in feces. This enhancement in the biodegradation of AHs is most probably a function of the relaxed specificity of the TBZ-degrading enzyme, a multi-component carbazole dioxygenase (Vasileiadis et al., 2020), which is also capable to achieve the transformation of other AHs of the same chemical group, as clearly demonstrated in our liquid culture studies discussed above. Still the moderate capacity of the bacterial consortium to rapidly remove TBZ from fecal samples is not in accord with the results of Papadopoulou et al. (2018) who showed that the bacterial consortium effectively remediated soils naturally contaminated with TBZ (up to 400 mg kg^{-1}).

This discrepancy could be possibly attributed to the higher organic matter content of feces (39.6–51.3% for sheep feces, (Moral et al., 2005; Elouear et al., 2016) respect to soils (2.5% in the soil tested in the study of Papadopoulou et al., 2018), which enhances the sorption of lipophilic chemicals like the BZs tested (Kim et al., 2010), thus limiting their bioavailability for biodegradation by the inoculated and indigenous microbial community. Alternatively, the bacterial inoculum, which is dominated by proteobacteria (Vasileiadis et al., 2020), known to prevail in the soil microbiome (Delgado-Baquerizo et al., 2018), was most probably outcompeted by the established indigenous microbial community of feces, which is distinct from the soil microbiome, and it is dominated by Bacteroidetes and Firmicutes (Mamun et al., 2019). The difficulty of exogenous inocula to compete with the indigenous microbial community of complex environmental matrices like soils and feces is a problem that often compromises the performance of bioaugmentation (Cycon et al., 2017).

In feces ABZ was transformed rapidly to ABZ-SO and further to ABZ-SO_2, which constituted the main derivative at the end of the incubation. Little is known regarding the persistence and transformation of ABZ in feces. Liu and Chen (2018) observed that ABZ undergoes different oxidations and hydrolysis transformations leading to the formation of various metabolic products amongst which ABZ-SO and ABZ-SO_2 were the major ones. These, like ABZ, could be further hydrolyzed to more polar and mobile transformation products increasing the risk for environmental contamination (Porto et al., 2020). In a recent study Turek-Szytow et al. (2020) showed that the dissipation of ABZ in chicken fecal material was 62% after 35 days, and addition of inorganic peroxide mixtures accelerated its dissipation to 91%. We showed for the first time that ABZ is not persistent itself in feces, but it is rapidly oxidized to its sulfoxide and sulfone which could persist in fecal material and eventually reach soils or aquatic bodies.

Regarding FBZ, in feces it was oxidized to a low extent to FBZ-SO$_2$ which is more mobile than FBZ (Porto et al., 2020). The moderate persistence of FBZ in feces regardless of bioaugmentation contradicts studies by Kreuzig et al. (2007) in pig feces where 80% of the ^{14}C-labeled FBZ persisted for more than 100 days.

In parallel we assessed the role of the indigenous microbial community of feces (i) as degraders of BZs and (ii) as competitors of the exogenous microbial inoculum, by following the degradation of these compounds in fumigated samples. Fumigation of the fecal material magnified the positive effect of bioaugmentation in the biodegradation of BZs. This was partially the result of the higher biodegradation of BZ compounds in bioaugmented fumigated vs non- fumigated samples (depicted by the generally higher degradation rates in the former), verifying our initial suggestion about the role of the indigenous fecal microbiota as strong competing agents to the exogenously introduced bacterial inoculum. Little is known about the potential negative influence of the indigenous fecal microbiota on bioaugmentation efficiency. However similar studies in soil have stressed the potential antagonistic role of the indigenous microbiota to exogenous inocula which fail to establish and provide adequate pollutant removal (Llado et al., 2013). Further, the magnified positive effect of fumigation in bioaugmentation efficiency is also associated with the reduced contribution of the indigenous fecal microbiota in the degradation of BZ in fumigated samples. Indeed, comparison between fumigated and non-fumigated samples without bioaugmentation showed a general increase in BZ persistence upon fumigation suggesting that the indigenous fecal microbiota contributes to the biodegradation of BZ compounds, although abiotic degradation processes also have a role in the dissipation of BZs in feces. However, the level of biodegradation imposed solely by the indigenous microbial community cannot lead to an effective removal of the BZ AHs studied in the frame of a 45–60-day storage period often employed to animal feces before application in agricultural soils.

5. Conclusions

A bacterial consortium able to rapidly transform TBZ exhibited a structure-based selectivity in the degradation of other BZ AH compounds with preferential degradation of those which carried thioalkyl substituents in their benzyl moiety of their BZ ring. When used for the bioaugmentation of fecal material contaminated with BZ AH compounds, the bacterial consortium accelerated the removal of these compounds regardless of the levels of AHs in the fecal material. The bioaugmentation capacity of the bacterial consortium was further enhanced when released from the competition of the indigenous fecal microbiota, which also contributed to some extent to BZ biodegradation. The removal of veterinary drugs from fecal material by aerobic (i.e. com- posting) or anaerobic (i.e. digestion) biological processing has showed erratic results on antibiotics (Ho et al., 2013; Feng et al., 2017), while their efficiency towards AHs remains a black box. Bioaugmentation of veterinary drugs has been so far tested in agricultural soils targeting antibiotics like sulfonamides (Hirth et al., 2016) and tetracyclines (Hong et al., 2020) rather than AH compounds which follow an analogous environmental dispersal route. Our findings provide pioneering evidence that bioaugmentation with benzimidazole-degrading inocula poses a potential solution for the remediation of feces. Bioaugmentation could be applied on piles of feces inside the livestock farm or in manure piles stored outside the livestock farm, mitigating the potential environmental dispersal of BZ AHs. Further studies will focus on the isolation of novel BZ microbial degraders, beyond TBZ, to enable a tailored-made bioaugmentation of fecal material.

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Chapter 5

Isolation of soil bacteria able to rapidly degrade the anthelminthic veterinary drug Albendazole.

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

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1. Introduction

Infections by gastrointestinal nematodes (GINs) are considered a major threat for grazing animals worldwide, leading to serious effects on their welfare and productivity (Mavrot et al., 2015; Kaplan, 2020). The main strategy for prevention and treatment of GINs is the use of anthelminthic (AH) compounds (McKellar and Jackson, 2004; Kaplan, 2020). Benzimidazoles (BZs) is one of the most widely used classes of synthetic AHs (Horvat et al., 2012). Their benzimidazole ring constitutes a very important pharmacophore moiety in drug discovery (Zhou et al., 2016), that has been associated with various biological activities like anticancer, antibacterial, antifungal, anti-inflammatory, antihistaminic, antioxidant, antihypertensive, and anticoagulant (Tunçbilek, Kiper and Altanlar, 2009). BZs act as inhibitors of mitosis by binding on tubulin and thus preventing microtubule formation (Mckellar and Scott, 1990; Bansal and Silakari, 2012). Several BZs are used as AHs, like albendazole (ABZ), ricobendazole, fenbendazole (FBZ), flubendazole (FLU) and mebendazole (MBZ) (Mckellar and Scott, 1990).

