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Response Of Soil Nitrification To The Veterinary Pharmaceuticals Monensin, Ivermectin And Zinc Bacitracin

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology
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RESPONSE OF SOIL NITRIFICATION TO THE VETERINARY PHARMACEUTICALS MONENSIN, IVERMECTIN AND ZINC BACITRACIN

(Thesis format: Monograph)

by

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Graduate Program in Biology

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Abstract

Pharmaceutical residues can reach agricultural land through amendment with animal or human waste. Since 2010, a series of replicated plots received annual applications of ivermectin, monensin and zinc bacitracin, either singly or in a mixture, at 0.1 mg/kg and 10 mg/kg concentrations. I collected soil samples before and after the fourth annual application of pharmaceuticals and assayed them for functional changes and *amoA* gene abundance, a gene needed for ammonia oxidation. In 2013, I exposed the soils to 100 mg/kg in a laboratory experiment which resulted in acceleration of nitrification. Under 10 mg/kg treatments in the field the abundance of ammonia-oxidizing bacteria was suppressed, while ammonia-oxidizing archaea increased, suggesting that bacteria are more sensitive to these pharmaceuticals, and that archaea can expand to occupy the partially vacated niche. None of the pharmaceuticals at the guideline level of 0.1 mg/kg had any effect on soil function or ammonia oxidizing organisms.

Keywords

Co-Authorship Statement

A manuscript submitted to Environmental Toxicology and Chemistry on October 10th 2014 was co-authored with Dr. Hugh Henry, Dr. Ed Topp, and Romain Marti. Dr. Henry and Dr. Topp provided materials and equipment needed to conduct the experiments through competitive funding of the AAFC Growing Forward 1 and Growing Forward 2 programs, and NSERC Discovery Grant. R. Marti designed the new set of primers used for archaeal qPCR analysis, and helped with qPCR troubleshooting and method optimizations. All three contributed to the manuscript writing and editing.

Acknowledgments

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List of Abbreviations

AMO – Ammonia monooxygenase
AO – Ammonia oxidizers
AOA – Ammonia oxidizing archaea
AOB – Ammonia oxidizing bacteria
D0, D7, D30, fall – sampling times
DGGE – Denaturing gradient gel electrophoresis
DNA – Deoxyribonucleic acid
EDTA – ethylenediaminetetraacetic acid
ERA – Environmental Risk Assessment
ISO – The International Organization for Standardization
IVER – Ivermectin
MIX – Mixture of the 3 studied drugs, ivermectin, monensin, and zinc bacitracin
MON – Monensin
NOEC – No observed effect concentration
OECD – Organization for Economic Co-operation and Development
OEHHA – Office of Environmental Hazard
PCR – Polymerase Chain Reaction
PEC – Predicted Environmental Concentration
qPCR – Quantitative Polymerase Chain Reaction
VICH – Veterinary International Conference on Harmonization
ZBAC – Zinc bacitracin

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1. Introduction

1.1. Soil: composition and function

Soils have specific physical and chemical characteristics, such as particle size and composition, organic matter content, diffused gases (e.g. O_2 and CO_2), pH, temperature, and water content (Tate III 2000). The combination of these factors can create favorable or adverse conditions for life. Unfavorable conditions can prevent plant life, however even small amount of nutrient allows for microbial growth. Microorganisms are very good at adapting their environments to their needs, and can modify the physical and chemical properties of soil, making it possible for them to thrive under conditions that would be stressful for other life forms, including plants (Paul and Clark 1989).

Soil is organized vertically in layers, called horizons, with the top layer generally being the most recent, while deeper layers represent further past (Tate III 2000). The most studied layers are the "O" (top organic layer, most pronounced in forest soils due to accumulating leaves and foliage), "A", and "B" layers (underneath). The "A" layer houses the greatest diversity and abundance of soil dwelling macro- and microorganisms, which decline in the "B" layer (Tate III 2000). Most non-forest soils have little organic matter present (>5%), and carbon and nitrogen are present in their mineral form; they are called mineral soils because of this high mineral content (Tate III 2000). Mineral soils can be classified based on the size of soil particles (sand, silt, clay), which facilitates inter-study comparisons (Soil textural triangle, http://www.nrcs.usda.gov). Most soils in Southwestern Ontario, Canada originated from material deposited during last glacial retreat, and contains high proportion of mineral matter (Tate III 2000). The majority of the land in this region has been used for agricultural purposes.

The physical and chemical elements of soil are modified by the biota present, which promotes community growth and development, resulting in the formation of interconnected trophic networks (Scharriba et al. 2012). Soil contains macro- (>2mm), meso- (0.1-2mm) and microorganisms (<0.1mm) which contribute to soil physical structure and function (Griffiths 1965). The total number of microorganisms living in soil is not known, but it is estimated at 40 million cells per gram forest soil (Whitman et al.

1998). All soil dwelling organisms contribute to soil functions such as decomposition of organic matter and nutrient turnover (Griffiths 1964). Soil macroorganisms include small soil dwelling animals such as earthworms, millipedes, beetles, and ants, that move through the soil column and mix it while they graze on microorganisms or mesoorganisms (Paul and Clark 1989). Mesoorganisms such as Collembola, mites, and nematodes migrate through soil feeding on decaying matter and microorganisms. Their movement disturbs soil particles, it may expose new nutrients and allows gases and water to mix in the soil column. Microorganisms can take advantage of these new conditions to proliferate. Bacteria and fungi are primarily responsible for breaking down dead plant matter and other microorganisms are responsible for various steps in nutrient cycling (Paul and Clark 1989, Tate III 2000).

1.2. Carbon and nitrogen in soils

Microorganisms in soil are considered the natural agents for human and animal organic waste disposal (Paul and Clark 1989), because of their ability to recycle carbon (C) and nitrogen (N) bound in organic matter. They break the nitrogen and carbon into mineral C and N that can enter the soil nutrient pool, which is very important for microorganism proliferation and plant growth. Together with carbon, other nutrients and trace minerals are released during decomposition for use by soil organisms and plants.

Carbon exists in the gaseous form in the air (as CO_2), and is incorporated into the soil ecosystem through CO_2 uptake by plants and bacteria (Hutchinson et al. 2007) and subsequent incorporation (immobilization) into living tissues. Once carbon is bound in the organic form, it remains there until the tissue dies, at which point C is recycled through the decomposition of dead tissues by soil decomposing organisms. Carbon can be stored in organic form in tissues for extended periods of time, especially if the species is long lived one. Even after tissue death, carbon can be trapped in the organic form, due to different levels of degradability of plant material (Terry et at. 1979). Materials such as starches are readily hydrolyzed and used by soil microorganisms, while molecules like cellulose, lignins, and keratins are only partially decomposed (Attiwill 1986, Tate III 2000) due to their strong molecular bonds.

Fungi and bacteria living in the soil are the major contributors to organic matter decomposition, and they breakdown organic matter into simpler molecules that are accessible to plants and other microorganisms. Dissipation (oxidation of organic matter) generates energy that microorganisms use for growth. During the process of energy production, soil microorganisms use molecular O_2 as an electron acceptor and yield CO_2 and H_2O. The amount of CO_2 released is proportional to the amount of degraded matter; therefore the rate of microbial decomposition is traceable by monitoring the CO_2 emitted from the soil (Lehmann and Miller 1999). About 70% of plant residue added to soils is degraded, while the remainder is incorporated into microbial biomass, or remains undegraded (Tate III 2000).

Nitrogen is abundant in the environment, but most of it exists in the atmosphere in the form of nitrogen gas (N_2). Itis only accessible to a small number of organisms (nitrogen fixing microorganisms), which add it to the nitrogen pool in the soil (Equation 1). Dead plant matter also contains nitrogen that is released during decomposition. The soil nitrogen pool therefore consists of nitrogen from N_2 fixation and N obtained through decomposition of plant matter, or in the case of managed agricultural land from fertilizer. Decomposers (fungi and bacteria) use tissue-bound N, mineralize it into ammonia (NH_3), and release it into the soil N pool, making it accessible to other organisms. Ammonia present in the soil is converted to ammonium (NH_4^+, the protonated form of ammonia) at typical soil pH values. Ammonium is converted to nitrite (first step of nitrification) by ammonia-oxidizing organisms. Nitrification is initiated by the insertion of an atom of oxygen into ammonia by the ammonia monooxygenase (AMO) enzyme. Further oxidation by hydroxylamine oxidoreductase results in nitrite (McTavish et al. 1993, Vajrala et al. 2013). Nitrite is quickly converted to nitrate by nitrifying bacteria, and readily used by plants. This form is easily converted back to N_2 by denitrifying organisms (Ellis et al. 1996), or lost to the environment through runoff from the soil surface or leaching to the ground water (Friedland et al. 1997).

Equation 1. Simplified nitrogen cycle in soil

$$N_2 \xrightarrow{fixation} organic\ N \xrightarrow{decomposition} NH_4^+ \xrightarrow{nitrification\ 1} NO_2^- \xrightarrow{nitrification\ 2} NO_3^-$$
$$\xrightarrow{uptake} organic\ N$$
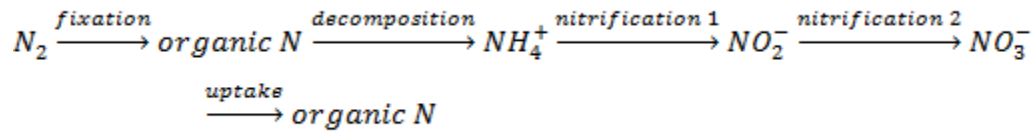
1.3. Ammonia oxidizing (AO) organisms

The conversion of soil ammonium to nitrite is catalyzed by a very narrow range of organisms called ammonia oxidizers (AO), which use ammonia as their only energy source (Stein et al. 2012). The ammonia-oxidizing microorganisms can be distinguished from other soil microorganisms through molecular analysis of the *amoA* genes, which encode the ammonia monooxygenase (AMO) enzyme. Two groups of microorganisms in soils, bacteria and archaea, possess AMO and are capable of converting NH_4^+ to nitrite (Rotthauwe et al. 1997, Francis et al. 2005). Both ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA) are active in soils. It is well established through *amoA* gene counts in soil that AOA dominate in NH_4^+ limited environments that contain high concentrations of organic nitrogen, while AOB thrive in nitrogen rich ecosystems such as agricultural land after fertilizer addition (Stein et al. 2012, Sonthiphand et al. 2013). The individual contributions of AOA and AOB to soil nitrification are however largely unknown. These organisms are difficult to culture in the laboratory, with only a few representatives having been described (Tourna et al. 2008, Martens-Habbena et al. 2009). The difficulty in isolating and cultivating these soil microorganisms might be due to their diverse nutritional needs, their need for coexistence with other organisms, or other conditions that cannot yet be recreated in the laboratory.

1.4. Unmanaged vs. managed (agricultural) soils

Ammonia-oxidizing archaea are usually more abundant than ammonia-oxidizing bacteria in cultivated soils (Leininger et al. 2006, Adair and Schwartz 2008, Tourna et al.

2008, O'Sullivan et al. 2011, Dias et al. 2012, Taylor et al. 2013). The cultivation of soil for crop production creates annual cycles of disturbances that might be responsible for the differences between the distributions of AOA and AOB organisms in unmanaged versus managed soils. In unmanaged soils, plant and animal remains decompose *in situ* to release nutrients (such as C and N) that other organisms can use. However, in the agricultural context, plant residues are rarely returned for decomposition and nutrient cycling, and nutrients are exported in the harvested crop. The nitrogen is depleted readily and needs to be replenished through fertilization before the next crop is planted (Vitousek et al. 1997, Edmeades 2003, Galloway et al. 2004). When inorganic nitrogen fertilizer is added to soil, plants and microorganisms compete for that nitrogen (Inselsbacher et al. 2010). While microorganisms are better than plants at acquiring ammonia in the first hours after fertilizer addition, plants are better at holding on to N due to their longer turnover rates (Schimel and Bennett 2004). Therefore, the majority of fertilizer applied to fields is used by crops and subsequently removed from the site.

In Ontario it is common practice to apply animal waste (manure) or human waste (biosolids) as fertilizer on agricultural fields. The re-use of otherwise wasted material is economically important, because it provides a quick and easy way of disposal of farm animal and human waste, and it saves landfill space, while providing a source of N to crops. The manure and biosolids contain organic nitrogen, which is not readily accessible to plants and needs to be mineralized by soil microorganisms into inorganic nitrogen. These amendments also contain phosphate and potassium, and they increases soil texture and water holding capacity (Eldridge et al. 2008).

1.5. Risk of applying manure and biosolids to agricultural fields

Despite the potential economic gains of using soil amendments, the potential impacts of applying waste material to fields need to be carefully considered. The land application of municipal biosolids (organic material recycled from sewage) and manures from medicated animals introduces veterinary and human pharmaceuticals, endocrine-active substances and personal care products into soil (Thiele-Bruhn 2003, Lorenzen et al. 2004, Borgmann et al. 2007, Sabourin et al. 2012). In manure, the type and amounts of

veterinary pharmaceuticals introduced at the moment of land application varies according to what is permitted for use in a given production system, the health status of the animals, and how the manure is stored and handled prior to application (Pope et al. 2009).

Antimicrobial pharmaceuticals are designed to work well in very small concentrations on the bacterial and parasitic organisms that attack livestock (Kumar et al. 2005, Toth et al. 2011, Jechalke et al. 2014). They are often excreted in their unchanged bioactive forms (Al-Ahmad et al. 1999, Thiele-Bruhn 2003, Sarmah et al. 2006), and thus manures can contain veterinary pharmaceuticals, including parasiticides and antimicrobial agents used for prophylaxis or therapy and for growth promotion (HC 2001, Sarmah et al. 2006, Liebig et al. 2010b). Because the majority of the pharmaceuticals excreted by medicated animals are still in their active form, they can negatively affect the soil bacteria that come in contact with them, with potential negative effects on N processing in soil. Manures typically contain pharmaceuticals in combination, and therefore mixture toxicity effects are of scientific and regulatory concern (Kemper 2008, van Gestel 2012, Altenburger et al. 2013). Among the possible mixture effects (additive, no effect, synergistic) the most concern centers on the synergistic effects of these compounds (multiplication of the effects of a single pharmaceutical). Singly the pharmaceuticals can be present at levels too low to be toxic, but combined and multiplied, the effects can be detrimental to soil microorganisms, and their services in soil.

Pollution with pharmaceuticals can have long term effects on soil organisms, but in heavily polluted environments soil microorganisms can become tolerant of the pollution, at a community scale (Schmitt et al. 2004). This response is called pollution-induced community tolerance (PICT) and it results from more tolerant organisms multiplying and replacing the more susceptible organisms. This resulted in a change in community structure, and sometimes soil function (Nannipieri et al. 2003, Schmitt et al. 2004, Demoling and Baath 2008, Aaen et al. 2011). The difference between susceptibility and tolerance of organisms can be due to intrinsic resistance, ability to tolerate low concentrations of pharmaceuticals, structural differences preventing negative effects of pharmaceuticals; or acquired resistance through acquiring resistance genes from other microorganisms or the environment. In the case of ammonia oxidizing organisms, bacteria and archaea are structurally distinct from one another; therefore it is possible that

the pharmaceuticals designed to target bacterial infections in farm animals may not necessarily affect archaea in the same way. Archaea have a different cell wall structure, biochemical pathways, and enzymatic activity from bacteria (Schleper and Nicol 2010) although not much is known about the soil achaea due to the difficulty in cultivating the organisms (only one soil AOA organisms has been cultivated in the laboratory; Martens-Habbena et al. 2009).

1.6. Regulations surrounding use of veterinary pharmaceuticals

The degree to which each new pharmaceutical is tested before approval depends on its predicted environmental concentration (PEC). The predicted level of pharmaceuticals reaching the environment is tested against a tiered environmental risk assessment (ERA), where the no effect concentration level is set as 0.1 ppm (1 parts per million is equal to 1 mg pharmaceutical per kg soil). A concentration of 0.1 ppm is the International Co-operation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH; http://www.vichsec.org) trigger value, and indicates the conservative cut-off amount of pharmaceutical in the environment expected to cause no detectable negative effects (NOEC = no observable effect concentration) on soil organisms or processes (VICH 2000, Montforts 2005, Schmitt et al. 2010) (overview of Canadian participation in VICH can be found at http://www.hc-sc.gc.ca/dhp-mps/vet/legislation/guide-ld/vich/index-eng.php). High loads of pharmaceuticals administered to animals can lead to increases in the amount of pharmaceuticals present in the manure.

If the PEC present in the soil treated with manure is below the VICH trigger value, no further tests are required for the specified pharmaceutical. However, if the amount expected, or already present exceeds 0.1 ppm, it triggers a Phase II of ERA, requiring additional soil fate studies on non-target species (Van Den Brink et al. 2005, VICH 2005, Tarazona et al. 2010). These are specified one-species tests measuring the lethality of the pharmaceutical, taking into account such endpoints as mortality, development, and reproduction (species used and corresponding OCED regulations summarized in (van Gestel 2012)). Soil incubations (microcosms) measuring functional
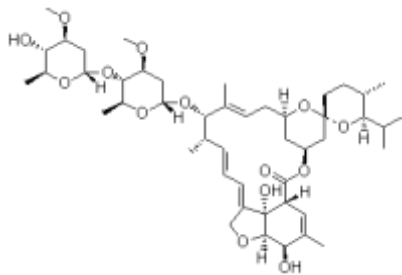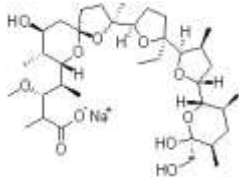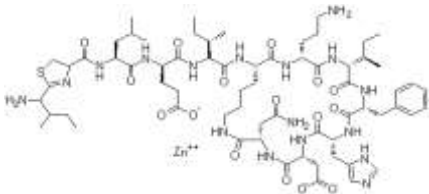
endpoints (such as mineralization or nitrification) are also used to monitor the microbial responses to pharmaceuticals (Van Beelen and Doelman 1997, OECD 2000, ISO 2012). The use of soil incubation experiments for functional toxicity studies can be coupled with molecular analysis (for example qPCR, quantitive polymerase chain reaction; PCR-DGGE, PCR based denaturating gradient gel electrophoresis, and others) as an indicator of the effects of these pharmaceuticals on the abundance and community structure of soil microorganisms (OEHHA 2009).