BZs are only partially metabolized by animals and thus excreted through feces and urine. Depending on their administration mode, 60 and 90% of the dose is excreted to urine and feces (Halley, Jacob and Lu, 1989; Gottschall, Theodorides and Wang, 1990; Horvat et al., 2012; Aksit et al., 2015). Fecal material is either left on the floor of livestock farms or stockpiled and subsequently used as manures in agricultural settings. Both these practices, combined with the proven persistence of BZ AHs in feces and manures (Prchal et al., 2016; Silveira Porto et al., 2021) could lead to the dispersal of these AHs in soil and their further translocation to other environmental compartments.

ABZ constitutes the most heavily used benzimidazole AHs in livestock farming. It was reported to be present in sheep feces at levels up to 12.8 and 7.7 mg kg^{-1} with lower levels of ABZ transformation products, albendazole sulfoxide (ABZ-SO) and albendazole sulfone (ABZ-SO$_2$), also detected. Lagos et al., (2021) reported that in sheep fecal material total ABZ residues (parent compound combined with ABZ-SO and ABZ-SO$_2$) showed a DT$_{50}$ of 13 days. Once in soil ABZ is rapidly transformed to ABZ-SO, which also carries AH activity (Belew et al., 2021), and then to the AH inactive ABZ-SO$_2$ with DT$_{90}$ values for the total ABZ residues ranging from 41.5 to > 365 days (Lagos et al., 2022). From soil ABZ can be either taken up by plants (Stuchlíková Raisová et al., 2017) and through grazing back to the animals at sublethal levels which favor the development of drug resistance in GINs (Navrátilová et al., 2021) or in the form of its polar transformation products ABZ-SO, ABZ-SO$_2$ and ABZ-SO$_2$ amine could leach to groundwater (Silveira Porto et al., 2021). Indeed, ABZ and its transformation products constituted the most frequently detected AHs in groundwater and surface water systems in Ireland (Mooney et al., 2021). Considering the proven toxicity of ABZ onto non-target soil (e.g. earthworm *Eisenia fetida*) (Gao et al., 2007) and aquatic organisms (e.g. crustacean *Daphnia magna* and fish *Danio rerio*) its environmental dispersal should be mitigated.

Several treatments of fecal material like composting or anaerobic digestion have been used to reduce the load of manures to veterinary drugs. These approaches have shown variable results, so far only tested for the removal of antibiotics (Selvam et al., 2013; Berendsen et al., 2018). Recently Turek-Szytow et al (2020) suggested that treatment of manures with inorganic peroxide mixtures (PM) could effectively eliminate ABZ, although it is expected that such reactive methods could also alter the properties of manures. One interesting, promising, low-cost and non-invasive mitigation approach is bioaugmentation of fecal material or even contaminated soils with microorganisms capable of

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degrading ABZ and its derivatives. First attempts by Hirth et al, (2016) and Hong et al., 2020) reported interesting results on the removal of the veterinary antibiotics sulfamethazine and tetracycline from soil. In our earlier work we used a thiabendazole (TBZ) -degrading bacterial consortium for the bioaugmentation of feces contaminated with ABZ and other benzimidazole AHs and noted a moderate acceleration in the removal of thiabendazole, its original substrate, but a less efficient still significant removal of ABZ (Lagos *et al.*, 2021). This led us to hypothesize that specialized microbial inocula tailored to the degradation of ABZ will be more efficient in the bioaugmentation of contaminated matrices. Hence, we aimed to isolate bacteria able to rapidly degrade ABZ. This was achieved through enrichment cultures from a selected soil collected from a livestock farm with regular use of ABZ which showed accelerated rates of degradation of ABZ in previous studies (Lagos et al., 2022 and 2023).

2. Materials and Methods

2.1. Chemicals and growth media

An analytical standard of ABZ (98%, Tokyo Chemical Industry©, Zwijndrecht, Belgium) was used in media preparation and for analytical purposes. Analytical standard of ABZ-SO (98% purity) was also purchased from Tokyo Chemical Industry© (Zwijndrecht, Belgium), while ABZ-SO$_2$ (97% purity) was purchased from Santa Cruz Biotech© (Heidelberg, Germany). A mixture of ABZ, ABZ-SO and ABZ-SO$_2$ in acetonitrile (1000 mg L^{-1}) were used for preparing serial dilutions ranging from 10 − 0.025 mg L^{-1} which were used to construct calibration curves for residue quantification by HPLC.

Selective mineral salts media (MSM) and its nitrogen amended version (MSMN), supplemented with ABZ as the sole C and N or as the sole C source respectively, were used for the isolation of ABZ-degrading bacteria. MSM and MSMN were prepared as described before (Karpouzas and Walker, 2000). Growth media were spiked with a 5000 µg ml $^{-1}$ filter-sterilized solution of ABZ in DMSO (Molecular Biology Grade, Sigma Aldrich©) aiming to a final concentration of 5 µg ml^{-1} of ABZ in the medium. DMSO levels in the medium never exceeded 0.1%. Growth media were also amended with 0.05% of Tween 20 to enhance ABZ solubility, as suggested in our earlier studies (Lagos et al., 2021). Agar plates of the aforementioned media plus ABZ and Tween 20 were prepared by addition of 15 g L^{-1} agar.

2.2. Enrichment cultures and isolation of albendazole-degrading bacteria

To isolate ABZ-degrading bacteria, we employed enrichment cultures in MSM and MSMN supplemented with ABZ. A soil from a livestock unit in Lesvos Island, Greece, 39°16'21.4"N 26°15'55.7"E, with history of ABZ administration and high degradation capacity towards ABZ (Lagos *et al.*, 2022) was used for bacteria isolation. Prior to the onset of the enrichment cultures, the soil was repeatedly treated with ABZ (5 µg g $^{-1}$), three times on 15-day intervals to stimulate and activate the microbial community able to degrade ABZ. After completing the pre-treatment, 0.5 g of soil were used to inoculate triplicate bottles per medium (20 ml), while duplicated non-inoculated samples containing the same volume of each medium were used as abiotic controls. All cultures were incubated in an orbital shaker in the dark at 25°C. The degradation of ABZ was measured by analyzing samples at regular interval by HPLC as described below. At the point where degradation of ABZ was >70% an aliquot of each culture (0.5 ml) was transferred in fresh triplicate cultures. The same procedure was repeated for four cycles in total and at the point of 65-70% degradation of ABZ in the fourth enrichment cycle, a serial dilution was prepared and spread on MSM or MSMN agar plates amended with ABZ (5 µg ml $^{-1}$). The plates were then placed for incubation at 25°C. After 3-4 days of incubation growing colonies were selected and transferred in the corresponding liquid media. The capacity of the selected colonies to degrade ABZ was determined at 7 days via HPLC. Aliquots of cultures which showed a high degradation capacity (>70% degradation in 7 days) were sub-cultured in fresh liquid media, to confirm their degradation capacity. Only cultures exhibiting >60% degradation in 7 days were considered as positive and they were all derived from MSMN. The selected cultures were plated on MSMN + ABZ agar plates to check purity. They were then processed for DNA extraction, and further molecular analysis as described below.