1.7. Studied pharmaceuticals and their uses

Three pharmaceuticals commonly used to treat farm animals in North America that were chosen for my study are monensin (MON), ivermectin (IVER), and zinc bacitracin (ZBAC). All three of these pharmaceuticals have different modes of action, and potentially different target organisms in soil (Table 1). Both IVER and MON are isolated from soil *Streptomyces* species (*S. avermitilis* and *S. cinnamoniensis,* respectively), while ZBAC is isolated from *Bacillus* species (*B. subtilis*, and *B. licheniformis*). While IVER and MON have bactericidal properties on their own, ZBAC needs to be bound to a metal ion to show bactericidal activity (Ming and Epperson 2002). Given that these pharmaceuticals originate from soil dwelling microorganisms, it is expected that resistance or tolerance to these compounds already exist at low levels in the environment (D'Costa et al. 2011, Bernier and Surette 2013) and this resistance can spread under heavy pharmaceutical applications.

Ivermectin (IVER) is a broad spectrum antiparasitic pharmaceutical, used to kill ecto- and endoparasitic infections in sheep, cattle, and pigs. Ivermectin is excreted primarily in faeces (Beynon 2012) as a parent (unchanged and active) pharmaceutical (Halley et al. 1989, Eržen et al. 2005). A negative effect on non-target invertebrate soil species was reported for IVER shortly after its widespread use. The negative effect was confirmed during single species toxicity testing in the laboratory and field

Table 1. Studied pharmaceuticals and their properties

	Ivermectin	Monensin	Zinc bacitracin
structure			
formula	$C_{48}H_{74}O_{14}$	$C_{36}H_{61}O_{11}$ Na	$C_{66}H_{101}N_{17}O_{16}Zn$
melting point	155 °C	269°C	250 °C
Solubility (solvent)	50 g/L (2-butanone)	50 g/L (methanol)	5.1 g/L (water)
action	antiparasitic	bactericidal	bactericidal

(Liebig et al. 2010b, O'Hea et al. 2010, Blanckenhorn et al. 2013), where IVER was shown to cause mortality (at 5ppm) and reduce reproduction (at 0.02ppm) in soil invertebrates (Jensen et al. 2009).

Monensin (MON) is an ionophore antibiotic (i.e. disrupts ion transport through membranes), with antiparasitic properties and it is commonly used in cattle and poultry for growth promotion and prevention of bacterial infections. It is excreted in its active form in faeces (Donoho 1984, Sassman and Lee 2007). Following the ban of pharmaceuticals as growth promoters in the European Union (UN) in 2006 (EC 2003), its use is mainly in North America. MON has reduced reproduction of soil invertebrates, but at a much higher level (100ppm) than IVER (Jensen et al. 2009).

Zinc bacitracin (ZBAC) is in its biologically active form as a complex of the heavy metal zinc and the antibiotic bacitracin (Ming and Epperson 2002). It is used in poultry and swine as a growth promoter. It acts by disrupting cell wall synthesis (preventing cross linking of peptidoglycans, which are numerous in the cell wall of gram positive bacteria but absent in eukaryotic cells). Similar to IVER and NON, it is found in its active form in animal manure (Donoso et al. 1970). When zinc is released from bacitracin (Drabløs et al. 1999) it can be considered a secondary contaminant in the soil treated with ZBAC. Metal pollution was shown to disrupt community function in soils with high levels of heavy metals such as copper and zinc (Mertens et al. 2010, Ruyters et al. 2013), and it can cause a PICT response in soil communities (Baath et al. 1998).

1.8. Microcosm incubations

Microcosms represent a community, place or situation that represents a miniature of something much larger, but encapsulating its characteristic qualities or features. Laboratory constructed microcosms are open or closed simplified ecosystems containing living organisms assembled in a simplified environment (Huhta 2006). Their function is to study processes that drive population and ecosystem ecology, but in a laboratory setting, with the intention that the results can then be extrapolated to the outside environment. In microcosms one has precise control over abiotic variables, and the manipulation of a single parameter is possible, while keeping other factors constant.

Microcosms are short term and therefore require less resources; however, due to space restrictions they can sometimes over-simplify the natural world, and extrapolation of the results therefore can be difficult (Fraser and Keddy 1997, Browder 2004, Drake and Kramer 2012). However, the ease of use of these simplified systems makes them useful tools for studying natural processes.

In studies of pharmaceutical toxicity in soils, microcosms are used to determine functional changes. The pharmaceuticals are added to determine the effects they have on the measured parameters (Lehmann and Miller 1999, Offre et al. 2009). For pharmaceutical dissipation tests, the dissipation of a studied pharmaceutical is monitored over time (Girardi et al. 2011).

1.9. Objectives and hypothesis

In the present study, I examined effects of the antiparasitic IVER and the antibiotics MON and ZBAC both singly and combination (MIX), using functional assays (mineralization and nitrification in soils) and molecular techniques (*amoA* gene abundance and structure of AOA and AOB communities). I hypothesized that the studied pharmaceuticals would affect the abundance of soil microorganisms and functions they carry out in the soil. Therefore, I predicted that: 1. Increasing exposure concentrations (0.1, 10, and 100 ppm) will have an increasingly negative effect on function and AO community structure. 2. Pharmaceuticals will cause a negative effect shortly after addition to soils, but no long term effect will be present (the community will recover from the disturbance, returning to the pre-disturbance levels of function and abundance). 3. In mixture, single pharmaceutical effects will multiply to exert a synergistic effect on the function and structure of soil.

2. Materials and Methods

2.1. Chemicals

Methanol, 2-butanone, formamide, IVER, MON sodium salt, and ZBAC salt were purchased from Sigma-Aldrich (Toronto, ON). Stock solutions of IVER, MON and ZBAC were prepared fresh before addition to soil using 2-butanone, methanol, and sterile water, respectively.

Molecular reagents (PCR, qPCR, and cloning reagents) were purchased from Agilent Technologies (Toronto). Primers were ordered from Sigma-Genosys (Toronto), and diluted to 10 μM with Tris buffer (Agilent Technologies). TAE buffer, 40% acrylamide and urea for DGGE analysis were purchased from BioRad Laboratories (Toronto). Radioactively labeled pharmaceuticals for dissipation studies were purchased from American Radiolabeled Chemicals, Inc. (ARC, St Louis, MO).

2.2. Long term field experiment (addition of 0.1 and 10 mg/kg of pharmaceuticals)

A long term field experiment to evaluate the effects of selected veterinary pharmaceuticals on soil properties was undertaken on the Agriculture and Agri-Food Canada research farm in London, Ontario (43°01'49.5"N, 81°12'23.8"W). The location has a humid continental climate characteristic of much of the mid-Eastern coast of North America and loam soils with low organic matter content (2-3%) and pH of 6.9-7.6. In 2010 the experiment was started, with the plots receiving pharmaceuticals that could reach agricultural land through the application of animal manures or municipal biosolids, including macrolide antibiotics, fluoroquinonole antibiotics, antiviral pharmaceuticals, antimycotic agents, antineoplastic pharmaceuticals, and the pharmaceuticals evaluated in the present study (IVER, MON, ZBAC).

The experimental procedures and field operations are described in Topp et al. (2013). Briefly, a series of plots (2 m^2, isolated by fiberglass frames) were treated every spring since 2010 with two concentrations (0.1 mg/kg and 10 mg/kg) of IVER, MON, or

ZBAC, and a mixture (MIX) of all three pharmaceuticals. Each year since 2010, the plots have been treated annually in the spring (3 annual treatments applied prior to 2013 pharmaceutical application). Each treatment had four replicated plots organized in a random block design, totaling 40 plots. One kilogram portions of soil were taken from each plot, amended in the laboratory with appropriate concentrations of pharmaceuticals according to the experimental protocol, brought out to the field, and incorporated into the top 15 cm using a mechanized rototiller (sterilized with 70% ethanol between treatments). Within 24 h of the pharmaceutical incorporation all plots were seeded with soybean (*Glycine max*, variety: Harosoy). While the actual path the pharmaceuticals reach agricultural fields is through manure application, the manure step was omitted in this study to be able to eliminate potential confounding effects due to other chemical and microbial constituents in manure.

2.2.1.   Soil sampling of field plots

Soils were sampled in May 2013 before the pharmaceutical addition (D0, representing a long term effect of 3 years of annual applications of pharmaceuticals), and in June 2013 at seven (D7) and thirty (D30) days after the addition of pharmaceuticals. Additional sampling to validate the methods was done in fall 2012 (approximately 6 months following the 2012 pharmaceutical addition to the plots; referred to as "fall" throughout this document).

At each of field sampling times, six soil cores (0-15 cm from the surface) were taken from each of the replicate plots with a sterilized (70% ethanol) T-corer and mixed together in a polyethylene bags. The samples were brought to the lab, sieved (2 mm maximum particle size), and adjusted to 15% moisture with sterile water. The water level in the sample was measured using MB45 Ohaus digital moisture analyzer (VWR, Toronto, ON). All soils were processed within 24 hours of collection, except the D0 soils, which were frozen at -20 ˚C prior to analysis. The sieved and moisture-adjusted field soils were used in the microcosm incubation experiment to assess soil function.

2.3. *Short term laboratory experiment (addition of 100 mg/kg of pharmaceuticals)*

Incubations were performed in the laboratory where control soil (i.e. never treated with the studied pharmaceuticals) was treated with 100 mg/kg of each pharmaceutical and their mixture in triplicate mason jars (500 g soil each). Several kilograms of soil was sieved and adjusted to 15% moisture content. Five hundred gram portions of soil were dispensed into a series of 1-L glass mason jars and amended with pharmaceuticals as follows. Stock solutions were made using 1 mg of pharmaceutical in 10 ml of solvent. Approximately one gram of soil was taken from each jar and placed into an aluminum foil boat, and 250 µL of stock solution was added to the soil aliquot. The solvent was allowed to evaporate for 10 minutes, after which the amended portion was mechanically mixed with the rest of the soil for 5 minutes. Mason jars were sealed with a screw-cap lid, and incubated at 30 ^{o}C. Every week the microcosms were opened for 5 minutes to allow gas exchange. Seven days following pharmaceutical addition (high D7) half the soil was removed from each microcosm for functional analysis and DNA extraction for molecular analysis. Following 30 days of incubation (high D30) the remaining soil was taken and the incubation terminated. A scintillation vial containing 10 mL of water was placed in each microcosm. Soil not used in experiments was frozen at -20 ^{o}C.

2.4. *Functional assays*

The Organization for Economic Co-operation and Development guidelines (OECD 2000) lists mineralization and nitrification as sensitive endpoints for soil toxicity testing. Here, mineralization was measured in both the laboratory, using microcosms with radioactively labeled plant material (Section 2.4.1), and in the field, using plant-based bait lamina strips (Sec. 2.4.2). Nitrification was measured through microcosm incubation and analysis of ammonium and nitrate extracted from the soil (Section 2.4.3). In this study, microcosms consisted of a mason jar (8 cm diameter, 20 cm high), small jar containing soil (4 cm diameter, 4 cm high), and vial of water (scintillation vial containing 10ml of water) to keep soil from drying. The lid on mason jar was tightly closed to eliminate gas exchange with the outside.

2.4.1. Rye mineralization

Soils collected from field (fall, D0, D7, and D30) and laboratory (high D7, high D30) were mixed with a known amount of ^{14}C labeled plant material ("rye", *Secale cereal*) and placed in microcosms. The rye was grown in 2008 in a greenhouse, with radiolabeled carbon ($^{14}CO_2$), which was incorporated into the plant tissues. The material was harvested, dried and chopped, and was stored frozen at -80 °C. The amount of radioactivity present in the plant material was determined by oxidation of triplicate 1 g portions of the material (Biological Oxidizer OX-500, R.J. Harvey Instrument Co., Hillsdale, NJ).

Rye was mixed with 25 g soil (moist weight) to obtain 1 200 000 Bq per jar of soil. Each small jar containing rye spiked soil was placed in a Mason jar with a scintillation vial with water and a scintillation vial with an alkali trap (7 mL of 1 M sodium hydroxide). The trap was exchanged on days 1, 3, 7, 14, 21, and 28. Ten mL of scintillation liquid ("cocktail", UniverSol™, MP Biomedicals, Montreal, QB) was added to each extracted trap, shaken lightly, and counted 24 hours after trap extraction in a LS 6500 Multi-purpose Scintillation Counter (Beckman Coulter TM, USA).

The cocktail was needed to allow for counting of the captured radioactivity. Namely, the solvent molecules reacted with β particles emitted from radioactive decay of ^{14}C to release UV light which, upon absorption by cocktail molecules, emitted blue light, which was counted (cpm, counts per minute) with the liquid scintillation counter (LSC). The LSC then corrected the cpm values to dpm (disintegrations per minute) based on counting efficiency (Auto DMP counting method, one of the options programed into LSC). The rate of ^{14}C-CO_2 production was calculated for each microcosm by plotting cumulative ^{14}C-CO_2 production against microcosm incubation time up to 28 days and fitting a curve to the data using Sigma Plot (Version 10, Systat Software Inc., Chicago, IL).

2.4.2.   Bait lamina

To test for a treatment effect on soil faunal activity, an experiment to evaluate the decomposition of organic matter was conducted *in situ* in fall 2012 using bait lamina strip tests (*Terra Protecta* GmbH, Berlin, Germany). The bait lamina strips were 10 cm × 0.5 cm × 0.01 cm, with 16 perforations spaced 0.5 cm apart and filled with a mixture called 'bait', containing plaster, activated coal and bran flakes (http://www.terra-protecta.de/englisch/ks-info-en.htm).

Ten bait strips were inserted vertically in the centre of each control and treated plot (40 plots total) in two parallel lines (lines and individual strips spaced 10 cm apart). The strips were oriented such that hole #1 was close to the surface, and hole #16 was the deepest. To avoid bait loss during insertion, a guide hole was premade in the soil with a metal tool of similar dimensions to the lamina strip, prior to the strip insertion.

The strips remained in the soil for 3 weeks, after which they were carefully pulled out. If the soil was wet and obscured the view of the holes, the strip was gently dipped in water to remove adhering soil while minimizing loss of the remaining bait (physical wiping of the strip with paper towels could have dislodged the bait). The holes were scored as active or inactive based on the amount of remaining bait (with bait pierced through counted as active). Activity was measured as the total number of pierced holes per treatment.

2.4.3.   Determination of nitrification potential

One hundred and thirty grams (wet weight) of field soils (fall, D0, D7, D30) and laboratory soils (high D7, high D30) were placed in microcosms (see section 2.2.1) and incubated at 30 °C for 28 days. Subsamples of 15 g (wet weight) were taken out on days 0, 1, 3, 5, 7, 14, and 28 and extracted for analysis of inorganic nitrogen species (Drury *et al.,* 2008). Briefly, 75 mL of 2 M KCl was added to the 15-g soil samples and shaken in Nalgene bottles for 1 h on a wrist action shaker (Burrell Scientific, Model 75, Pittsburgh, PA) at 385 rpm. Extracts were poured through GF/A grade microfiber filters (Whatman™, VWR, Toronto, ON) under vacuum. The supernatants were collected in

scintillation vials that were stored at -20 ^{o}C until colorimetric analysis for ammonium and nitrate + nitrite. These nutrient analyses were performed using a SmartChem 140 discrete auto-analyzer, (Westco Scientific Instruments, Brookfield, CT). During analysis. nitrate was reduced to nitrite by passing it through an open tubular cadmium reductor (OTCR) coil, which formed a colored dye upon reaction with N-(naphthyl)-ethylenediamine dihydrochloride (SmartChem 140 Method 375-100E-2). The nitrite component present in the soil samples was negligible, because nitrite only exists transiently in the soil. Ammonium was quantified through reaction with phenol and hypochlorite to form indophenol blue (USEPA Method 350.1).

2.5. Molecular methods.

In addition to functional assays, molecular methods were used to examine changes in community composition in respect to the different pharmaceutical treatments. Changes in total 16S rDNA and *amoA* genes were monitored. The genes were quantified using qualitative methods and visualized using denaturing gradient gel electrophoresis, which resolves DNA molecules of equal length on the basis of melting behavior.

2.5.1.   DNA extraction

DNA was extracted from 250 mg of soil (wet weight) using the PowerSoil DNA extraction kit (MO BIO laboratories, Inc., VWR, Toronto) and quantified with a Nanodrop 1000 instrument (ThermoScientific, Toronto, ON). Extracts (corresponding) were diluted 10-fold to a final concentration of 1-2 ng DNA/µl with DNAse-free reagent water (MO Bio Laboratories, Toronto, ON) and used as template in the PCR. For all of the samples, the 10x dilution removed enough inhibitors to allow amplification when used in the PCR reaction. Extracted and diluted DNA was stored at -20˚C until analysis.

2.5.2. *Amplification of DNA*

The polymerase chain reaction (PCR) was used for amplification of extracted DNA with primers for total 16S rDNA (GM5F/907R, (Muyzer et al. 1993)), AOA (Crenamo A23f/Crenamo A616r, (Tourna et al. 2008)) and AOB ((*amoA*1F/*amoA* 2R) ((Rotthauwe et al. 1997)).The 25 μl PCR reaction consisted of 5μl SYBR Green 5x buffer, 1.5 μl $MgCL_2$ (200 μM), 0.2 μl of dNTP (25μM), GoTaq Flexi Polymerase (Thermo Fisher Scientific, Ottawa, ON), 1 μl of each primer (10 mM) and 2μl of 10 fold diluted DNA sample, and remainder filled with molecular grade water. Five microliters of PCR product was electrophoretically resolved on 1.5% agarose gel (70 min at 220V), and stained with GelRed Nucleic Acid Stain (Cedarlane®, Burlington, ON) to confirm the product was of the expected size. The primer information and conditions of the PCR reactions are summarized in Table 2.

2.5.3. *Quantitative PCR*

For quantitative analysis (qPCR) of total 16S rDNA, the BACT2 primer set described previously (Suzuki et al. 2000) was used with 2x Brilliant SYBR® qPCR Master Mix (Agilent Technologies, Toronto, ON). Reactions contained 12.5 μL buffer, 0.75uL of each primer (10 mM), 2uL of 10 fold diluted template DNA, and 0.75 μL TM1389F probe (HEX-CTTGTACACACCGCCCGTC-BHQ1, HEX: 2′,4′,5′,7′-tetrachloro-6-carboxy-4,7-dichlorofluorescein succinimidyl ester; BHQ1: Black Hole Quencher-1; 10 mM), and molecular grade water for a total of 25uL.

The abundance of *amoA* gene for AOA and AOB was determined with the following primers: the AOB *amoA* -specific primers (*amoA*-1F/*amoA*-2R) used were the same as in the PCR reaction (Rotthauwe et al. 1997). The AOA *amoA*-specific primers (Cre374-F: TAATTGGCGGAACATTGGTT, and Cre495-R: CATGTATGGAGGCAATGTCG; Figure 1) were designed and validated in the present study as described in Marti et al. (2014). Reactions contained 12.5 μL 2x Brilliant SYBR® Green qPCR Master Mix (Agilent Technologies, Toronto, ON), 0.75 uL of

Table 2. PCR primer information

Target	Primer pair	Conditions	Product size (bp)	Reference
Archaeal *amoA*	Crenamo A23f/Crenamo A616r	95^o(10min), 95° (45s), 57° (60s), 72° (45s)x39cycles	620	Tourna et al., 2008
Bacterial *amoA*	*amoA*1F/*amoA*-2R	95° (10min), 95° (45s), 55° (60s), 72° (45s)x39cycles	490	Rotthauwe et al 1997
16S rDNA	GM5F/907R	95° (10min), 95° (15s), 59° (20s), 72° (40s)x39cycles	600	Muyzer et al 1993
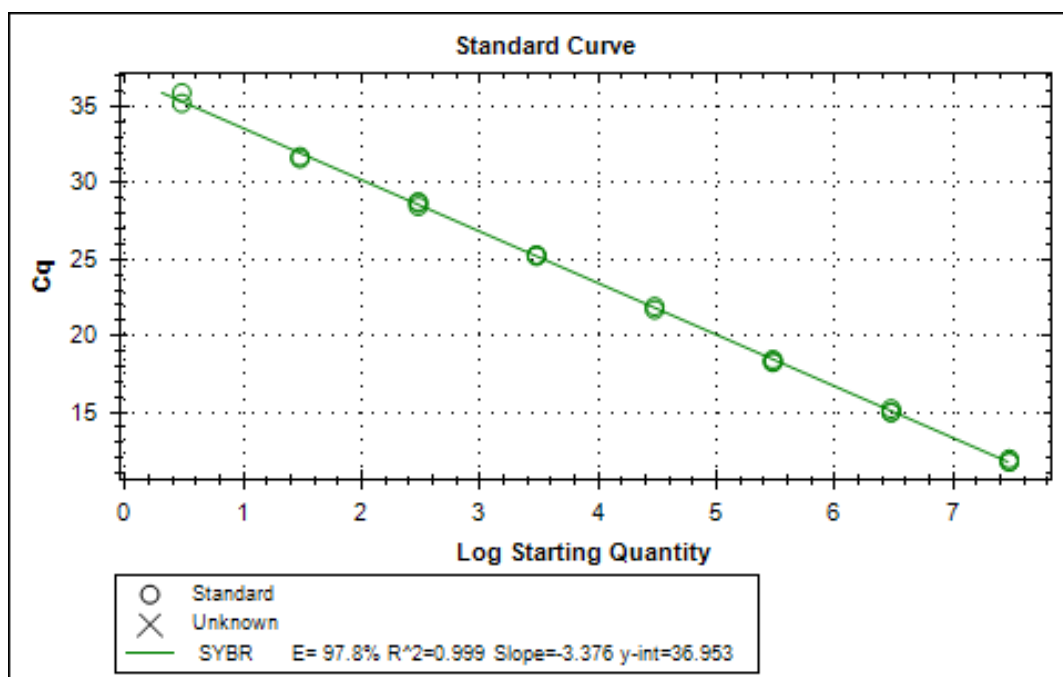
Figure 1. Standard curve for new archaeal *amoA* primers, Cre374-F/Cre495-R. Cq represents the cycle that the amplified DNA reached a threshold quantity (between 0-40 cycles). The starting quantity corresponds to the known quantity of DNA from the standard curve dilutions.

each primer (10 mM), and 2 uL of 10 fold diluted template DNA, and molecular grade water to bring the volume up to 25 uL. Standard curves were created using 10 fold dilutions of plasmid containing 10^6 to 10^0 copies per well of archaeal or bacterial *amoA* insert, or 16S rDNA insert. The plasmid was prepared by cloning PCR products into competent *E.coli* cells using the StrataClone cloning kit (Agilent Technologies, Toronto, ON), and linearized with NotI-HF™ restriction enzyme (New England BioLabs, Whitby, ON). Qualitative PCR conditions and efficiency of the standard curve reactions summarized in Table 3.

Melting curve analysis for *amoA t*argets ranged from 65˚C to 95˚C and was added at the end of the 40 cycles, and revealed a single product peak and low primer dimer concentrations. Amplifications were performed with a CFX96 Real-Time System (BioRad, Toronto, ON) and using the Biorad CFX manager v3.0. Each qPCR sample was run in triplicate. Negative controls without DNA template were performed in triplicate for each run. The *amoA* product of the qPCR reaction for the control and high mixture treatments at D30 after pharmaceutical addition was sequenced in order to determine the relationship between the two communities.

2.5.4. PCR-DGGE method

Denaturing gradient gel electrophoresis (DGGE) analysis used PCR products of *amoA* and 16S rDNA, with one modification: the bacterial forward primer had a 33 base pair GC tail (Muyzer et al. 1993) added to the 5' end. Fifteen microliters of the PCR products were loaded directly (without purification) on to 6% polyacrylamide DGGE gel (100% denaturing mixture consisted of 7M urea and 40% deionized formamide) of 50-65%, 55-65%, and 35-50% for 16 rDNA, bacterial *amoA* and archaeal *amoA*, respectively. Gels were run at 90 V in 60˚C 1xTris acetate EDTA (TAE) buffer for 16 hours , stained with SYBR Gold (nucleic acid stain, Life Technologies, Burlington, ON) and visualized with Molecular Imager Gel Doc™ XR (BioRad, Toronto, ON). Dominant bands were cut from the DGGE gel, cloned following a previously described protocol (Section 2.3.3.) and sequenced.

Table 3. Qualitative PCR primer information

Target	Primer pair	Conditions	Efficiency	Product size (bp)	Reference
Archaeal *amoA*	Cre374-F/Cre495-R	95° (600s), 95° (15s), 57° (20s), 72° (40s)x39cycles, 65-95 melting curve	97.6	122	*this paper*
Bacterial *amoA*	*amoA*1F/*amoA*-2R	95° (600s), 95° (15s), 58° (20s), 72° (40s)x39cycles, 65-95 melting curve	92.9	490	Rotthauwe et al 1997
Bacterial 16S rDNA	BACT1369F/PROK1492R	95° (600s), 95° (15s), 59° (40s), 72° (40s)x39cycles	109	174	Suzuki et al., 2001

2.5.5.   *Cloning and sequencing of DNA products*

The bacterial and archaeal *amoA* products of the qPCR reaction (control and 10 mg/kg mixture treatments at D30 after field pharmaceutical addition) were purified (QIAquick PCR purification kit, Agilent Technologies, Toronto), and measured using a Nanodrop 1000 instrument (ThermoScientific, Toronto, ON). The prepared DNA mix was then used in a cloning procedure with competent *E.coli* cells using a StrataClone cloning kit (Agilent Technologies, Toronto, ON). The colonies containing the desired insert were picked and amplified to obtain a large quantity of DNA (between 45 and 60 ng/µL) to be sent for sequencing (Robarts, and on site sequencing). The resulting sequences were then analyzed and graphed.

2.6. *Pharmaceutical dissipation*

Pharmaceutical dissipation experiments were conducted using control soils (never treated with pharmaceuticals) that were sieved (2mm) and adjusted to 15% moisture. The radioactively labeled pharmaceuticals ivermectin-B1a[24,25-^{3}H] (1.85 TBq/mmol in ethanol) and monensin [9-^{3}H] (185 GBq/mmol) were purchased from American Radiolabeled Company (ARC, 99% purity, Saint Louis, MO). The stock solutions were diluted with ethanol to make working solutions (6 000 000 Bq per 1mL) before addition to soil, and both were stored at -20°C.

Triplicate small jars containing 50 g of control soil each were supplemented with approximately 10,000 dpm/g of ivermectin or monensin. Approximately 1g of soil was taken out of the jar onto a foil boat and prepared pharmaceutical solution was added to it. The soil was left for 10 min to allow the solvent to evaporate and mechanically mixed with the rest of the soil. Small jars containing spiked soil were placed inside mason jars and incubated at 30°C for 50 days. Microcosms contained a vial of water, but no alkali trap, because no radiolabeled CO_2 was emitted (tritium compounds do not contain radiolabel ^{14}C).

Subsamples (5g of soil) were taken from microcosms on days 1, 3, 7, 21, 35 and 50, and extracted with methanol three times. Briefly, 15ml of methanol (HPLC grade,

Caledon, USA) was added to each 5g sample, shaken for 30 min and centrifuged for 10 min at 13 000 rpm (Labofuge 6000, Heraeuz Christ) and poured through a filtered funnel (0.2 mm). This was repeated two more times. Following the third extraction, the filtrate was evaporated under a stream of nitrogen gas, re-suspended in methanol and a subsample of this preparation was added to 10ml of cocktail and counted using LS 6500 Multi-purpose Scintillation Counter (Beckman Instruments, Irvine, CA, USA). The generated data were fitted to first order kinetics curve and used to estimate the half-lives (time needed for 50% of added substance to dissipate) of the pharmaceuticals.

Radiolabeled zinc bacitracin was not available; therefore 100 mg/kg of unlabeled ZBAC (bacitracin zinc salt, 99% pure, Sigma-Aldrich, Toronto) was added to soil, incubated, extracted and analyzed as described above. ZBAC amended soils were extracted with multiple solvents (Table 6), and analyzed using LC-MS/MS. However the amount of zinc bacitracin in the samples was below detection. A follow-up experiment revealed poor solubility of the pharmaceutical in all of the solvents used except the phosphate buffer. Zinc bacitracin was suspended in this buffer at 2 and 10 ppm and left in for 3 h, and the pharmaceutical was analyzed to confirm that ZBAC could be detected through the LC-MS/MS method. LC-MS analysis was performed on a Thermo Q-Exactive coupled to an Agilent 1290 HPLC system. Samples were run in full scan mode to monitor for the parent ion in addition to the use of the MS/MS data for quantification. Samples were separated using an Agilent Zorbax eclipse plup C18 RPHD column (2.1 x 50mm, 1.8 micron) using a water:acetonitrile gradient of 100 to 0 over 5 min at a flow rate of 0.3ml/min. The MS parameters were as follows: mass range 100-1000; 35000 resolution; sheath gas 25; aux gas 15; spray voltage 4.3; capillary temperature 260 $^{\circ}$C; aux gas 425 $^{\circ}$C.

2.7. Data analysis

All analysis and graphs were done using SigmaPlot (Version 10, Systat Software Inc., Chicago, IL). Nitrification rates were estimated on the basis of N accumulated as nitrate over the 28-day incubation and calculated as the rate of accumulation per day of incubation. Significant differences were established at a significance level of $P < 0.05$

using one way ANOVAs with Holm-Sidak post hoc tests. If data was violating the normality or equal variance assumptions of the ANOVA test, a log transformation was conducted. For data that were not parametric after log transformation, Kruskal-Wallis ANOVAs on ranks were performed followed by Dunn's post hoc tests.

The *amoA* or 16S gene abundance was calculated as the copy number of archaeal *amoA*, bacterial *amoA*, or 16S rDNA per g soil (wet weight). Statistically significant differences were designated as $P < 0.05$ using SigmaPlot software (as above) using one way ANOVAs with Holm-Sidak post hoc tests. Non-parametric data was log transformed, but if the transformation did not improve the variance or normality, Kruskal-Wallis ANOVAs on ranks with Dunn's post hoc tests were performed.

The intensity of the DGGE bands was not considered as a potential response to the treatments, because of an artifact of PCR procedure, that can unequally overestimate the high abundance sequences and underestimate or exclude the low abundance sequences. Therefore only presence/absence of bands was considered to assess changes in AO community structure.

The resulting sequences were analyzed using BioEdit Sequence Alignment Editor (Hall, 1999) and run through the BLAST online search engine to identify the fragments. The sequence data were then aligned with ClustalW method using MEGA6 (version 6.0.5, Tamura 2013). Relationships between sequences were visualized as a phylogenetic tree.

3. Results

3.1. Long term field experiment

3.1.1.   Functional responses

The mineraliztion experiment with bait lamina, conducted six months after the 2013 pharmaceutical addition, showed no significant differences in activity of macroorganisms compared to control (Figure 2). Similatily, no differences   in mineralization using radioactiverye were observed at any of the four (fall, D0, D7, and D30) sampling times (Table 5).

There was no significant short term effect on nitrification potential (compared to control soil) after the fourth annual application (D7 and D30) or six months after 3 annual applications (fall) of each pharmaceutical used singly or in mixture at either of the two concentrations (0.1mg/kg and 10mg/kg); however, at D0 (prior to 2013 pharmaceutical application, and year after last pharmaceutical addition) the mixture at 10 mg/kg concentration increased the nitrification rate (F = 3.4, df = 8, 31, P<0.05; Table 4).

The ammonium concentration in field soil was low (Table 6) and it rapidly depleted during the microcosm incubations, reaching the baseline level (0.1 µg N/g soil) within 1-3 days (Appendix 7). The amount of ammonia was significantly higher in 10 mg/kg ZBAC and MIX treatments seven days after drug addition (H = 22.7, df = 8, 10, P<0.05). However the correlation between the nitrification rate and initial ammonia concentrations was not significant (Figure 3).

3.1.2.   Abundance of total bacteria (qPCR) and community structure (DGGE)

There were no differences between the quantities of total bacteria under the different pharmaceutical treatments compared to the control soils at any sampling points (Figure 4). The structure of the community as per thr DGGE banding pattern also revealed no changes in structure (Appendix 1).

Table 4. Mean potential nitrification rate (± SD) in soils treated with different concentrations of ivermectin (IVER), monensin (MON), zinc bacitracin (ZBAC), and mixture of the 3 (MIX) and incubated for 28 days. For laboratory experiment (100 mg/kg) N=3 for control and treatment, for field experiment (0.1 and 10 mg/kg) $N_{control} = 8$, $N_{treatment} = 4$.

Treatment		Potential nitrification rate (ng of nitrate + nitrite N accumulated/gram soil/day)										
		Days post pharmaceutical application										
concentration	pharmaceutical	7			30			180			360		
0 mg/kg	CONT	186	±	20	870	±	125	N/A			N/A		
100 mg/kg	IVER	192	±	44	910	±	18	N/A			N/A		
	MON	281	±	18	724	±	32	N/A			N/A		
	ZBAC	462	±	157*	1038	±	67	N/A			N/A		
	MIX	720	±	33*	948	±	67	N/A			N/A		
0 mg/kg	CONT	771	±	191	412	±	72	680	±	296	622	±	157
10 mg/kg	IVER	731	±	31	485	±	33	610	±	72	785	±	232
	MON	759	±	129	403	±	30	571	±	115	761	±	150
	ZBAC	855	±	167	476	±	30	541	±	228	751	±	69
	MIX	866	±	75	447	±	59	485	±	331	1037	±	231*
0.1 mg/kg	IVER	749	±	45	410	±	45	610	±	24	576	±	131
	MON	548	±	209	369	±	55	544	±	113	662	±	178
	ZBAC	561	±	135	431	±	126	549	±	79	809	±	159
	MIX	756	±	223	405	±	30	660	±	156	803	±	268

Note: Asterisks represent significant differences ($P<0.05$) of the treatment compared to the control soil. N/A means the samples were not analysed at that time point. 180 and 360 days corresponds to "fall" and "D0"
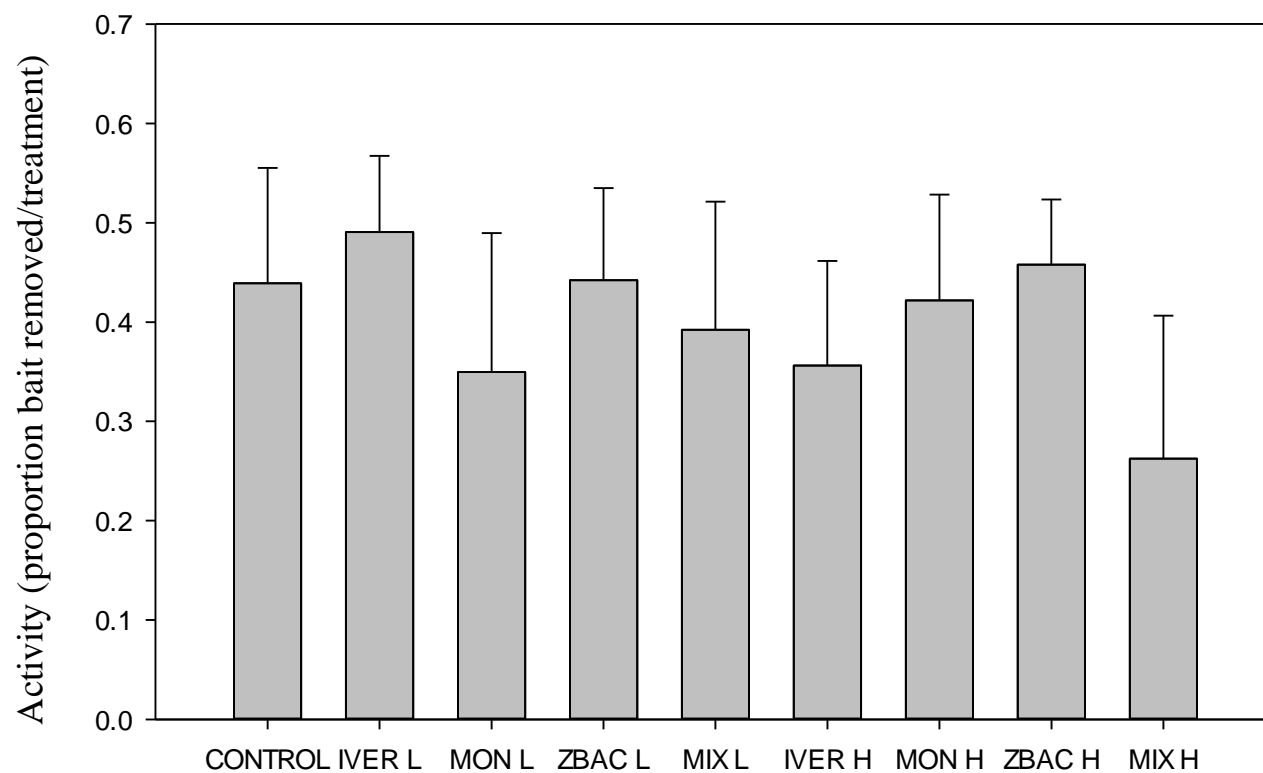
Figure 2. Bait lamina strip activity (mean ± SD) in field plots six months after pharmaceutical addition ("fall") at two different concentrations ("L" = 0.1 mg/kg; "H" = 10 mg/kg) of ivermectin (IVER), monensin (MON), zinc bacitracin (ZBAC), or mixture of the 3(MIX).$N_{control}$ = 8, $N_{treatment}$ = 4. No significant differences were detected

Table 5. Mineralization (mean ± SD) in soils treated with different concentrations of ivermectin (IVER), monensin (MON), zinc bacitracin (ZBAC), and mixture of the 3 (MIX) at the end of 28-day incubation with radioactive rye. For laboratory experiment (100 mg/kg) N=3 for control and treatment, for field experiment (0.1 and 10 mg/kg) $N_{control} = 8$, $N_{treatment} = 4$.