2.3. Albendazole residue analysis

ABZ was extracted from liquid media by mixing 0.5 mL of culture with 0.5 mL of acetonitrile. The mixture was vigorously vortexed for 30 sec, then filtered through 0.45-µm PTFE hydrophobic syringe filters and directly analyzed in a Shimadzu HPLC–DAD system equipped with a Grace Smart RP C18 column (150 mm × 4.6 mm) (Shimadzu Corporation, Japan) as described before (Lagos *et al.*, 2022). Fortification tests at three concentration levels (0.1, 1 and 10 mg L^{-1}) showed mean percentage recoveries for ABZ, ABZSO, ABZSO$_2$, of 91.7%, 90.4%, 95.2% respectively.

2.4. Molecular identification of the albendazole-degrading bacteria

DNA extraction from the isolated bacteria was performed with the Nucleospin® Tissue kit (Marcherey–Nagel, Germany). Briefly, the near full-length (1500 bp) 16S rRNA gene of bacterial cultures was amplified with primers 8f–1512r (Felske *et al.*, 1997) as described by Perruchon et al., (Perruchon *et al.*, 2016). The identity of the isolated bacteria was determined via cloning the PCR products, using the pGEM®- T easy plasmid vector, and sequencing of the full length 16S rRNA gene. Three clones for each isolate were Sanger sequenced and the obtained sequences were edited manually and analyzed for best match with the Basic Local Alignment Search Tool (BLAST, v.2.9.0) (Altschup *et al.*, 1990). The closest relatives obtained plus an outgroup sequence were aligned with the Muscle software (Notredame, Higgins and Heringa, 2000). Uninformative blocks and misalignments were removed with the GBlocks software (Talavera and Castresana, 2007), and the sequence alignment obtained was utilized for the construction of maximum likelihood trees generated according to the general time reversible model, with gamma rate heterogeneity and accounting for

invariable sites, using the PhyML software (v.3.1) (Guindon and Gascuel, 2003). The sequences of the clones which studied were submitted in GeneBank NCBI, database with the accession numbers OP604271 to OP604273.

3. Results And Discussion

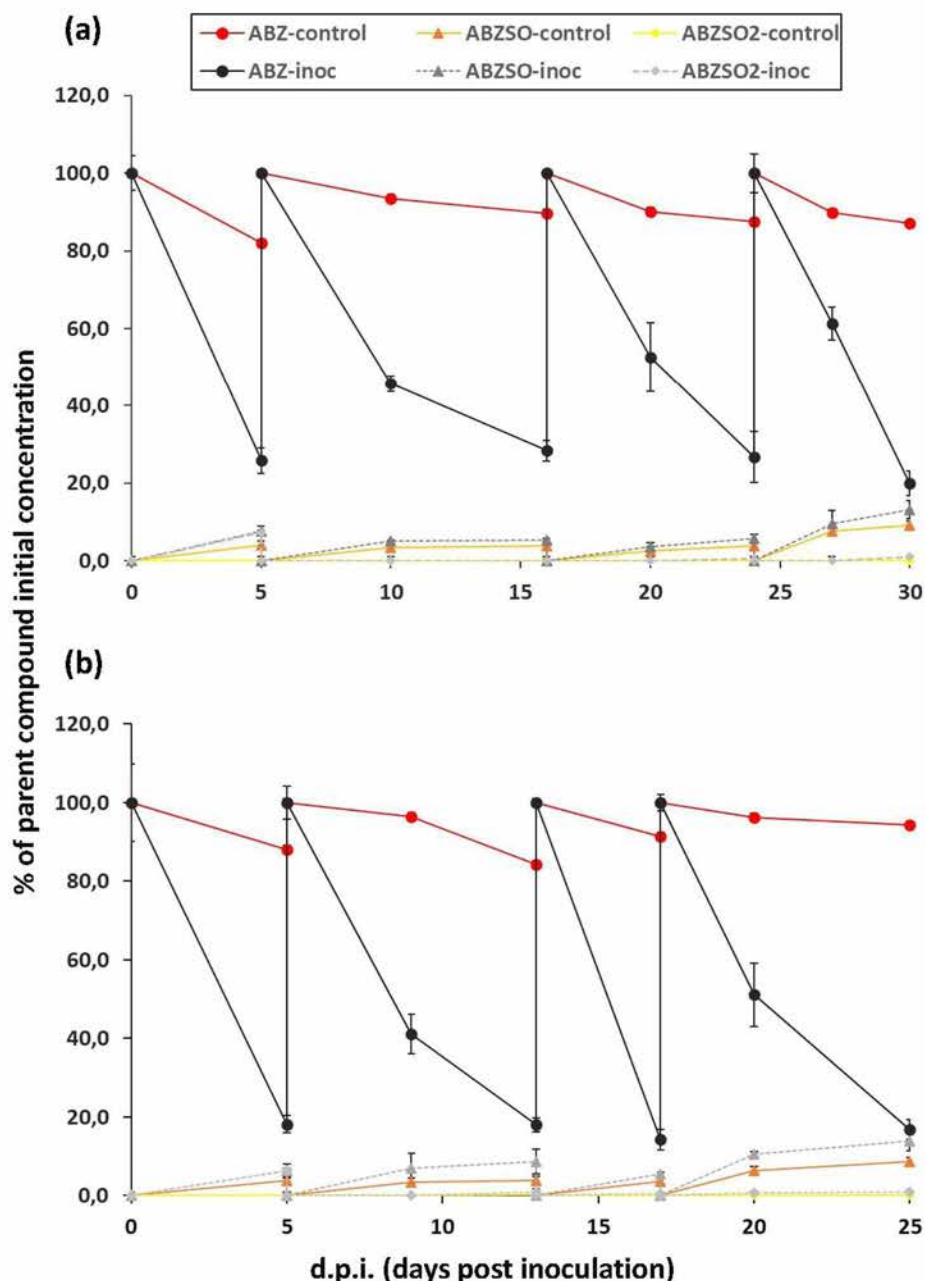## 3.1. Enrichment cultures in MSM and MSMN media

Figure 5.1 Degradation of albendazole (ABZ) and the formation and degradation patterns of its transformation products, albendazole sulfoxide (ABZ-SO) and albendazole sulfone (ABZ-SO$_2$) in four successive enrichment cycles in selective media MSMN (a) and MSM (b) either inoculated (inoc) or not inoculated (control) with a soil exhibiting enhanced biodegradation of ABZ. Each value is the mean of three replicates ± the standard deviation of the mean.