Treatment		Mineralization (% radioactivity recovered after 28 day incubation)			
		Days post pharmaceutical application			
concentration	pharmaceutical	7	30	180 (fall)	360 (pre-application)
0 mg/kg	CONT	36 ± 7	36 ± 3	N/A	N/A
100 mg/kg	IVER	31 ± 4	32 ± 3	N/A	N/A
	MON	33 ± 2	28 ± 2	N/A	N/A
	ZBAC	32 ± 2	51 ± 18	N/A	N/A
	MIX	26 ± 3	29 ± 4	N/A	N/A
0 mg/kg	CONT	42 ± 10	38 ± 10	30 ± 8	30 ± 8
10 mg/kg	IVER	36 ± 3	38 ± 4	35 ± 16	27 ± 9
	MON	45 ± 3	38 ± 5	47 ± 16	28 ± 4
	ZBAC	33 ± 7	42 ± 6	36 ± 7	32 ± 6
	MIX	32 ± 5	38 ± 3	43 ± 18	29 ± 7
0.1 mg/kg	IVER	41 ± 1	34 ± 6	25 ± 10	30 ± 4
	MON	42 ± 3	36 ± 6	27 ± 5	30 ± 4
	ZBAC	34 ± 6	34 ± 4	59 ± 12	39 ± 9
	MIX	40 ± 10	37 ± 8	35 ± 4	31 ± 3

Note: Asterisks represent significant differences (P<0.05) of the treatment compared to the control soil. N/A means the samples were not analysed at that time point. 180 and 360 days corresponds to "fall" and "D0"

Table 6. Ammonia (mean ± SD) detected in soil samples under different concentrations of ivermectin (IVER), monensin (MON), zinc bacitracin (ZBAC), and mixture of the 3 (MIX) measured at the time of soil collection. For laboratory experiment (100 mg/kg) N=3 for control and treatment, for field experiment (0.1 and 10 mg/kg) $N_{control}$ = 8, $N_{treatment}$ = 4.

Treatment		Ammonia present in soils (ng of ammonia N /gram soil)			
		Days post pharmaceutical application			
concentration	pharmaceutical	7	30	180	360 (pre-application)
0 mg/kg	CONT	143 ± 88	2791 ± 71	N/A	N/A
100 mg/kg	IVER	80 ± 62	2894 ± 68	N/A	N/A
	MON	3401 ± 432*	2993 ± 100	N/A	N/A
	ZBAC	33 ± 4	2966 ± 101	N/A	N/A
	MIX	6173 ± 641*	3014 ± 117	N/A	N/A
0 mg/kg	CONT	48 ± 42	429 ± 149	3650 ± 1185	1678 ± 149
10 mg/kg	IVER	160 ± 97	566 ± 58	3519 ± 443	770 ± 58
	MON	95 ± 76	537 ± 161	2730 ± 99	2470 ± 161
	ZBAC	193 ± 31*	455 ± 294	3677 ± 501	4352 ± 294
	MIX	248 ± 22*	542 ± 160	2612 ± 1562	1889 ± 160
0.1 mg/kg	IVER	86 ± 22	199 ± 105	3361 ± 297	500 ± 221
	MON	71 ± 38	542 ± 188	3257 ± 393	1187 ± 1181
	ZBAC	52 ± 42	52 ± 96	3343 ± 859	474 ± 161
	MIX	115 ± 42	115 ± 96	3662 ± 560	1377 ± 239

Note: Asterisks represent significant differences (P<0.05) of the treatment compared to the control soil. N/A means the samples were not analysed at that time point. 180 and 360 days corresponds to "fall" and "D0"
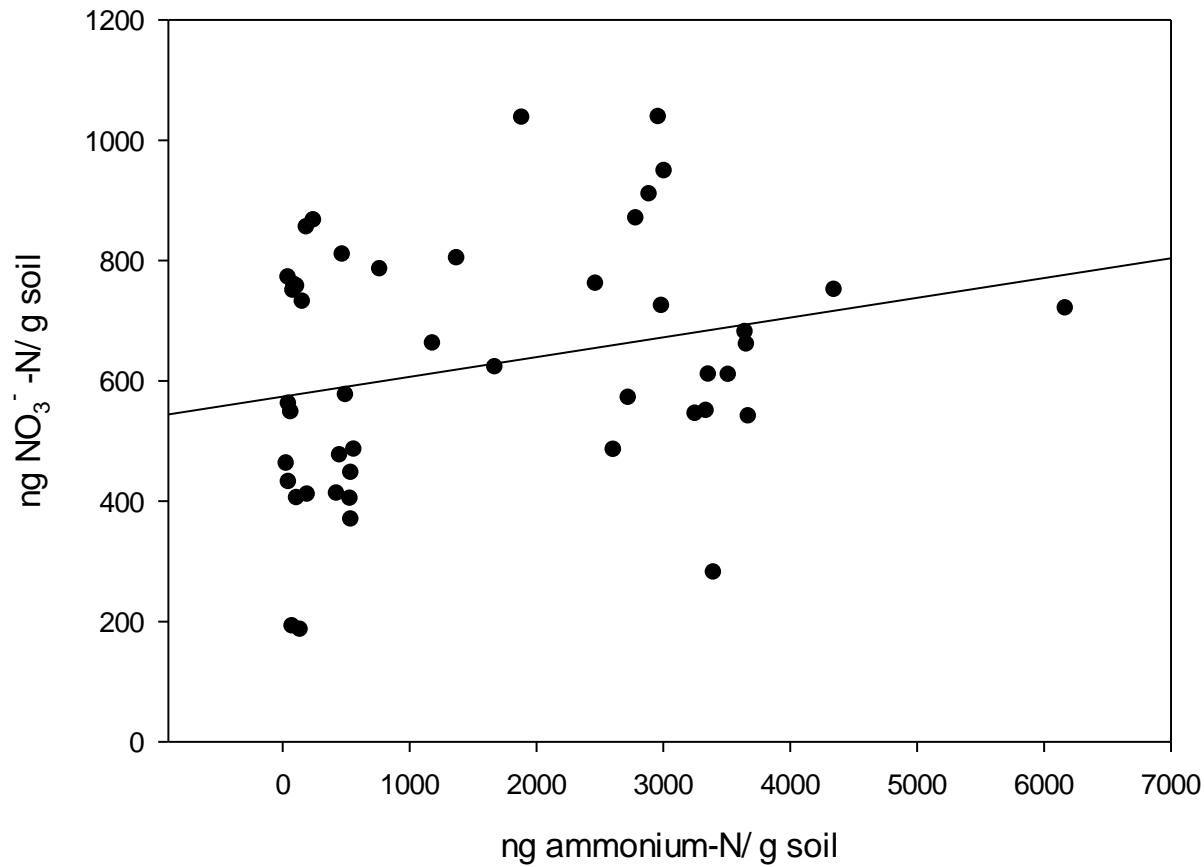
Figure 3. Relationship between nitrification potential rate and soil ammonium concentration at the start of the assay, pooling all of the data from the field and laboratory experiments. Indicated is the best fit linear regression, which had a coefficient of determination (r^2) of 0.106.
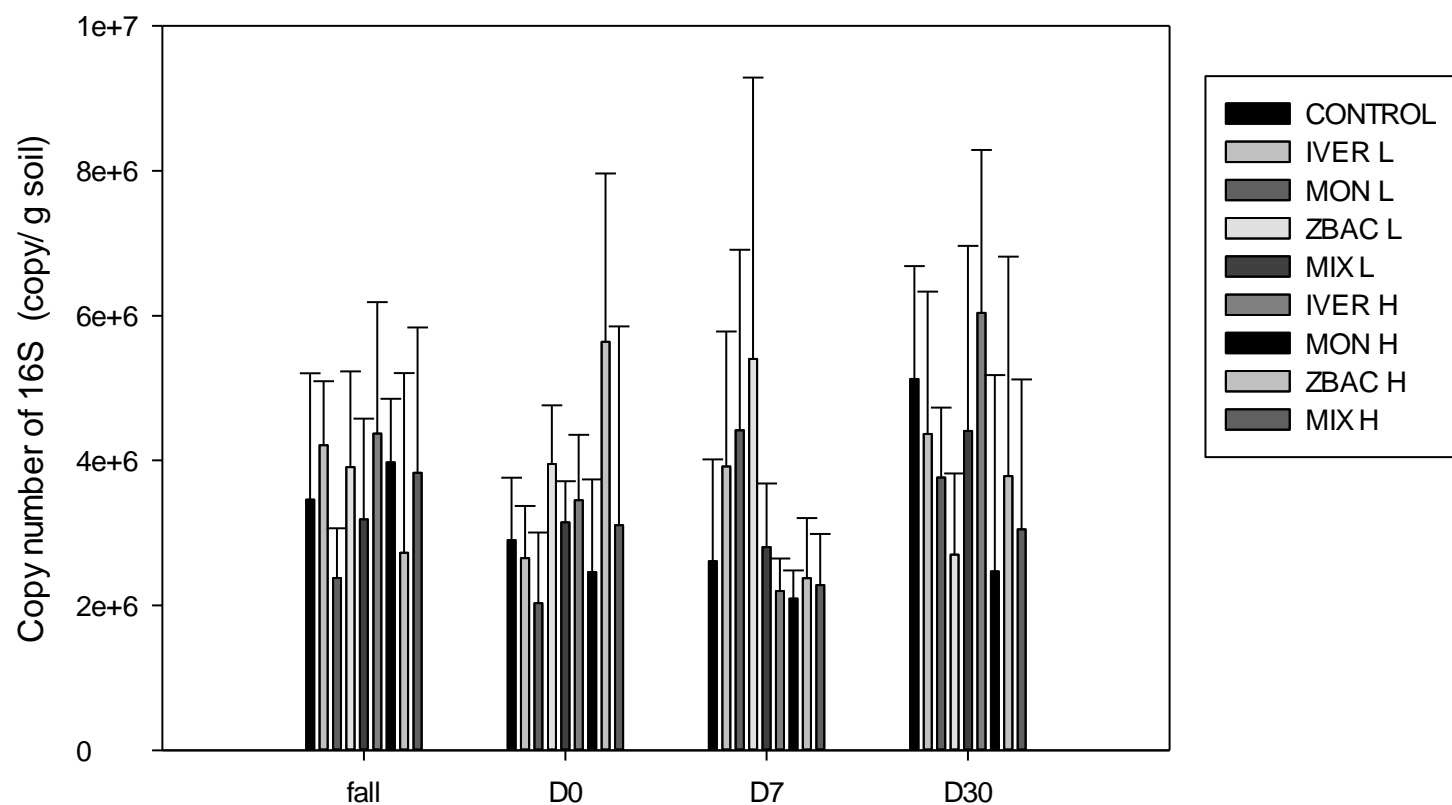
Figure 4. Abundance of 16S rDNA (mean ± SD) in field soil samples at two different concentrations ("L" = 0.1 mg/kg; "H" = 10 mg/kg) of ivermectin (IVER), monensin (MON), zinc bacitracin (ZBAC), or mixture of the 3 (MIX). $N_{control}$ = 8, $N_{treatment}$ = 4. No differences were detected.

3.1.3. *Abundance and community structure of ammonia oxidizing organisms*

No long term effects were seen in the PCR-DGGE profiles of AOA (Appendix 3) or AOB (Appendix 4). The DGGE banding pattern for archaea did not change after pharmaceutical addition, but bacterial DGGE banding had some differences between control and treatments at D30 after pharmaceutical addition. There were also changes in *amoA* copy number in AOA and AOB within 30 days of the treatment with pharmaceuticals (Figure 5 and 6). Notably, the quantity of bacterial *amoA* decreased significantly 7 days after receiving any of the pharmaceuticals, or mixture, at the 10 mg/kg dose (F = 13.3, df = 8, 31, P<0.001; Figure 6), and remained significantly lower 30 days after application of ZBAC and MON at 10 mg/kg treatment (H = 25.0, df = 8, P<0.001). The archaeal *amoA* vas not significantly different between control and treatments, but increased significantly at D30 for all soils receiving 10 mg/kg (F = 11.1, df = 8, 29, P<0.001; Figure 5). Expressed as a ratio, AOA /AOB changed from 0.25 at D0 to 0.5 at D7 and 2 at D30 with 10mg/kg pharmaceutical addition, reflecting a decrease in AOB *amoA* copy numbers and a subsequent increase of AOA at D30. We saw a 5 fold difference between AOA and AOB, with the bacteria remaining as the dominant ammonia oxidizer.

Cloned qPCR products (3 clones from each replicate treatment) at the control and high mixture treatment 30 days post pharmaceutical application in the field were sequenced and revealed little diversity in archaeal or bacterial sequences (Figure 7 and 8). The sequences did not form distinct control-only or mixture-only clades, but were intermixed with each other.