The degradation of ABZ in enrichment cultures in MSM and MSMN is presented in Figure 5.1. In the first enrichment cycle in both growth media degradation of ABZ was over 70% after 6 days. In MSM, the degradation of ABZ was stable in all enrichment cycles with over 70% degradation of ABZ after 6 days (Figure 1a). On the other hand, the degradation of ABZ in MSMN was accelerated along enrichment cycles (Figure 5.1b), except of the fourth enrichment cycle where we observed a slower degradation rate. Abiotic degradation of ABZ in the non-inoculated controls in both media never exceeded 20% (Fig. 5.1), suggesting that the degradation of ABZ observed in the inoculated cultures is microbially driven. In both media the degradation of ABZ

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along the enrichment process was coupled with the formation of ABZ-SO, as a main transformation product, while low levels of ABZ-SO$_2$ were also detected. The sum of these derivatives never exceeded 15% of the initial amount of the parent compound implying the formation of other transformation products that were not monitored in our study. The levels of ABZ-SO and ABZ-SO$_2$ formed were slightly higher in the inoculated compared to the non-inoculated cultures suggesting the involvement of both abiotic and biotic mechanisms in their production. Previous studies have shown that ABZ-SO and ABZ-SO$_2$ are produced both via abiotic (Liou and Chen, 2018) and biotic processes (Prasad *et al.*, 2008; Prasad, Girisham and Reddy, 2009, 2010).

3.2. Isolation and screening of albendazole-degrading bacteria

After the completion of enrichment cultures and plating, in total twenty and twelve morphologically distinct bacterial colonies were selected from MSMN+ABZ and MSM+ABZ agar plates respectively and screened for their ability to degrade ABZ (Figures 2 & 3). Four colonies which showed more than 50% degradation of ABZ, were selected from MSM cultures (Figure 5.2a) but their degrading capacity was not verified in a second round of cultivation and testing (Figure 5.2b). In case of MSMN, five colonies which presented > 60% degradation of ABZ after 7 days of incubation were selected for further testing (Figure 5.3a). From these only two cultures, named C3
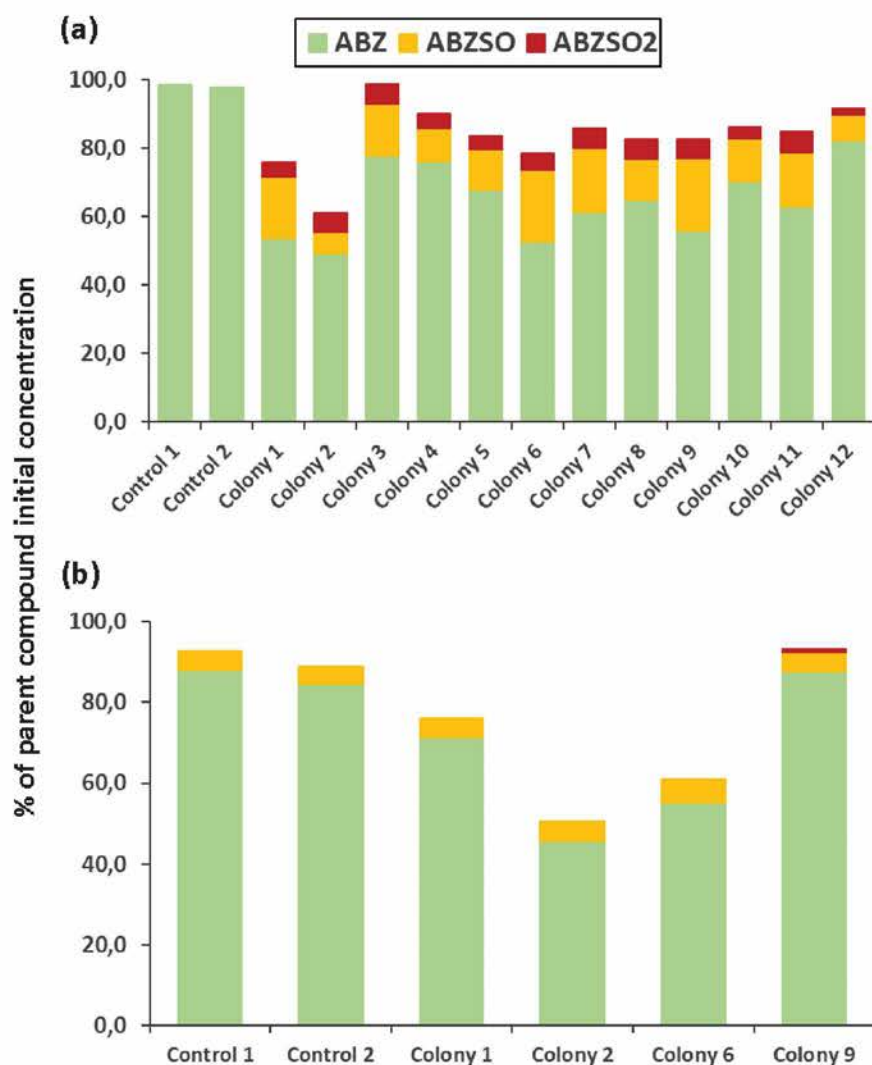
Figure 4.2 Degradation level of albendazole (ABZ) and formation of its transformation products, albendazole sulfoxide (ABZSO) and albendazole sulfone (ABZSO2) in MSM liquid cultures inoculated with selected colonies and in non-inoculated controls. (a) Degradation of ABZ by colonies obtained from a first round of selection after seven days of incubation (b) Colonies showing promising degradation of ABZ in the first screening were tested again for their degradation capacity.

and C13, maintained their high degradation capacity and exhibited > 60% degradation of ABZ after 7 days of incubation (Figure 5.3b). In agreement with the transformation pattern of ABZ in enrichment cultures, both isolates transformed ABZ to ABZ-SO, while small amounts of ABZ-SO$_2$ were also produced. In line with our findings, previous studies with fungal and bacterial isolates tested for their degrading capacity against ABZ also identified ABZ-SO and ABZ-SO$_2$ as the sole transformation products