3.2. *Short term laboratory experiment*

3.2.1. *Functional responses*

Mineralization did not change seven or 30 days after the treatment with 100 mg/kg concentrations of the studied pharmaceuticals (Table 4). The no-change in this
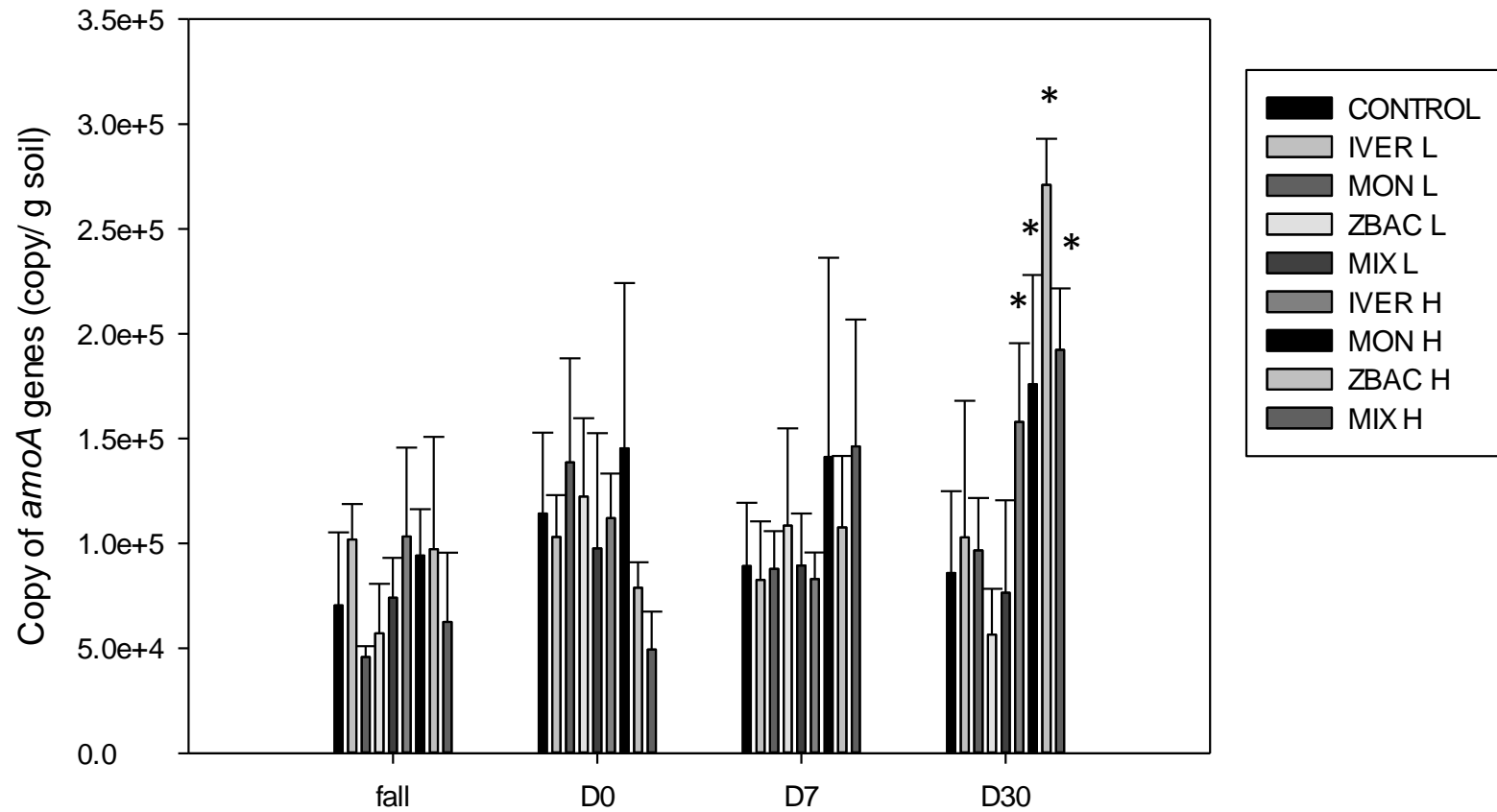
Figure 5. Archaeal *amoA* copy number (mean ± SD) in field soils at two different concentrations ("L" = 0.1 mg/kg; "H" = 10 mg/kg) of ivermectin (IVER), monensin (MON), zinc bacitracin (ZBAC), and mixture of the 3 (MIX). $N_{control} = 8$, $N_{treatment} = 4$. Asterisks represent treatments that were significantly different ($P<0.05$) from control.
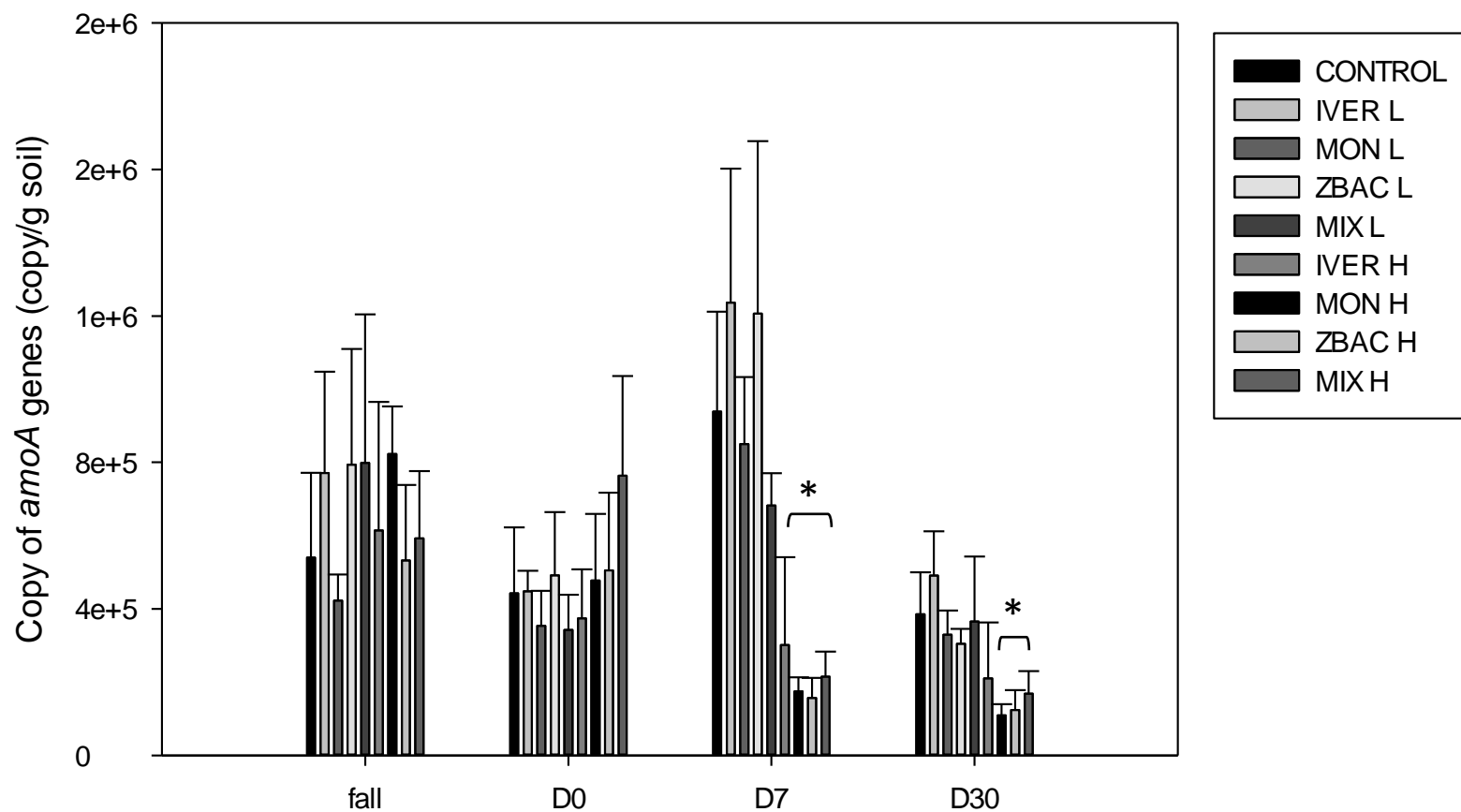
Figure 6. Bacterial *amoA* copy number (mean ± SD) in field soils at two different concentrations ("L" = 0.1 mg/kg; "H" = 10 mg/kg) of ivermectin (IVER), monensin (MON), zinc bacitracin (ZBAC), or mixture of the 3 (MIX). $N_{control}$ = 8, $N_{treatment}$ = 4. Asterisks represent treatments that were significantly different ($P < 0.05$) from control.
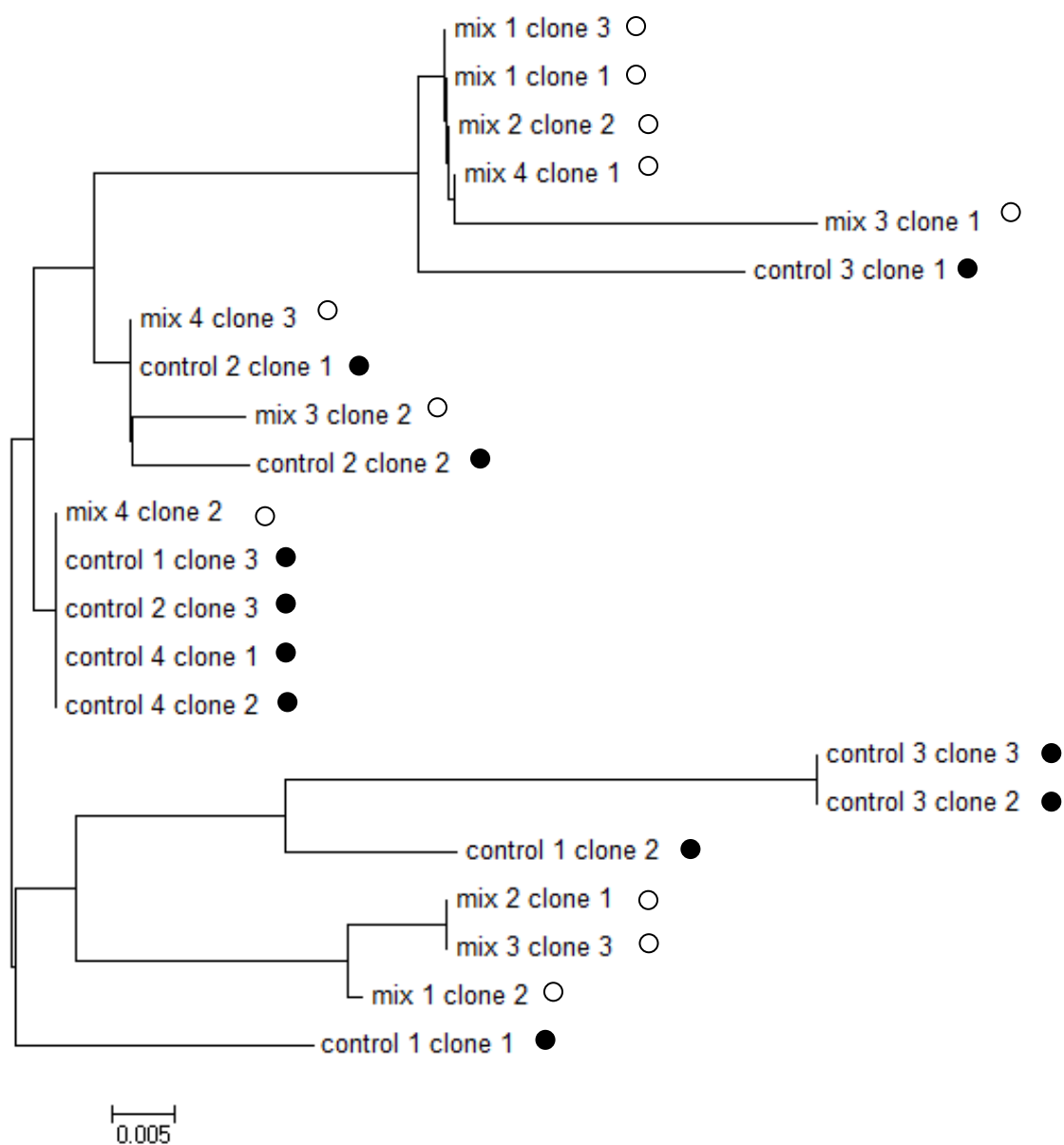
Figure 7. Relationship of archaeal *amoA* sequences extracted 30 days after pharmaceutical addition in 2013 from control plot (control 1-4, marked with black circles) and 10 mg/kg mixture (mix 1-4, marked with white circles). The scale bar represents 0.005 nucleotide substitution per 100 nucleotides.

Figure 8. Relationship of bacterial *amoA* sequences extracted 30 days after pharmaceutical addition in 2013 from control plot (control 1-4, marked with black circles) and 10 mg/kg mixture (mix 1-4, marked with white circles). The scale bar represents 0.05 nucleotide substitution per 100 nucleotides.

endpoint contrasted with the nitrification rate at 100mg/kg concentration. The nitrification was significantly higher in ZBAC and MIX (F = 186.9, df = 4, 10, P<0.001) seven days after the pharmaceutical addition, when compared to control. This difference was not present at D30. Starting concentrations of ammonia present in soil 7 days after pharmaceutical addition was significantly sigher in MON and MIX treatments (F = 176.1, d = 4, 10, P<0.001), but was depleted fast during the microcosm incubations (Appendix 6) and no differences were detected at day 30 after pharmaceutical addition (Table 6).

3.2.2. Abundance and community structure of total bacteria and amoA

There was no significant difference between quantity of total bacteria under the different treatments (Figure 9), nor was the DGGE banding pattern of 16S rDNA different between the treatments and control (Appendix 2). Similarily, DGGE analysis of bacterial or archaeal *amoA* genes showed no difference in community structure between the treatments and the control (Appendix 5). There was no detected change in the *amoA* gene copy number 30 days post-application of pharmaceuticals (Figure 10 and 11), but a significant increase in *amoA* under IVER treatmnet was detected at D7 in archaeal population (F = 4.7, df = 4, 10, P<0.001).

3.3. Pharmaceutical dissipation

The radioactive analysis of IVER and MON showed that they were degraded within one month of application to the soil (IVER $t_{1/2}$ = 15 days, MON $t_{1/2}$ = 20 days). ZBAC could not be extracted from the soils, and dissolved poorly in most of the solvents used (methanol, ethanol. ethyl acetate, acetonitrilie). It was dissolved in potassium buffer (what is in it), and detected at 1 and 10 mg/kg concentrations in solution, but extraction efficiency from soil was very low (below 10% at D0). It is most likely tightly bound to soil particles, and therefore unavailable.
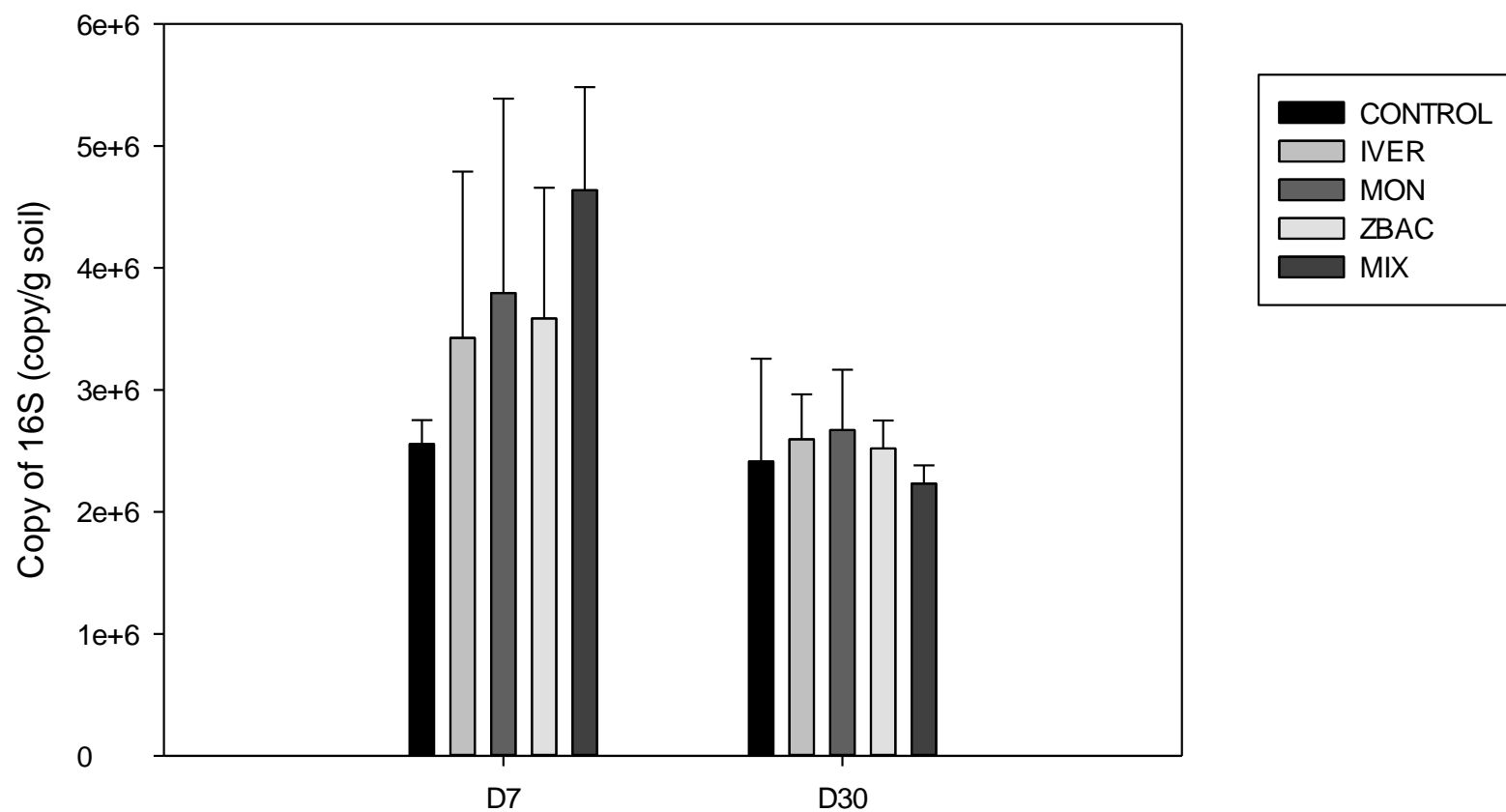
Figure 9. Abundance of 16S rDNA (mean ± SD) in laboratory soil samples at two different concentrations ("L" = 0.1 mg/kg; "H" = 10 mg/kg) of ivermectin (IVER), monensin (MON), zinc bacitracin (ZBAC), or mixture of the 3 (MIX). No differences were detected. $N_{control}$ = 3, $N_{treatment}$ = 3. No differences were detected.
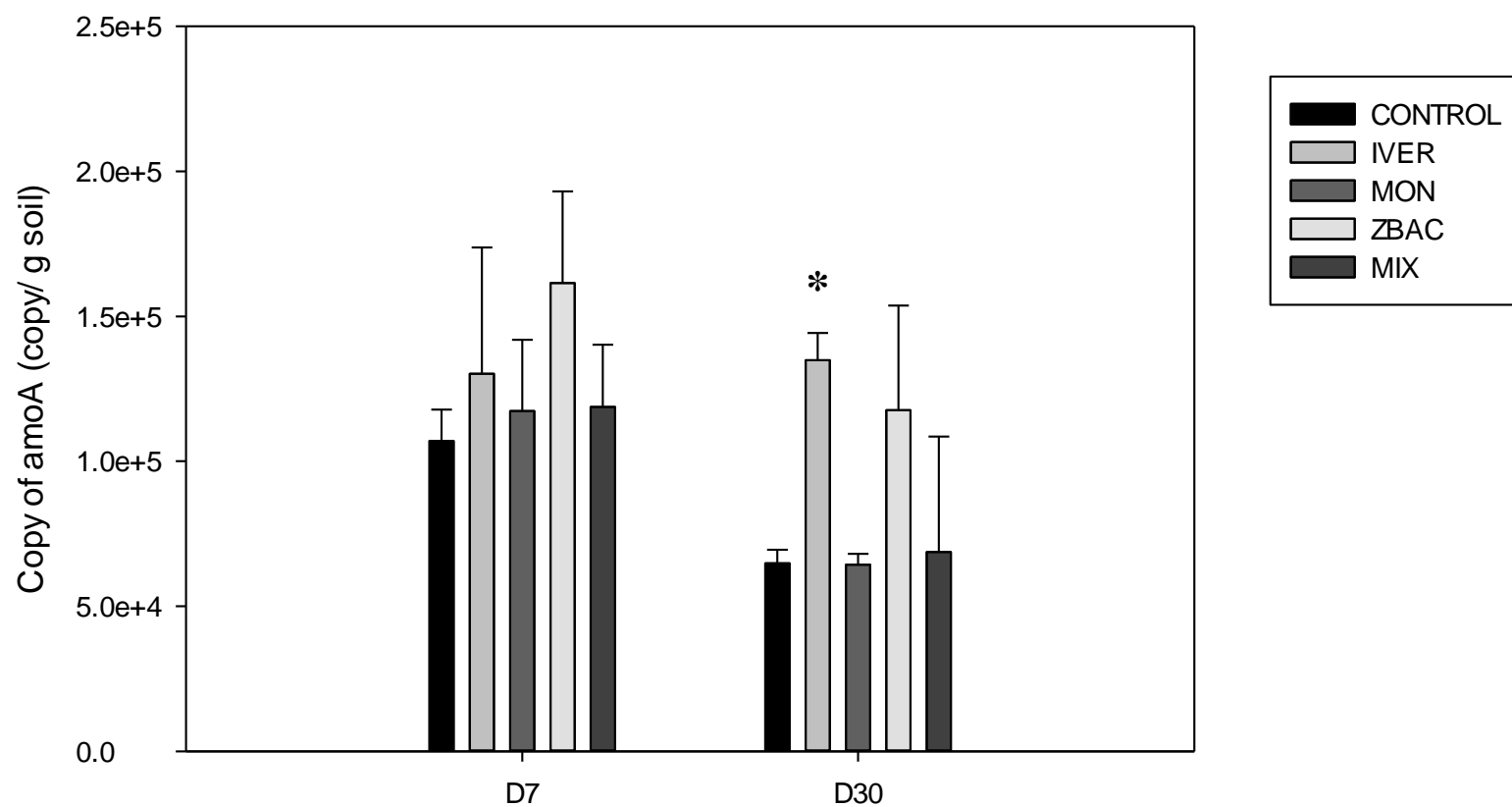
Figure 10. Abundance of archaeal *amoA* (mean ± SD) in soils treated with 100 mg/kg concentration of ivermectin (IVER), monensin (MON), zinc bacitracin (ZBAC), or mixture of the 3 (MIX). $N_{control} = 3$, $N_{treatment} = 3$. Asterisks represent treatments that were significantly different ($P<0.05$) from control.
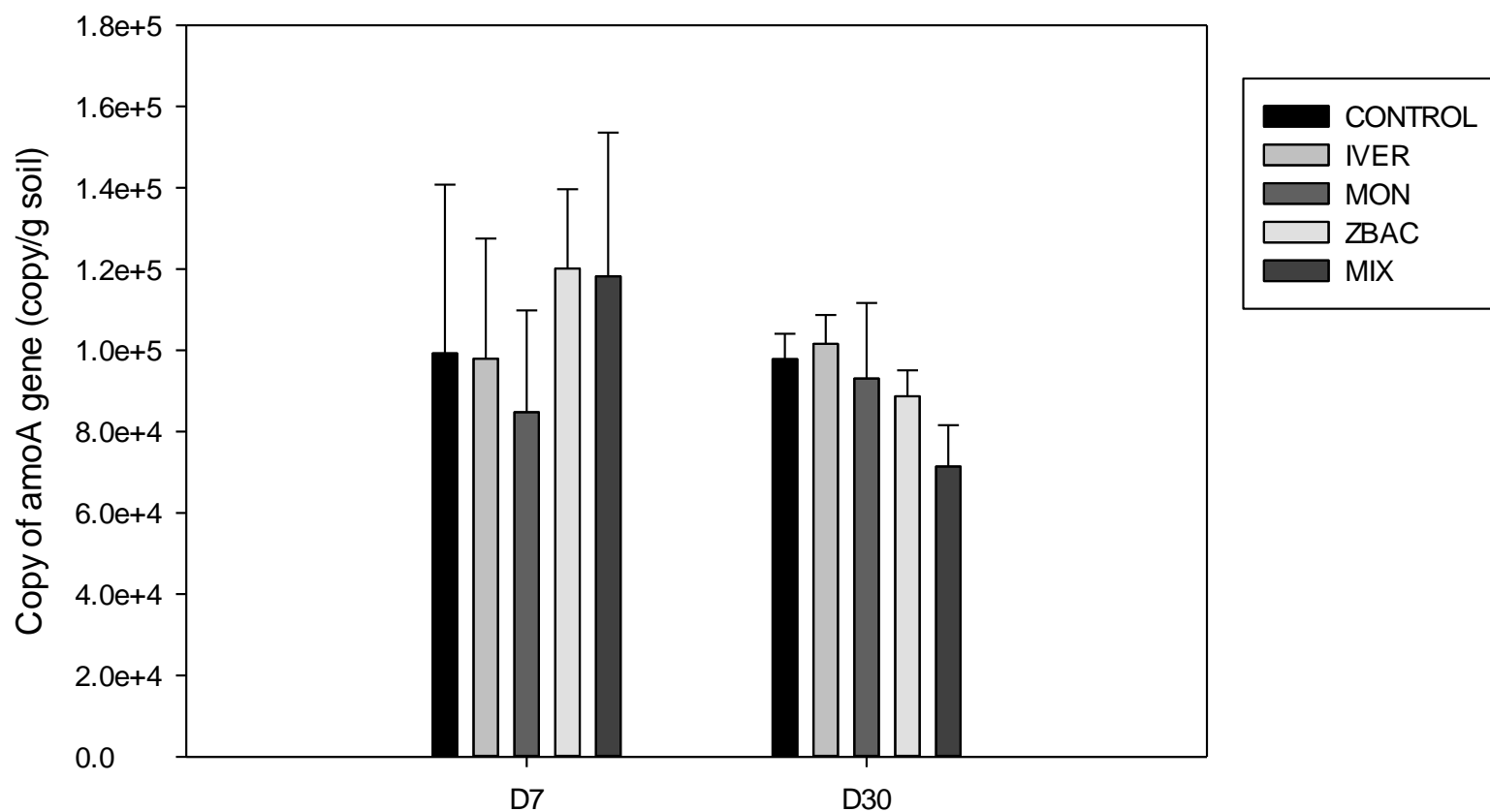
Figure 11. Abundance of bacterial *amoA* (mean ± SD)in soils treated with 100 mg/kg concentration of ivermectin (IVER), monensin (MON), zinc bacitracin (ZBAC), or mixture of the 3 (MIX). Ncontrol = 3, Ntreatment = 3. No differences were detected.