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of ABZ (Prasad et al., 2009, 2010). In contrast Prasad et al (2008) observed, besides ABZ-SO and ABZ-SO$_2$, the formation of a new N-methylated derivative, produced by the degradation of ABZ by a *Cunninghamella blakesleeana* fungal strain.
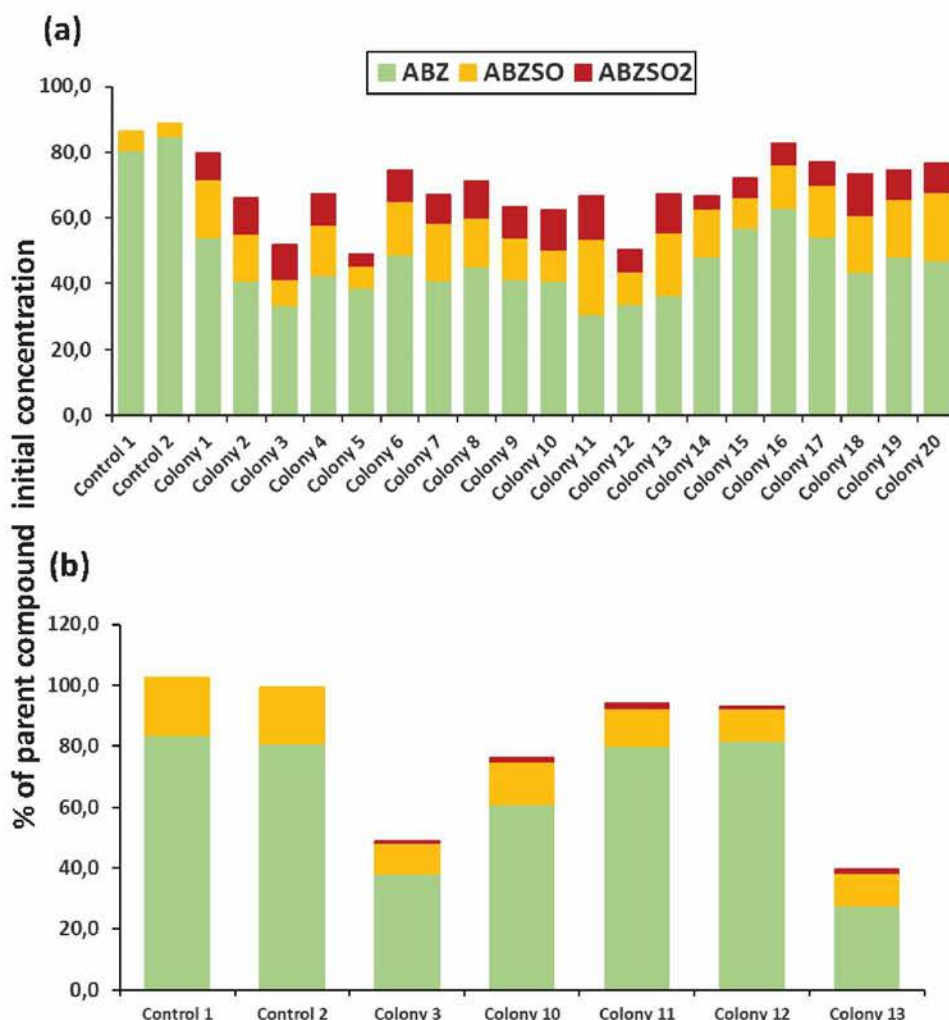

Figure 4.3 Degradation level of albendazole (ABZ) and formation of its transformation products, albendazole sulfoxide (ABZ-SO) and albendazole sulfone (ABZ-SO$_2$) in MSMN liquid cultures inoculated with selected colonies and in non-inoculated controls. (a) Degradation of ABZ by colonies obtained from a first round of selection after seven days of incubation (b) Colonies showing promising degradation of ABZ in the first screening were tested again for their degradation capacity.

3.3. Identification of albendazole-degrading bacteria

Based on their degradation capacity against ABZ the two isolates were further identified via molecular means. Clone libraries, prepared from cultures C3 and C13, revealed that the phylotypes represented in these cultures showed highest sequence match to the 16S rRNA gene sequence of bacteria of the genus *Acinetobacter*. Phylogenetic analysis based on the full-length 16S rRNA gene sequence verified the assignment of the two bacterial isolates to the genus *Acinetobacter*. Specifically, clones from culture C3 grouped with species *Acinetobacter oleivorans* and *Acinetobacter calcoaceticus*, while clones obtained from culture C13 were phylogenetically closer to *Acinetobacter pittii* (Figure 5.4). However, the low bootstrap values do not allow the assignment of the two isolates to the species level. Bacteria of the genus *Acinetobacter* are ubiquitous in soil, and they are characterized as metabolically versatile bacteria able to catabolize a wide range of natural compounds, implying active participation

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in nutrient cycling (Jung and Park, 2015). They are also known as efficient degraders of xenobiotic aromatic compounds like phenolic derivatives, quinones, pyridines, indoles (Paller et al, 1995; Ying et al., 2007; Zhang et al., 2021) and pesticides. For example, *A. calcoaceticus* and *A. oleivorans* strains were able to degrade the insecticide fipronil (Uniyal *et al.*, 2016), while Zhan et al. (2018) and Singh et al. (2004) isolated *Acinetobacter* stains able to degrade pyrethroids and atrazine respectively.

To date there are a few reports of microorganisms able to degrade ABZ. Lei et al (2013) isolated a *Rhodococcus* strain that was able to degrade ABZ and use it as a C source, in agreement with our *Acinetobacter* isolates that degraded ABZ only in MSMN where the AH served as a sole C source. Prasad et al (2010) screened several bacterial strains for their capacity to oxidize ABZ to ABZ-SO and identified *Enterobacter aerogenes*, *Klebsiella aerogenes*, *Pseudomonas aeruginosa* and *Streptomyces griseus* stains as active degraders of ABZ. Besides bacteria, Prasad et al (2008; 2009) also isolated fungal degraders of ABZ like a *Fusarium moniliforme* strain and a *Cunninghamella blakesleeana* strain.
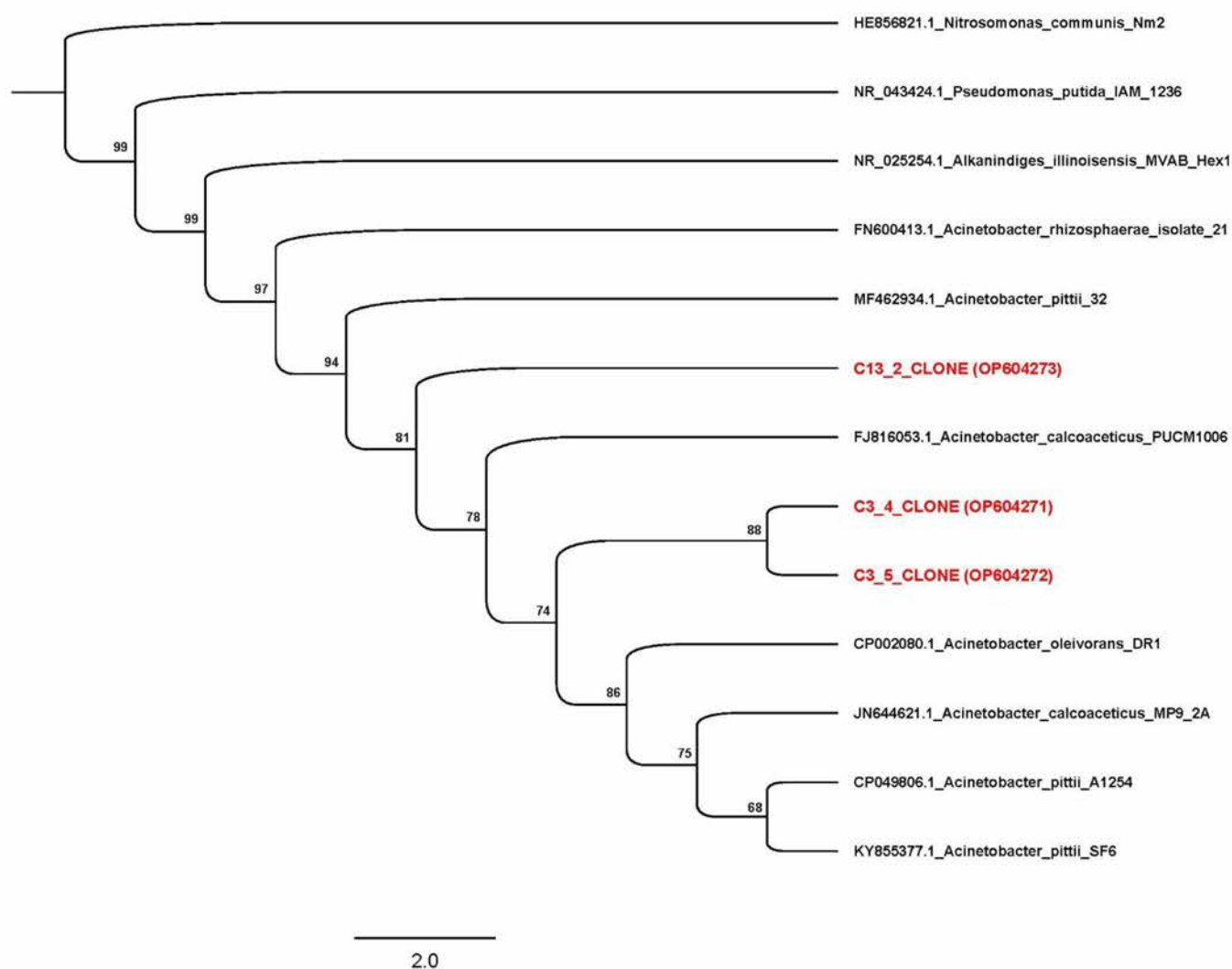

Figure 4.4 Phylogenetic analysis of the sequences from three selected clones which indicated with red colour based on the complete 16S rRNA gene sequence. All sequences were grouped within the genus Acinetobacter. Thousand bootstrap replicates were run with PhyML following the GTRGAMMAI (General Time Reversible with GAMma rate heterogeneity and considering Invariable sites) model. The bootstrap support is expressed in scale from 0 to 100. The NCBI accession numbers of each clone are indicated.

4. Conclusions

We report the isolation of two soil bacterial isolates, identified as *Acinetobacter* spp., that were able to degrade the synthetic benzimidazole AHs ABZ. Our isolates were able to transform ABZ to ABZ-SO and ABZ-SO$_2$, although the formation of other transformation products was not monitored. Whole genome sequencing analysis and further transcriptomic or proteomic analysis will provide insights into the transformation pathway and the genetic mechanism driving the transformation of ABZ in these bacterial isolates. This information is essential before the use of these bacteria as inocula for the bioaugmentation of contaminated fecal material and soils.

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Chapter 6

General Discussion

Discussion

Gastrointestinal nematodes (GINs) constitute a major threat for the welfare and productivity of livestock world-wide. Infections from GINs, which are called helminthiases, are prevented, and treated mainly by administrating synthetic anthelmintics (AHs) for more than 60 years now. However, AHs are only partially metabolized by animals, and they are largely released (40-90% of administrated dose) in feces at concentrations ranging from $\mu g\ Kg^{-1}$ to mg Kg^{-1} levels. These compounds reach grassland soils through the direct deposition of feces or urine by grazing animals but most importantly in agricultural soils through the application of contaminated manures. From there they could be further transported to other environmental compartments and also taken up by plants getting into the trophic chain. However, little is currently known regarding their interactions with soil microorganisms which have a pivotal role in soil ecosystem functioning. Two hypotheses have been formulated to determine the nature of this interaction: (a) AHs will have a toxic effect on soil microbial communities with reciprocal undesirable effects on ecosystem functioning, or (b) AHs will trigger microbial adaptation mechanisms enabling the evolution and dispersal of novel catabolic traits facilitating the accelerated biodegradation of AHs in soil. Thus, the main objective of this thesis was to investigate the interactions between three synthetic AHs, the benzimidazole (BZ) albendazole (ABZ) and the macrocyclic lactones (ML) ivermectin (IVM) and eprinomectin (EPM), and soil microorganisms, and to evaluate microbial degradation of AHs as a mean to decreasing environmental exposure to AHs. The experimental part of this thesis was divided in four main parts which constituted the four chapters of this thesis. First (Chapter 2), we investigated the role of basic processes, meaning biodegradation and adsorption, to the environmental fate of AHs in soils putting emphasis on the role of soil microbiota in the degradation of these compounds. Secondly, (Chapter 3) we studied the possible effects of AHs on the function and diversity of soil microorganisms in selected soils from Chapter 2 and explored the possibility that, beyond ecotoxicity effects, the continuous exposure of soils to AHs stimulate acclimation of the soil microbiota towards their enhanced biodegradation. Subsequently (Chapter 4), we evaluated the potential of a bacterial consortium able to degrade TBZ to be used as a wider bioaugmentation agent for the depuration of manures contaminated with BZ AHs. Finally (Chapter 5), considering the results of Chapter 4, we isolated soil bacteria with the ability to degrade ABZ (as tailored-made inoculum), the most ecotoxicologically relevant molecule from the ones tested and also prone to biodegradation.