4. Discussion

The pharmaceutical concentrations of 0.1, 10, and 100 mg/kg soil were chosen to encompass a regulatory threshold level that was relevant with respect to expected environmental concentrations, through to excessive exposures that are expected to have an effect. The 0.1 mg/kg concentration is a cut-off for Tier 1 assessment and is often considered below the no observed effect concentration (NOEC), and therefore chosen as a guideline and regulatory measure of pharmaceutical concentration. The concentration of 10 mg/kg was considered as an effect concentration that will have an impact on studied processes, while 100 mg/kg is very high and chosen as an inhibitory concentration for this study.

4.1. High pharmaceutical concentration, short term exposure

Under laboratory conditions, an unrealistically high concentration of pharmaceuticals was added to the soil to achieve an effect concentration that would affect the measured endpoints. The lab experiment increased the concentration of pharmaceuticals 10 times from the field exposure and therefore the pharmaceutical amount could reach stress or toxic levels able to disrupt soil processes, as shown. Short term (up to 30 days) exposure to 100 mg/kg pharmaceuticals affected the functional endpoints, but had little impact on 16S rDNA or *amoA* gene abundance.

The mixture of pharmaceuticals negatively affected mineralization of radioactive rye at 7 days post application compared to the untreated control. This effect was not present 30 days after pharmaceutical addition. In contrast to a negative effect on mineralization, ZBAC and MIX treatments had a positive effect on nitrogen potential rates 7 days after pharmaceutical addition compared to control. Both MIX and ZBAC followed a similar pattern, indicating that zinc bacitracin present in the mixture might be the driving force behind the mixture effect in this incubation. As with mineralization, the effect was not seen 30 days after pharmaceutical addition, therefore the effects these pharmaceuticals exert on soil organisms are short-lived.

The fast return to control rates in the mineralization experiment (within 30 days of pharmaceutical addition) can be explained by fast pharmaceutical dissipation. For example, pharmaceuticals can be inactivated through biodegradation (microbial breakdown), photolysis (degradation using light), or sorption to soil (attaching to soil particles), all of which decrease pharmaceutical concentrations and limit bioavailability. Dissipation rates (expressed as half-lives in this study) of MON were higher than previously published values (Carlson and Mabury 2006, Sassman and Lee 2007), however some discrepancies between half-lives have been reported. Monensin dissipation has varied between 3 and 13 days depending on whether the experiment was carried out in the field, or in the laboratory (Yoshida et al. 2010), with field conditions increasing the pharmaceutical dissipation rates. In the case of ivermectin, the half-life depended on whether the experiment was carried out in the presence of soil microorganisms and light (Mougin et al. 2003). IVER is photodegradable (Mougin et al. 2003), but in the dark dissipation relies on microbial dissipation only, and is much slower (21 days compared to 230 days). The half-life of 15 days found here for IVER was shorter than those previously reported (Mougin et al. 2003, Levot 2011). Other factors such as presence of manure (Al-Rajab et al. 2009), depth in the soil column (Santoro et al., 2008), or soil type (Sabourin et al., 2010) were considered as potential sources of variation on pharmaceutical dissipation in the environment. Soil sorption can also be a reason a particular pharmaceutical is removed from the soil quickly after its addition, decreasing the estimated half-life. However sorption to soil does not always reduce bioavailability (Ingerslev and Halling-Sorensen 2000, Thiele-Bruhn 2003). It was shown that adding 20 mg/kg of ciprofloxacin to soil did not inhibit the mineralization function, because up to 88% of the pharmaceutical was strongly bound to soil (Girardi et al. 2011). Here, ZBAC was strongly attached to soil and assumed to be unavailable to microorganisms, while a high recorded extraction rate from faeces (92-93%) was previously reported (Donoso et al. 1970, Wicker et al. 1977, Frøyshov et al. 1986). ZBAC was not soluble in tested solvents, and only soluble in phosphate buffer at low concentration, therefore its half-life could not have been determined. Because the studied pharmaceuticals dissipate rapidly in soils (Sarmah et al. 2006, Liebig et al. 2010a), they are active against microorganisms for only a short time. Their negative effects were seen at the 100 mg/kg concentration,

showing that they are capable of inhibiting microbial processes if added to soil at high enough levels.

Another reason for changes in nitrification occurring at day 7 but not day 30 after pharmaceutical addition was nitrogen depletion in the soil. Ammonium levels were very low at the start of the experiment, and other sources of nitrogen had to be available for the increased nitrification rate. Antimicrobial bacitracin added to the soil could have dissociated from the zinc bacitracin complex and negatively affected sensitive groups of microorganisms and released ammonium, which increased the nitrification. Another possibility is that heavy metal zinc killed sensitive organisms in soil. Heavy metals can select for tolerant species at the expense of sensitive species, resulting in pollution induced community tolerance (Schmitt et al. 2006, Fechner et al. 2011). The soil used in the experiments had 4.6-5.5 mg Zn/kg soil (A&L Canada Laboratories Inc., Soil test report, 2012) in the absence of ZBAC addition. The 100 mg/kg treatment doubled the concentration of zinc in soils (adding approximately 4.4 mg of zinc per kg soil). Prior exposure to zinc heavy metal (between 30 and 780 mg/kg) has improved rather than diminished nitrification in soil (Rusk et al. 2004), while Mertens et al. (2009) showed that nitrification is restored to control levels in soil within 2 years of zinc contamination. The organisms living in the experimental soil might therefore already have been tolerant to zinc prior to the treatment, and thus experienced no negative effect after pharmaceutical addition. Initial amount of ammonium in the soil did not correlate well with the increase in nitrification rates, and no change in abundance was detected. Therefore, it appears that zinc and bacitracin were unlikely to increase soil ammonium-N by killing sensitive organisms.

Another possible reason for the stimulation of nitrification by ZBAC was that ZBAC carried sufficient N to increase the nitrification rate. As the only pharmaceutical that contained N (16% of compound by MW), it might have stimulated nitrification. However, this was unlikely since initial ammonium concentrations were not correlated with nitrification rates, indicating that ammonium-N was not rate limiting in the assay. Certain microorganisms are able to grow on pharmaceuticals, as was found with under long-term application of sulfamethazine (Topp et al. 2013).

The decrease in mineralization and increase in nitrification was not accompanied by changes in community structure for the 100 mg/kg treatments based on DGGE banding profiles using 16S rDNA gene primers, or 16S rDNA gene abundance measured by quantitative PCR. Similarly, no differences in bacterial *amoA* gene copies were seen at the concentration of 100 mg/kg at 7 or 30 days after pharmaceutical addition, despite observed changes in mineralization and nitrification rates. In a study by Ollivier et al. (2013), a single application of sulfamethazine contaminated manure on a field did not result in great changes in AOA or AOB abundance, but the second application caused a 15-fold increase in AOA abundance compared to AOB (Ollivier et al. 2013). Therefore a multiyear contamination with 100mg/kg of the studied pharmaceuticals might result in changed *amoA* abundance. However, this high concentration of pharmaceuticals is highly unrealistic in the environment.

Pharmaceutical concentrations found in the field vary with pharmaceutical type, persistence and the quantities used in animal productions. For example, topsoil fertilized with poultry litter from commercial farms contained 5-183 µg monensin/kg soil (Sun et al. 2013). A cumulative PEC_{soil} of 63.4 µg monensin/kg was derived for soil receiving six consecutive applications of manure (Hansen et al. 2009) and 50 µg monensin/kg following a single application of poultry litter (Žižek et al. 2011). In a study undertaken in Nebraska, liquid swine manure contained 320 + 31 mg bacitracin A /kg dry weight (Joy et al. 2014). Manure from a pig finishing operation in China contained 51 + 2.3 mg bacitracin/kg dry weight (Zhou et al. 2013). In both instances no residues were detected in soils treated with these manures, suggesting that ZBAC was sequestered or dissipated. Ivermectin can be delivered through injection, pour on and oral drench (Lumaret et al. 2012). Ivermectin-treated cattle excreted 0.31 to 0.81 mg ivermectin/kg dung dry weight, and pasture soil immediately under dung pats had ivermectin concentrations of up to 0.085 mg/kg soil dry weight (Römbke et al. 2010). Liebig et al. (2010b) derived a steady state (considering dissipation rate and manure application practice) PEC_{soil} of 2.67-6.22 µg ivermectin/kg soil dry weight for soil receiving manure from weaner pigs (Liebig et al. 2010b). Similar to pharmaceuticals, heavy metals can also cause change in community composition and microorganism abundance. Under the 100 mg/kg treatment, the soil received about 4.4 mg/kg of zinc metal.

Laboratory additions of 100 mg/kg increased the concentration of pharmaceuticals, changing functional endpoints in soil, therefore validating that these pharmaceuticals can cause a negative effect in soils. The change in function was not, however, accompanied by changes in 16S rDNA or *amoA* gene abundance. The difference seen could be attributed to the soils being treated with a single application of pharmaceuticals, as opposed to the multi-year exposure present in the field. After pharmaceutical addition, the soil was incubated in the laboratory at a constant temperature, and not exposed to the temperature fluctuations that are present outside (Tourna et al. 2008), nor changing day/night cycles, or other environmental factors, like rain or frost. Therefore studying toxicity of pharmaceuticals under field conditions needs to be considered.

4.2. Intermediate levels of pharmaceuticals

At the intermediate field exposure dose of 10 mg/kg, there was no long term (as evaluated in 2012-2013 using pre-application samples) effect on measured endpoints for single pharmaceutical exposures, signifying that this level pharmaceutical exposure over 3 seasons did not disturb soil nitrification. Mixture exposure increased the nitrification rate over the long term (one year after application), but not the short term (within 30 days of pharmaceutical application). Both the bait lamina study and the radioactive rye mineralization experiments showed that single pharmaceuticals or mixture had no significant effects on mineralization. These two mineralization experiments tested the same soil, but due to soil preparation (moisture adjustment and sieving) prior to the radioactive rye addition, some soil dwelling organisms were excluded from that experiment. Specifically, bait lamina strips added to the field soil were exposed to micro-, meso- and macroorganisms, while the rye incubation soil was sieved to a maximum particle size of 2 mm. Therefore the mixture might have had small negative effects on soil macroorganisms, but not on meso- or microorganisms. Short term (within 30 days of addition) effects of 10mg/kg pharmaceutical addition to soil did not change the functions measured or 16S rDNA gene abundance, but relative abundance of the *amoA* gene for ammonia oxidizing archaea and bacteria changed. Similarly, it was found that tylosin at

10 mg/L changed the structure of the bacterial community, but did not change the denitrification rate (Roose-Amsaleg et al. 2013), while a shift in community composition and a decreased rate of substrate mineralization was detected for tylosin at 50 mg/kg (Demoling and Baath 2008). When looking at the responses of agricultural soil microorganisms to sulfachloropyridazine, a concentration of only 7.3 mg/kg resulted in changes in community composition due to sensitive organisms being replaced by tolerant species (Schmitt et al. 2004).

The difference in the ratio of archaeal to bacterial *amoA* genes in soils receiving any of the pharmaceuticals or mixture at 10 mg/kg was reflective of both an increase in AOA abundance (30 days after addition) and decrease in AOB abundance (both 7 and 30 days after addition). AOB abundance decreased at 7 days post pharmaceutical addition, while AOA remained unchanged. The decrease in the abundance of ammonia-oxidizing bacteria at D7 with exposure to 10mg/kg, suggests these concentrations were toxic to AO bacteria. Because AOA grows faster than AOB (You et al. 2009), bacteria would be at a disadvantage. Higher observed susceptibility of AOB to pharmaceuticals compared to AOA could be due to structural differences between the two groups. Unlike bacterial cell walls, archaeal cell walls do not have peptidoglycan present (Schleper and Nicol 2010), and these peptidoglycans are a target for many pharmaceuticals (Khelaifia and Drancourt 2012). Therefore AOA would be less susceptible to a number of pharmaceuticals. Other factors such as light intensity (Merbt et al. 2012), or soil acidity (Gubry-Rangin et al. 2011) were shown to influence AO archaea. Merbt et al. (2012) showed that under constant light ($60\mu E/m^2/s$), AOA growth was more sensitive that AOB growth, and cycles of light and dark were needed for recovery. The molecular analysis of representative archaea from soil samples showed clustering into pH dependent clusters, where adaptation to different pH was evident (Gubry-Rangin et al. 2011). However in present study, the sequences from mixture and control soils did not form distinct clustering, but rather the sequences were intermixed suggesting that the community under 10 mg/kg concentration of the mixture did not result in community shift.

Another possibility for a decrease in AOB abundance is limited NH_4^+, which prevents AOB from further growth. The initial growth of ammonia oxidizing bacteria would decrease the available ammonium in soil, making the environment less favorable

for that group. By day 7 after pharmaceutical addition the amount of extractable ammonium in field soils decreased below the limit of detection. Lower nitrogen resources would mean AOB numbers would diminish quickly, while the space and released nutrients would be available to archaea. AOA have a competitive advantage in nitrogen limited environments (Km= 0.132 µM NH_4^+; Martens-Habbena et al. 2009), and often a decreasing amount of ammonium corresponds to increasing AOA abundance (Sauder et al. 2012), as opposed to high ammonium sites where AOB (Km= 15 µM NH_4^+; Martens-Habbena et al. 2009) has an advantage (Verhamme et al. 2011). It was noted in multiple studies that agricultural soils that receive inorganic N in the form of fertilizer have higher abundance of AOB organisms due to nitrogen rich manure addition (Glaser et al. 2010, Höfferle et al. 2010, Reed et al. 2010). Similarly, soil used for cattle overwintering with heavy animal presence had increased abundance of AOB (Radl et al. 2014).