In **Chapter 2** the role of soil microorganisms (biotic) and of soil physicochemical properties (abiotic), and their possible interactions, on the dissipation of ABZ, IVM and EPM was evaluated in selected pasture soils collected from livestock units of Lesvos Island. Comparative studies in fumigated and non-fumigated samples of a range of soils suggested a significant contribution of soil microorganisms in the dissipation of all three tested AHs based on their significantly higher DT_{50} values upon fumigation. ABZ exhibited low persistence in soil, with an average DT_{50} value of 0.3 days, which doubled to 4.3 days after fumigation, in line with previous studies (Wu & Hu, 2014). ABZ was quickly transformed to ABZ-SO and ABZ-SO_2, which were the major residual components. Considering that ABZ-SO possess anthelmintic activity (Belew et al., 2021) and both ABZ-SO and ABZ-SO_2 constituted the main transformation products of ABZ in our soils but also in animal tissues (Capece et al., 2009) and microbial cultures (Prasad et al., 2009, 2010), we calculated DT_{50} for the sum of the parent and its two oxidation products. In this case also, the dissipation of the total ABZ residues was significantly decelerated by the soil fumigation suggesting that soil microorganisms have an active and important role in the degradation not only of the parent compound but also of its TPs. However, the presence of $ABZSO_2$ in all soils at the end of this study may pose a potential risk for groundwater and surface water contamination, considering its higher water solubility and mobility in soils (Silveira Porto et al., 2021). IVM (ivermectin) and EPM (eprinomectin) showed moderate persistence in soil, with DT_{50} values of 34.5 and 30 days, respectively, which were significantly extended upon fumigation. In agreement with

our results, previous studies have showed similar results about persistence in soil of IVM and EPM and role of soil microbiota in their dissipation (Krogh et al., 2009; Litskas et al., 2013).

We further asked the question whether the repeated exposure of soils to AHs could lead to their accelerated microbial degradation. To address this, we selected soils with different levels of potential prior exposure to the studied AHs that might have triggered the evolution of novel microbial catabolic capacities leading to growth-linked microbial degradation of AHs and accelerated dissipation. However, we did not observe any significant enhancement in the degradation of the tested AHs in soils with presumed higher exposure to those compounds. These results suggest that AHs could not enhance their own biodegradation or that presumed exposure of soils to AHs was not adequate to enhance their biodegradation. Soil physicochemical properties, particularly soil total organic carbon (TOC) and pH, correlated with the persistence and adsorption of the studied AHs. TOC influenced the persistence of ABZ and IVM differently, with higher TOC promoting faster degradation of ABZ but retarding the degradation of IVM. Soil pH positively correlated with the persistence of IVM and EPM, suggesting a contribution of abiotic processes. Adsorption affinity followed the order IVM > ABZ > EPM, and TOC played a major role in the adsorption of IVM and EPM in soils, which has been demonstrated in several studies (Halley et al., 1993; Krogh et al., 2008; Litskas et al., 2011). The limited role of TOC in ABZ adsorption aligned with its positive effect on ABZ dissipation, while the strong adsorption of IVM in high TOC soils hindered its degradation. In summary, at Chapter 2 it is highlighted that soil microorganisms are key drivers of the dissipation of ABZ, IVM and EPM in soil complemented by further abiotic factors. Hence soil parameters like TOC and pH also affect the dissipation of AHs, although their contribution varies according to the compound studied.

In **Chapter 3** we tried to further shed light into the complex interactions between the soil microbiota and the studied AHs. We investigated whether repeated exposure of soils to AHs would lead to microbial adaptation and their enhanced biodegradation or to accumulation of AH residues and toxicity to the soil microbiota. For this purpose, soils which in Chapter 2 exhibited fast or slow degradation of the studied AHs were selected («fast» and «slow» soils respectively) and repeatedly treated with the three AHs. We hypothesized that this time the repeated exposure of the fast soils to AHs in the laboratory would be a scenario that could impose enhanced biodegradation of the studied compounds, while the same exposure scheme could alternatively result in accumulation of AHs and lead to potential ecotoxicity on the soil microbiota. Monitoring of the dissipation rates of the AHs verified our accelerated degradation hypothesis only for ABZ in the fast soil but not in the slow soil. Interestingly we noted that the formation and dissipation patterns of the two oxidation products, ABZ-SO and ABZ-SO$_2$, varied between soils. The slow dissipation rates and therefore accumulation of biologically active ABZ-SO in the "slow" soil could have inhibited the establishment of an active microbial community capable of degrading the parent ABZ, a phenomenon previously observed with pesticides (Motonaga et al., 1998; Robertson et al., 1998). On the other hand, our hypothesis for enhanced biodegradation was not verified for IVM and EPM neither in the fast nor in the slow soils. In contrast, we noted an accumulation of the residues of IVM and EPM in all studied soils.

In light of the accumulation of AH residues in several of the soils tested we investigated their effects on the soil microbiota. All AHs strongly and negatively affect the abundance of fungi and Crenarchaeota with ABZ being consistently the most inhibitory compound, which is not surprising considering the use of structurally similar BZ compounds as fungicides (Grogan, 2006). We further looked for potential effects of AHs on the diversity of bacteria, fungi and protists, their main predators in soil ecosystems (Geisen et al., 2018). None of the studied AHs had an effect on the α-diversity of bacteria, whereas ABZ had a strong negative effect on the α-diversity of fungi and protists. ABZ was again the AH with the stronger effect on the composition of bacteria and fungi community in both soils, unlike IVM and EPM whose effects were limited either to one of the two soils or were significant

only at the high dose rate. Unlike their prey, the community of protists was not affected by the AHs. These data constitute one of the most comprehensive dataset demonstrating potential effects of AHs on the different components of the soil microbiota, with only a few studies so far looking at this aspect (Ma et al., 2021; Papadopoulou et al., 2016a, 2018; Qiu et al., 2022). Our analysis identified specific microbial players like bacteria belonging to *Chitinophagaceae* and *Sphingomonas* that were negatively and positively associated with ABZ, respectively. The fungal community in all soils was dominated by *Sporormiella*, commonly found in animal dung. The presence of AHs, particularly ABZ, affected microorganisms involved in nutrient cycling, plant growth promotion, predation, and population control, with potential consequences for soil ecosystem functioning (Bailey et al., 2013; Seki et al., 2012).

Based on the effects of AHs on the abundance and diversity of broad microbial groups, we further focused on the potential effects of AHs on N cycling and especially on ammonia oxidizing microorganisms (AOMs), an important functional microbial group which is consider as an ideal bioindicator for assessing toxicity of chemicals to the soil microbiota (Feld et al., 2015; Vasileiadis et al., 2018). All AHs induced significant negative effects on the activity and abundance of AOMs with ABZ again being the AH with the most persistent and prominent effect compared to IVM and EPM. In general, despite its short persistence, ABZ was consistently the most toxic to broad phylogenetic microbial groups and AOMs. We speculate that this persistent inhibitory effect is driven not only by the parent compound but also by its transformation products like the bioactive ABZ-SO. Similar observations have been reported for pesticides where transformation products were equally or more toxic than the parent compound on AOM (Vasileiadis et al., 2018). Little is known about the effects of AHs on the soil microbiota and particularly on AOM. In the few studies available, Papadopoulou et al. (2016) reported no inhibitory effects of thiabendazole (TBZ) on the abundance and activity of AOB and AOA. Whereas Konopka et al. (2015) reported that IVM applied in soil as a mixture with the antibiotics monensin, and zinc bacitracin at high concentration levels (10 mg kg^{-1}) suppressed the abundance of AOB. Overall, our data suggest that AHs, once found in soil at environmentally relevant levels, could impose diverse effects on the abundance, diversity, and activity of soil microbiota, highlighting the potential impacts on soil ecosystem functioning. This was more prominent and consistent for ABZ whose increasing ecotoxicity on the soil microbiota worth investigating further.