With low amounts of N in soil, AOB numbers remained low and the AOA numbers increased, presumably due to their ability to thrive in low nitrogen environments and utilizing the space released by AOB. The functional redundancy of ammonia oxidizing organisms (discussed in Nannipieri et al. 2003) allowed for retained function in the soil at 10 mg/kg, despite changing numbers of AOA and AOB, as detected by quantitative PCR of the *amoA* gene.

4.3. Regulatory threshold concentrations

There were no effects of any treatment, either long term (after 3 years of applications) or acute (7 and 30 days after application), for the environmentally relevant and regulatory concentration of 0.1 mg/kg on any measured functional endpoints. This held true for both single pharmaceutical and mixture exposures.

Pharmaceuticals were most likely applied at sub-inhibitory concentrations (NOEC) that did not affect microbial populations, even for the mixture treatment. This result could be a side effect of existing resistance pathways, or tolerance mechanisms. Natural tolerance to naturally derived antibiotics exists in soils (Thiele-Bruhn 2003, D'Costa et al. 2011), especially for soil-derived chemicals. For example, some botanically

derived pesticides have no effect on microorganisms, as opposed to synthetics that were not derived from nature but created in laboratory (Spyrou et al. 2009).

The threshold concentration of 0.1 mg/kg, the cut off for Environmental Risk Assessments, is conservative enough not to disrupt soil processes or cause any detectable changes in *amoA* gene abundance or community structure of soil organisms under IVER, MON and ZBAC treatments. Based on the current study, the three studied pharmaceuticals were dissipated in the soils rapidly, but other pharmaceuticals can be persistent in the environment, and accumulate over the years of exposure (Tamtam et al. 2011, Vazquez-Roig et al. 2012). Therefore, long term dissipation studies of different pharmaceuticals in the field should always be carried out, and lab experiments might underestimate the long term effect pharmaceuticals can have on soil organisms.

Conclusion

At an environmentally reasonable and regulatory threshold concentration of 0.1 mg/kg, there was no effect of the studied pharmaceuticals on mineralization or nitrification, either in the long or short term. This scenario, however, was tested on an artificial system, where pharmaceuticals were added to the soil directly in order to control the concentrations. Using manure mixed with pharmaceuticals is needed to verify that this would happen during normal farming practice.

Manure acts as a source of nutrients to soil microorganisms and it has sorption characteristics (it can bind the pharmaceuticals to the organic matter present in the manure) that can make pharmaceuticals bio-unavailable. Therefore adding veterinary pharmaceuticals with the manure would potentially decrease any negative effects observed in this study. However, number of different pharmaceuticals can be added to the soil at once with each manure application, due to multiple veterinary pharmaceuticals used in animal production. The mixture of these pharmaceuticals can affect the soil microorganisms, where single drug did not. Therefore it is important to measure additional endpoints that were beyond the scope of this study. One could look at other soil functions (other steps in nitrogen cycle in the soil, or other nutrient cycling), or biodiversity (displacement of species, direct counts or molecular analysis) to assess if the pharmaceuticals affect other groups of microorganisms or their function.

It is possible that if our mixture contained more than the 3 studied pharmaceuticals at 0.1 mg/kg concentration, it could have an impact on studied functions and abundance of ammonia oxidizers, especially bacteria. It is unknown if ammonia oxidizing archaea would at all respond to the veterinary pharmaceuticals. Little is known about that group in soil; further study of archaeal physiology from soil-isolated microorganisms is needed to understand how they interact with pharmaceuticals in the environment. It is essential to get more information about the soil dwelling organisms, their function, and how that function in soils is affected by addition of manure containing veterinary pharmaceutical.

References

Aaen, K. N., P. E. Holm, A. Priemé, N. N. Hung, and K. K. Brandt. 2011. Comparison of aerobic and anaerobic [3H]leucine incorporation assays for determining pollution-induced bacterial community tolerance in copper-polluted, irrigated soils. Environmental Toxicology and Chemistry **30**:588-595.

Adair, K., and E. Schwartz. 2008. Evidence that Ammonia-Oxidizing Archaea are More Abundant than Ammonia-Oxidizing Bacteria in Semiarid Soils of Northern Arizona, USA. Microbial Ecology **56**:420-426.

Al-Ahmad, A., F. D. Daschner, and K. Kümmerer. 1999. Biodegradability of cefotiam, ciprofloxacin, meropenem, penicillin G, and sulfamethoxazole and inhibition of waste water bacteria. Archives of Environmental Contamination and Toxicology **37**:158-163.

Al-Rajab, A. J., L. Sabourin, A. Scott, D. R. Lapen, and E. Topp. 2009. Impact of biosolids on the persistence and dissipation pathways of triclosan and triclocarban in an agricultural soil. Science of The Total Environment **407**:5978-5985.

Altenburger, R., T. Backhaus, W. Boedeker, M. Faust, and M. Scholze. 2013. Simplifying complexity: Mixture toxicity assessment in the last 20 years. Environmental Toxicology and Chemistry **32**:1685-1687.

Baath, E., A. Frostegard, A. Tunlid, and M. Daiz-Ravina. 1998. Microbial community-based measurements to estimate heavy metal effects in soil: the use of phospholipid fatty acid patterns and bacterial community tolerance. Ambio **27**:58-61.

Bernier, S. P., and M. G. Surette. 2013. Concentration-dependent activity of antibiotics in natural environments. Frontiers in Microbiology **4**.

Beynon, S. A. 2012. Potential environmental consequences of administration of anthelmintics to sheep. Veterinary Parasitology **189**:113-124.

Blanckenhorn, W. U., N. Puniamoorthy, M. A. Schäfer, A. Scheffczyk, and J. Römbke. 2013. Standardized laboratory tests with 21 species of temperate and tropical sepsid flies confirm their suitability as bioassays of pharmaceutical residues (ivermectin) in cattle dung. Ecotoxicology and Environmental Safety **89**:21-28.

Borgmann, U., D. T. Bennie, A. L. Ball, and V. Palabrica. 2007. Effect of a mixture of seven pharmaceuticals on Hyalella azteca over multiple generations. Chemosphere **66**:1278-1283.

Browder, J. 2004. Review of Ecological Microcosms by Robert J. Beyers and Howard T. Odum. Ecological Modelling **178**:77-80.

Carlson, J. C., and S. A. Mabury. 2006. Dissipation kinetics and mobility of chlortetracycline, tylosin, and monensin in an agricultural soil in Northumberland County, Ontario, Canada. Environmental Toxicology and Chemistry **25**:1-10.

D'Costa, V. M., C. E. King, L. Kalan, M. Morar, W. W. L. Sung, C. Schwarz, D. Froese, G. Zazula, F. Calmels, R. Debruyne, G. B. Golding, H. N. Poinar, and G. D. Wright. 2011. Antibiotic resistance is ancient. Nature **477**:457-461.

Demoling, L. A., and E. Baath. 2008. The use of leucine incorporation to determine the toxicity of phenols to bacterial communities extracted from soil. Applied Soil Ecology **38**:34-41.

Dias, A. C. F., E. F. Hoogwout, M. D. C. Pereira e Silva, J. F. Salles, L. S. van Overbeek, and J. D. van Elsas. 2012. Potato cultivar type affects the structure of ammonia oxidizer communities in field soil under potato beyond the rhizosphere. Soil Biology and Biochemistry **50**:85-95.

Donoho, A. L. 1984. Biochemical studies on the fate of monensin in animals and in the environment. Journal of Animal Science **58**:1528-1539.

Donoso, J., G. O. Craig, and R. S. Baldwin. 1970. The distribution and excretion of zinc bacitracin-14C in rats and swine. Toxicology and Applied Pharmacology **17**:366-374.

Drabløs, F., D. G. Nicholson, and M. Rønning. 1999. EXAFS study of zinc coordination in bacitracin A. Biochimica et Biophysica Acta - Protein Structure and Molecular Enzymology **1431**:433-442.

Drake, J., and A. Kramer. 2012. Mechanistic analogy: how microcosms explain nature. Theoretical Ecology **5**:433-444.

EC. 2003. Regulation (EC) 1831/2003 of the European parlament and of the council of 22 September 2003 on additives for use in animal nutrition.

Edmeades, D. C. 2003. The long-term effects of manures and fertilisers on soil productivity and quality: A review. Nutrient Cycling in Agroecosystems **66**:165-180.

Eldridge, S. M., K. Y. Chan, Z. H. Xu, C. R. Chen, and I. Barchia. 2008. Plant-available nitrogen supply from granulated biosolids: Implications for land application guidelines. Australian Journal of Soil Research **46**:423-436.

Ellis, S., L. Dendooven, and K. W. T. Goulding. 1996. Quantitative assessment of soil nitrate disappearance and N2O evolution during denitrification: Nitrate disappearance during denitrification. Soil Biology and Biochemistry **28**:589-595.

Eržen, N. K., L. Kolar, V. C. Flajs, J. Kužner, I. Marc, and M. Pogačnik. 2005. Degradation of abamectin and doramectin on sheep grazed pasture. Ecotoxicology **14**:627-635.

Fechner, L. C., C. Gourlay-Francé, and M. H. Tusseau-Vuillemin. 2011. Low exposure levels of urban metals induce heterotrophic community tolerance: A microcosm validation. Ecotoxicology **20**:793-802.

Francis, C. A., K. J. Roberts, J. M. Beman, A. E. Santoro, and B. B. Oakley. 2005. Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. Proceedings of the National Academy of Sciences of the United States of America **102**:14683-14688.

Fraser, L. H., and P. Keddy. 1997. The role of experimental microcosms in ecological research. Trends in Ecology & Evolution **12**:478-481.

Friedland, A. J., E. K. Miller, J. J. Battles, and J. F. Thorne. 1991. Nitrogen deposition, distribution and cycling in a subalpine spruce-fir forest in the Adirondacks, New York, USA. Biogeochemistry **14**:31-55.

Frøyshov, Ø., S. Pedersen, and K. Hove. 1986. Absorption, metabolism and excretion of zinc 14C-bacitracin fed to young pigs. Journal of Animal Physiology and Animal Nutrition **55**:100-110.

Galloway, J. N., F. J. Dentener, D. G. Capone, E. W. Boyer, R. W. Howarth, S. P. Seitzinger, G. P. Asner, C. C. Cleveland, P. A. Green, E. A. Holland, D. M. Karl,

A. F. Michaels, J. H. Porter, A. R. Townsend, and C. J. Vörösmarty. 2004. Nitrogen cycles: Past, present, and future. Biogeochemistry **70**:153-226.

Girardi, C., J. Greve, M. Lamshöft, I. Fetzer, A. Miltner, A. Schäffer, and M. Kästner. 2011. Biodegradation of ciprofloxacin in water and soil and its effects on the microbial communities. Journal of Hazardous Materials **198**:22-30.

Glaser, K., E. Hackl, E. Inselsbacher, J. Strauss, W. Wanek, S. Zechmeister-Boltenstern, and A. Sessitsch. 2010. Dynamics of ammonia-oxidizing communities in barley-planted bulk soil and rhizosphere following nitrate and ammonium fertilizer amendment. FEMS Microbiology Ecology **74**:575-591.

Griffiths, E. 1965. Micro-organisms and soil structure. Biological Reviews **40**:129-142.

Gubry-Rangin, C., B. Hai, C. Quince, M. Engel, B. C. Thomson, P. James, M. Schloter, R. I. Griffiths, J. I. Prosser, and G. W. Nicol. 2011. Niche specialization of terrestrial archaeal ammonia oxidizers. Proceedings of the National Academy of Sciences of the United States of America **108**:21206-21211.

Hall, T.A. (1999) BioEdit: a user friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series **41**: 95-98

Halley, B. A., T. A. Jacob, and A. Y. H. Lu. 1989. The environmental impact of the use of ivermectin: environmental effects and fate. Chemosphere **18**:1543-1563.

Hansen, D. L., S. Ishii, M. J. Sadowsky, and R. E. Hicks. 2009. Escherichia coli Populations in Great Lakes Waterfowl Exhibit Spatial Stability and Temporal Shifting. Applied and Environmental Microbiology **75**:1546-1551.

HC. 2001. Drugs and Health products. Health Canada website (http://www.hc-sc.gc.ca/dhp-mps/index-eng.php) accessed August 12 2013.

Höfferle, Š., G. W. Nicol, L. Pal, J. Hacin, J. I. Prosser, and I. Mandić-Mulec. 2010. Ammonium supply rate influences archaeal and bacterial ammonia oxidizers in a wetland soil vertical profile. FEMS Microbiology Ecology **74**:302-315.

Huhta, V. 2007. The role of soil fauna in ecosystems: A historical review. Pedobiologia **50**:489-495.

Hutchinson, J. J., C. A. Campbell, and R. L. Desjardins. 2007. Some perspectives on carbon sequestration in agriculture. Agricultural and Forest Meteorology **142**:288-302.

Ingerslev, F., and B. Halling-Sorensen. 2000. Biodegradability properties of sulfonamides in activated sludge. Environmental Toxicology and Chemistry **19**:2467-2473.

Inselsbacher, E., N. Hinko-Najera Umana, F. C. Stange, M. Gorfer, E. Schüller, K. Ripka, S. Zechmeister-Boltenstern, R. Hood-Novotny, J. Strauss, and W. Wanek. 2010. Short-term competition between crop plants and soil microbes for inorganic N fertilizer. Soil Biology and Biochemistry **42**:360-372.

ISO. 2012. ISO 14238: Soil quality — Biological methods — Determination of nitrogen mineralization and nitrification in soils and the influence of chemicals on these processes. International Organization for Standardization, Geneva, Switzerland.

Jechalke, S., A. Focks, I. Rosendahl, J. Groeneweg, J. Siemens, H. Heuer, and K. Smalla. 2014. Structural and functional response of the soil bacterial community to application of manure from difloxacin-treated pigs. FEMS Microbiology Ecology **87**:78-88.

Jensen, J., X. Diao, and A. D. Hansen. 2009. Single- and two-species tests to study effects of the anthelmintics ivermectin and morantel and the coccidiostatic monensin on soil invertebrates. Environmental Toxicology and Chemistry **28**:316-323.

Joy, S. R., X. Li, D. D. Snow, J. E. Gilley, B. Woodbury, and S. L. Bartelt-Hunt. 2014. Fate of antimicrobials and antimicrobial resistance genes in simulated swine manure storage. Science of the Total Environment **481**:69-74.

Kemper, N. 2008. Veterinary antibiotics in the aquatic and terrestrial environment. Ecological Indicators **8**:1-13.

Khelaifia, S., and M. Drancourt. 2012. Susceptibility of archaea to antimicrobial agents: Applications to clinical microbiology. Clinical Microbiology and Infection **18**:841-848.

Kumar, K., S. C. Gupta, S. K. Baidoo, Y. Chander, and C. J. Rosen. 2005. Antibiotic uptake by plants from soil fertilized with animal manure. Journal of Environmental Quality **34**:2082-2085.

Lehmann, R. G., and J. R. Miller. 1999. Development of a soil ecotoxicology method: Preparation and soil degradation of 14C-labeled grass. Applied Soil Ecology **12**:93-98.

Leininger, S., T. Urich, M. Schloter, L. Schwark, J. Qi, G. W. Nicol, J. I. Prosser, S. C. Schuster, and C. Schleper. 2006. Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature **442**:806-809.

Levot, G. W. 2011. Degradation of diflubenzuron, ivermectin, cyromazine and temephos in soil following surface disposal of sheep dipping and jetting solutions. Animal Production Science **51**:996-1001.

Liebig, M., A. Alonso Fernandez, E. Blubaum-Gronau, A. Boxall, M. Brinke, G. Carbonell, P. Egeler, K. Fenner, C. Fernandez, G. Fink, J. Garric, B. Halling-Sorensen, T. Knacker, K. A. Krogh, A. Kuster, D. Loffler, M. A. Porcel Cots, L. Pope, C. Prasse, J. Rombke, I. Ronnefahrt, M. K. Schneider, N. Schweitzer, J. V. Tarazona, T. A. Ternes, and W. Traunspurger. 2010a. Environmental risk assessment of ivermectin: a case study. (Special Issue: Environmental risk assessment and management (ERAPharm).). Integrated Environmental Assessment and Management (IEAM) **6**:567-587.

Lorenzen, A., J. G. Hendel, K. L. Conn, S. Bittman, A. B. Kwabiah, G. Lazarovitz, D. Masse, T. A. McAllister, and E. Topp. 2004. Survey of hormone activities in municipal biosolids and animal manures. Environmental Toxicology **19**:216-225.

Lumaret, J. P., F. Errouissi, K. Floate, J. Römbke, and K. Wardhaugh. 2012. A review on the toxicity and non-target effects of macrocyclic lactones in terrestrial and aquatic environments. Current Pharmaceutical Biotechnology **13**:1004-1060.

Martens-Habbena, W., P. M. Berube, H. Urakawa, J. R. De La Torre, and D. A. Stahl. 2009. Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. Nature **461**:976-979.

Marti, R., Y. C. Tien, R. Murray, A. Scott, L. Sabourin, and E. Topp. 2014. Safely coupling livestock and crop production systems: How rapidly do antibiotic resistance genes dissipate in soil following a commercial application of swine or dairy manure? Applied and Environmental Microbiology **80**:3258-3265.

McTavish, H., J. A. Fuchs, and A. B. Hooper. 1993. Sequence of the gene coding for ammonia monooxygenase in Nitrosomonas europaea. Journal of Bacteriology **175**:2436-2444.

Merbt, S. N., D. A. Stahl, E. O. Casamayor, E. Martí, G. W. Nicol, and J. I. Prosser. 2012. Differential photoinhibition of bacterial and archaeal ammonia oxidation. FEMS Microbiology Letters **327**:41-46.

Mertens, J., K. Broos, S. A. Wakelin, G. A. Kowalchuk, D. Springael, and E. Smolders. 2009. Bacteria, not archaea, restore nitrification in a zinc-contaminated soil. ISME Journal **3**:916-923.

Mertens, J., S. A. Wakelin, K. Broos, M. J. McLaughlin, and E. Smolders. 2010. Extent of copper tolerance and consequences for functional stability of the ammonia-oxidizing community in long-term copper-contaminated Soils. Environmental Toxicology and Chemistry **29**:27-37.