Considering the persistence and toxicity of AHs in soils we tried to explore novel biological means to prevent the dispersal and accumulation of AHs in soils. Hence in **Chapter 4** we evaluated the potential use of bioaugmentation as a mean to remove BZ AH compounds from fecal material, thereby preventing their environmental dispersal. We hypothesized that a bacterial consortium able to degrade the anthelmintic benzimidazole TBZ (Perruchon et al., 2018) in soil and liquid cultures, would be able to effectively degrade other AH compounds of the same chemical group. We first showed in liquid culture experiments that this bacterial consortium was able to accelerate, besides TBZ, the degradation of other AH compounds, such as ABZ, and its oxidation analog ricobendazole (albendazole sulfoxide, ABZSO), but showed moderate degradation capacity against FBZ and limited degradation capacity for other benzimidazole compounds like FLZ and MBZ. We speculated that the bulky substituents of the benzyl moiety of the benzimidazole ring of MBZ, FLZ and FBZ do not facilitate optimum fitting in the active site pocket of the carbazole dioxygenase enzyme of *Sphingomonas* (the member of the consortium which is responsible for the degradation of TBZ) which shows high affinity for carbazole and TBZ, both lacking such substituents (Vasileiadis et al., 2018).

We further explored the potential of consortium to act as bioaugmentation agent when inoculated in fecal material contaminated with ABZ, TBZ and FBZ. We noted that under natural conditions the consortium only moderately enhanced the degradation of AHs, possibly due to the higher organic matter content and the presence of indigenous microbial communities in feces which probably

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hampered their proliferation and capacity to access AHs. This observation was not surprising considering the previously reported difficulty of exogenous inocula to compete with the indigenous microbial community of complex environmental matrices like soils and feces (Cycon & Piotrowska-Seget, 2015). Indeed, when the exogenous inoculum was added to fumigated feces, its degradation efficiency towards ABZ and FBZ was amplified most probably by reducing the competition from the indigenous microbiota for nutrients and space. By comparing and contrasting the dissipation patterns of AHs in inoculated and non-inoculated feces we observed that the indigenous fecal microbiota also contributed to the degradation of benzimidazole compounds, but their biodegradation alone was insufficient for effective removal within the typical storage period of animal feces before application in agricultural soils. Overall, these findings provided pioneering evidence for the potential use of bioaugmentation for the remediation of feces, hence diminishing the environmental dispersal of AH compounds.

By looking at the moderate performance of the TBZ – degrading bacterial consortium against the majority of AHs and considering the higher ecotoxicity of ABZ compared to the other AHs tested, we decided to isolate bacteria able to specifically degrade ABZ in an effort to create tailored-made inocula for the more efficient bioaugmentation of feces. Hence in **Chapter 5**, we aimed to use the soil exhibiting accelerated biodegradation of ABZ (Chapter 3) as a source for the isolation of bacteria able to rapidly degrade ABZ. Enrichment cultures and further subculturing and testing led to the isolation of two bacterial isolates which were able to effectively degrade ABZ in selective media where the AH constituted the sole energy source. Phylogenetic analysis based on the full-length 16S rRNA gene sequence showed that the two isolates belonged to the genus *Acinetobacter*, which encompass members with known metabolic versatility and ability to degrade various natural and xenobiotic compounds (Uniyal et al., 2016; Ying et al., 2007; Zhang et al., 2021). These bacteria will be further characterized and their potential use in bioaugmentation strategies as specialized microbial inocula would be explored.

Conclusions

Overall, our findings have shed light into the interactions of AHs albendazole, ivermectin and eprinomectin with the soil microorganisms, the factors driving these interactions and the potential use of soil microorganisms as a vehicle to avert the environmental dispersal of AHs. The main conclusions of the current thesis could be summarized as follows:

- Biotic and abiotic factors contribute to the dissipation of ABZ, IVM and EPM in soils, with the soil microbiota having the most important and critical role in the removal of the residues of these compounds from soil.

- Exposure of soils to AHs at concentration levels expected to be encountered in agricultural and grassland settings could have detrimental effects on the soil microbiota affecting important functional microbial groups like AOMs. These findings are expected to benchmark future studies on the toxicity of AHs on the soil microbiota and will pave the way for potential reconsideration of soil microbes as protection goals in the environmental risk analysis of AHs.

- Under the field exposure conditions tested in our study none of the AHs tested show any signs of enhanced biodegradation. However, under laboratory extreme exposure schemes ABZ was the sole AH that exhibited accelerated biodegradation, although the relevance of this phenomenon in realistic exposure schemes in soils is questionable.

- Bioaugmentation of feces with non-specialists benzimidazole-degrading inocula could pose a potential solution for the remediation of manures contaminated with BZs although their efficacy is challenged by the competition with the indigenous microbiota of feces.

- In the quest for development of more specialized tailored made inocula for the more efficient removal of ABZ from manures we isolated two soil bacteria, identified as *Acinetobacter* spp., that were able to rapidly degrade ABZ

Future perspectives

The current thesis highlighted the important role of microorganisms in the dissipation of AHs in soils while at the same time provided first evidence and concerns about their possible ecotoxicity on the soil microbiota, at diversity and functional level. It also provided promising tools for the potential bioaugmentation of manures as a treatment strategy for the mitigation of the environmental dispersal of benzimidazole AHs and in this respect reported the isolation of *Acinetobacter* spp. strains capable of degrading ABZ. Based on these findings, further issues could be followed up in the future like:

I. Whole genome sequencing analysis and further transcriptomic or proteomic analysis of ABZ-degrading *Acinetobacter* spp. isolates will provide insights into the transformation pathway and the genetic mechanism driving the transformation of ABZ. This information is essential before the use of these bacteria as inocula for the bioaugmentation of contaminated fecal material and soils.

II. Verify the toxicity of ABZ and of other AHs to AOM through *in vitro* assays with a range of soil derived AOB and AOA and explore the specific toxicity mechanisms towards this functional microbial group.

III. Expand toxicity assays and explore the potential toxicity of AHs to other key functional soil microbial groups like arbuscular mycorrhizal fungi at *in vitro* and in soil tests.

IV. Considering the demonstrated toxicity of ABZ on the soil microbiota, explore potential interactive effects of AHs with other pollutants which are commonly found in agricultural soils like pesticides and mostly microplastics which are considered emerging contaminants in agricultural settings expected to directly interact with hydrophobic AHs.

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