Ming, L. J., and J. D. Epperson. 2002. Metal binding and structure-activity relationship of the metalloantibiotic peptide bacitracin. Journal of Inorganic Biochemistry **91**:46-58.

Montforts, M. H. 2005. RIVM report 601500002/2005. The trigger values in the environmental risk assessment for (veterinary) medicines in the European Union: a critical appraisal.

Mougin, C., A. Kollmann, J. Dubroca, P. H. Ducrot, M. Alvinerie, and P. Galtier. 2003. Fate of the veterinary medicine ivermectin in soil. Environmental Chemistry Letters **1**:131-134.

Muyzer, G., E. C. De Waal, and A. G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology **59**:695-700.

Nannipieri, P., J. Ascher, M. T. Ceccherini, L. Landi, G. Pietramellara, and G. Renella. 2003. Microbial diversity and soil functions. European Journal of Soil Science **54**:655-670.

O'Hea, N. M., L. Kirwan, P. S. Giller, and J. A. Finn. 2010. Lethal and sub-lethal effects of ivermectin on north temperate dung beetles, Aphodius ater and Aphodius rufipes (Coleoptera: Scarabaeidae). Insect Conservation and Diversity **3**:24-33.

O'Sullivan, C. A., S. A. Wakelin, I. R. P. Fillery, A. L. Gregg, and M. M. Roper. 2011. Archaeal ammonia oxidisers are abundant in acidic, coarse-textured Australian soils. Soil Research **49**:715-724.

OECD. 2000. Test No. 216: Soil Microorganisms: Nitrogen Transformation Test. OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing.

OEHHA. 2009. Soil toxicity and bioassessment test methods for ecological risk assessment: Toxicity Test Methods for Soil Microorganisms, Terrestrial Plants, Terrestrial Invertebrates and Terrestrial Vertebrates.*in* Office of Environmental Health Hazard Assessment, California Environmental Protection Agency.

Offre, P., J. I. Prosser, and G. W. Nicol. 2009. Growth of ammonia-oxidizing archaea in soil microcosms is inhibited by acetylene. FEMS Microbiology Ecology **70**:99-108.

Ollivier, J., D. Schacht, R. Kindler, J. Groeneweg, M. Engel, B. M. Wilke, K. Kleineidam, and M. Schloter. 2013. Effects of repeated application of

sulfadiazine-contaminated pig manure on the abundance and diversity of ammonia and nitrite oxidizers in the root-rhizosphere complex of pasture plants under field conditions. Frontiers in Microbiology **4**, article 22.

Paul, E. A., and F. E. Clark. 1989. Soil microbiology and biochemistry. Academic press, San Diego, pg 11-162.

Pastor, J., and W. M. Post. 1986. Influence of climate, soil moisture, and succession on forest carbon and nitrogen cycles. Biogeochemistry **2**:3-27.

Pope, L., A. B. A. Boxall, C. Corsing, B. Halling-Sorensen, A. Tait, and E. Topp. 2009. Exposure Assessment of Veterinary Medicines in Terrestrial Systems. Pages 129-154 *in* M. Crane, A. B. A. Boxall, and K. Barrett, editors. Veterinary Medicines in the Environment. SETAC, Pensacola, FL.

Radl, V., A. Chroňáková, J. Čuhel, M. Šimek, D. Elhottová, G. Welzl, and M. Schloter. 2014. Bacteria dominate ammonia oxidation in soils used for outdoor cattle overwintering. Applied Soil Ecology **77**:68-71.

Reed, D. W., J. M. Smith, C. A. Francis, and Y. Fujita. 2010. Responses of ammonia-oxidizing bacterial and archaeal populations to organic nitrogen amendments in low-nutrient groundwater. Applied and Environmental Microbiology **76**:2517-2523.

Römbke, J., A. Coors, T. A. Fernández, B. Förster, C. Fernández, J. Jensen, J. P. Lumaret, M. T. P. Cots, and M. Liebig. 2010. Effects of the parasiticide ivermectin on the structure and function of dung and soil invertebrate communities in the field (Madrid, Spain). Applied Soil Ecology **45**:284-292.

Roose-Amsaleg, C., C. Yan, A. M. Hoang, and A. M. Laverman. 2013. Chronic exposure of river sediments to environmentally relevant levels of tetracycline affects bacterial communities but not denitrification rates. Ecotoxicology **22**:1467-1478.

Rotthauwe, J. H., K. P. Witzel, and W. Liesack. 1997. The ammonia monooxygenase structural gene *amoA* as a functional marker: Molecular fine-scale analysis of natural ammonia-oxidizing populations. Applied and Environmental Microbiology **63**:4704-4712.

Rusk, J. A., R. E. Hamon, D. P. Stevens, and M. J. McLaughlin. 2004. Adaptation of soil biological nitrification to heavy metals. Environmental Science and Technology **38**:3092-3097.

Ruyters, S., G. W. Nicol, J. I. Prosser, B. Lievens, and E. Smolders. 2013. Activity of the ammonia oxidising bacteria is responsible for zinc tolerance development of the ammonia oxidising community in soil: A stable isotope probing study. Soil Biology and Biochemistry **58**:244-247.

Sabourin, L., A. Al-Rajab, R. Chapman, D. Lapen, and E. Topp. 2010. Fate of the antifungal drug clotrimazole in agricultural soil. Environmental Toxicology and Chemistry **30**: 582-587.

Sabourin, L., P. Duenk, S. Bonte-Gelok, M. Payne, D. R. Lapen, and E. Topp. 2012. Uptake of pharmaceuticals, hormones and parabens into vegetables grown in soil fertilized with municipal biosolids. Science of the Total Environment **431**:233-236.

Santoro, A., C. Francis, N. Sieyes, and A. Boehm. 2008. Shifts in the relative abundance of ammonia-oxidizing bacteria and archaea across physicochemical gradients in subterranean estuary. Environmental Microbiology **10**: 1068-1079.

Sarmah, A. K., M. T. Meyer, and A. B. A. Boxall. 2006. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. Chemosphere **65**:725-759.

Sassman, S. A., and L. S. Lee. 2007. Sorption and degradation in soils of veterinary ionophore antibiotics: Monensin and lasalocid. Environmental Toxicology and Chemistry **26**:1614-1621.

Sauder, L. A., F. Peterse, S. Schouten, and J. D. Neufeld. 2012. Low-ammonia niche of ammonia-oxidizing archaea in rotating biological contactors of a municipal wastewater treatment plant. Environmental Microbiology **14**:2589-2600.

Scharroba, A., D. Dibbern, M. Hünninghaus, S. Kramer, J. Moll, O. Butenschoen, M. Bonkowski, F. Buscot, E. Kandeler, R. Koller, D. Krüger, T. Lueders, S. Scheu, and L. Ruess. 2012. Effects of resource availability and quality on the structure of the micro-food web of an arable soil across depth. Soil Biology and Biochemistry **50**:1-11.

Schimel, J. P., and J. Bennett. 2004. Nitrogen mineralization: Challenges of a changing paradigm. Ecology **85**:591-602.

Schleper, C., and G. W. Nicol. 2010. Ammonia-Oxidising Archaea – Physiology, Ecology and Evolution. Pages 1-41 *in* K. P. Robert, editor. Advances in Microbial Physiology. Academic Press.

Schmitt, H., T. Boucard, J. Garric, J. Jensen, J. Parrott, A. Péry, J. Römbke, J. O. Straub, T. H. Hutchinson, P. Sánchez-Argüello, Å. Wennmalm, and K. Duis. 2010. Recommendations on the environmental risk assessment of pharmaceuticals: Effect characterization. Integrated Environmental Assessment and Management **6**:588-602.

Schmitt, H., B. Martinali, P. Van Beelen, and W. Seinen. 2006. On the limits of toxicant-induced tolerance testing: Cotolerance and response variation of antibiotic effects. Environmental Toxicology and Chemistry **25**:1961-1968.

Schmitt, H., P. Van Beelen, J. Tolls, and C. L. Van Leeijwen. 2004. Pollution-induced Community Tolerance of Soil Microbial Communities Caused by the Antibiotic Sulfachloropyridazine. Environmental Science and Technology **38**:1148-1153.

Sonthiphand, P., E. Cejudo, S. L. Schiff, and J. D. Neufeld. 2013. Wastewater effluent impacts ammonia-oxidizing prokaryotes of the Grand River, Canada. Applied and Environmental Microbiology **79**:7454-7465.

Spyrou, I. M., D. G. Karpouzas, and U. Menkissoglu-Spiroudi. 2009. Do botanical pesticides alter the structure of the soil microbial community? Microbial Ecology **58**:715-727.

Stein, L., R. a. Roy, and P. F. Dunfield. 2012. Aerobic Methanotrophy and Nitrification: Processes and Connections. In: eLS. John Wiley & Sons Ltd, Chichester, Chichester.

Sun, P., D. Barmaz, M. L. Cabrera, S. G. Pavlostathis, and C.-H. Huang. 2013. Detection and quantification of ionophore antibiotics in runoff, soil and poultry litter. Journal of Chromatography A **1312**:10-17.

Suzuki, M. T., L. T. Taylor, and E. F. DeLong. 2000. Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5'-nuclease assays. Applied and Environmental Microbiology **66**:4605-4614.

Tamtam, F., F. van Oort, B. Le Bot, T. Dinh, S. Mompelat, M. Chevreuil, I. Lamy, and M. Thiry. 2011. Assessing the fate of antibiotic contaminants in metal contaminated soils four years after cessation of long-term waste water irrigation. Science of the Total Environment **409**:540-547.

Tamura K, Stecher G, Peterson D, Filipski A, and Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Molecular Biology and Evolution 30: 2725-2729.

Tarazona, J. V., B. I. Escher, E. Giltrow, J. Sumpter, and T. Knacker. 2010. Targeting the environmental risk assessment of pharmaceuticals: Facts and fantasies. Integrated Environmental Assessment and Management **6**:603-613.

Tate III, R. L. 2000. Soil microbiology. 2nd edition. John Wiley, New York, pg…..

Taylor, A., N. Vajrala, A. Giguere, A. Gitelman, D. Arp, D. Myrold, L. Sayavedra-Soto, and P. Bottomley. 2013. Use of Aliphatic n-Alkynes To Discriminate Soil Nitrification Activities of Ammonia-Oxidizing Thaumarchaea and Bacteria Applied and Environmental Microbiology **79**:6544-6551.

Terry, R. E., D. W. Nelson, and L. E. Sommers. 1979. Carbon cycling during sewage sludge decomposition in soils. Soil Science of America Journal **43**:494-499.

Thiele-Bruhn, S. 2003. Pharmaceutical antibiotic compounds in soils - A review. Journal of Plant Nutrition and Soil Science **166**:145-167.

Topp, E., R. Chapman, M. Devers-Lamrani, A. Hartmann, R. Marti, F. Martin-Laurent, L. Sabourin, A. Scott, and M. Sumarah. 2013. Accelerated biodegradation of veterinary antibiotics in agricultural soil following long-term exposure, and isolation of a sulfamethazinedegrading microbacterium sp. Journal of Environmental Quality **42**:173-178.

Toth, J. D., Y. Feng, and Z. Dou. 2011. Veterinary antibiotics at environmentally relevant concentrations inhibit soil iron reduction and nitrification. Soil Biology and Biochemistry **43**:2470-2472.

Tourna, M., T. E. Freitag, G. W. Nicol, and J. I. Prosser. 2008. Growth, activity and temperature responses of ammonia-oxidizing archaea and bacteria in soil microcosms. Environmental Microbiology **10**:1357-1364.

Vajrala, N., W. Martens-Habbena, L. A. Sayavedra-Soto, A. Schauer, P. J. Bottomley, D. A. Stahl, and D. J. Arp. 2013. Hydroxylamine as an intermediate in ammonia oxidation by globally abundant marine archaea. Proceedings of the National Academy of Sciences of the United States of America **110**:1006-1011.

Van Beelen, P., and P. Doelman. 1997. Significance and application of microbial toxicity tests in assessing ecotoxicological risks of contaminants in soil and sediment. Chemosphere **34**:455-499.

Van Den Brink, P. J., J. V. Tarazona, K. R. Solomon, T. Knacker, N. W. Van Den Brink, T. C. M. Brock, and J. P. Hoogland. 2005. The use of terrestrial and aquatic microcosms and mesocosms for the ecological risk assessment of veterinary medicinal products. Environmental Toxicology and Chemistry **24**:820-829.

van Gestel, C. A. M. 2012. Soil ecotoxicology: State of the art and future directions. ZooKeys **176**:275-296.

Vazquez-Roig, P., V. Andreu, C. Blasco, and Y. Picó. 2012. Risk assessment on the presence of pharmaceuticals in sediments, soils and waters of the Pego–Oliva

Marshlands (Valencia, eastern Spain). Science of the Total Environment **440**:24-32.

Verhamme, D. T., J. I. Prosser, and G. W. Nicol. 2011. Ammonia concentration determines differential growth of ammonia-oxidising archaea and bacteria in soil microcosms. ISME Journal **5**:1067-1071.

VICH. 2000. VICH GL 6. Environmental impact assessment (EIAS) for veterinary medicinal products (VMPS) - Phase I.

VICH. 2005. VICH GL 38. Environmental impact assessments (EIAs) for veterinary medicinal products (VMPs) - Phase II.

Vitousek, P. M., J. D. Aber, R. W. Howarth, G. E. Likens, P. A. Matson, D. W. Schindler, W. H. Schlesinger, and D. G. Tilman. 1997. Human alteration of the global nitrogen cycle: Sources and consequences. Ecological Applications **7**:737-750.

Whitman, W. B., D. C. Coleman, and W. J. Wiebe. 1998. Prokaryotes: The unseen majority. Proceedings of the National Academy of Sciences of the United States of America **95**:6578-6583.

Wicker, D. L., W. N. Iscrigg, and J. H. Trammell. 1977. The control and prevention of necrotic enteritis in broilers with zinc bacitracin. Poultry Science **56**:1229-1231.

Yoshida, N., M. J. L. Castro, and A. F. Cirelli. 2010. Degradation of monensin on soils: Influence of organic matter and water content. Chemistry and Ecology **26**:27-33.

You, J., A. Das, E. M. Dolan, and Z. Hu. 2009. Ammonia-oxidizing archaea involved in nitrogen removal. Water Research **43**:1801-1809.

Zhou, L. J., G. G. Ying, S. Liu, R. Q. Zhang, H. J. Lai, Z. F. Chen, and C. G. Pan. 2013. Excretion masses and environmental occurrence of antibiotics in typical swine and dairy cattle farms in China. Science of the Total Environment **444**:183-195.

Žižek, S., R. Hrženjak, G. T. Kalcher, K. Šrimpf, N. Šemrov, and P. Zidar. 2011. Does monensin in chicken manure from poultry farms pose a threat to soil invertebrates? Chemosphere **83**:517-523.
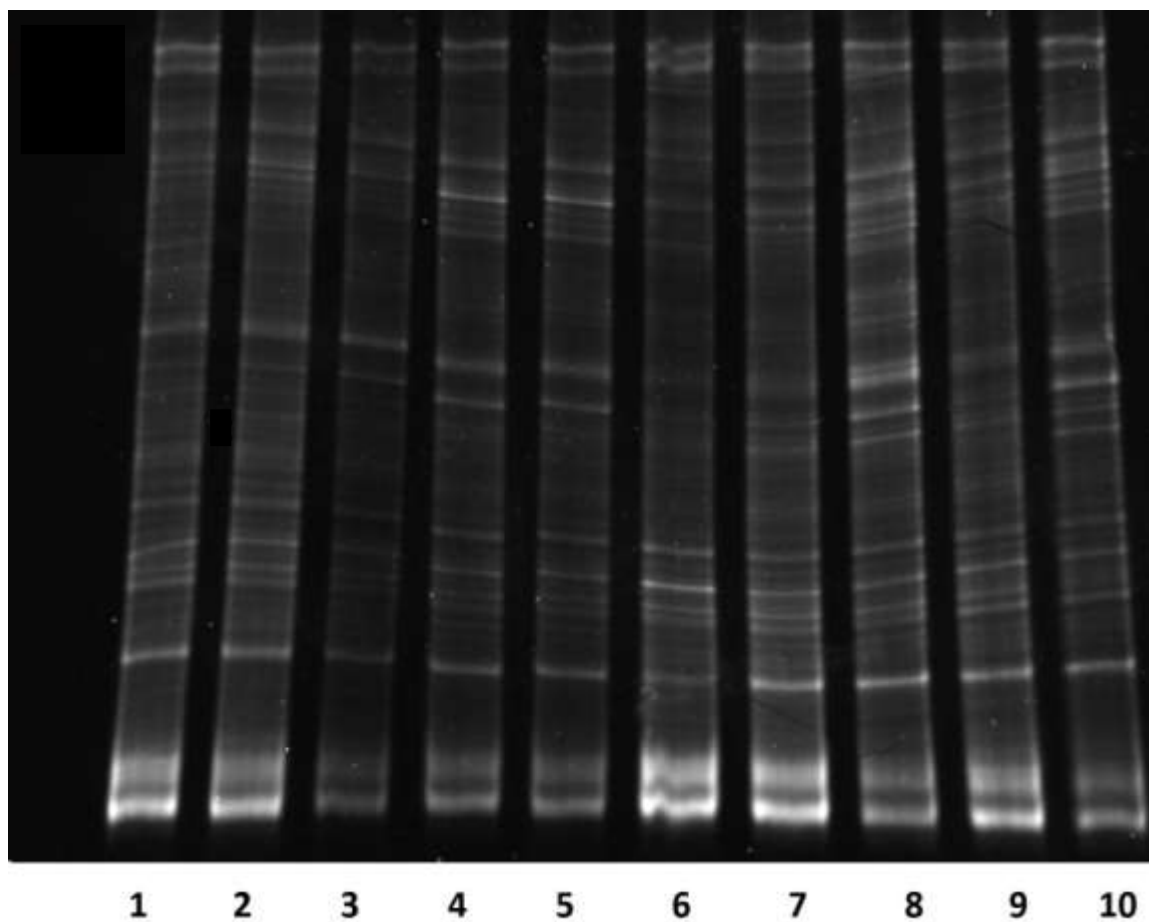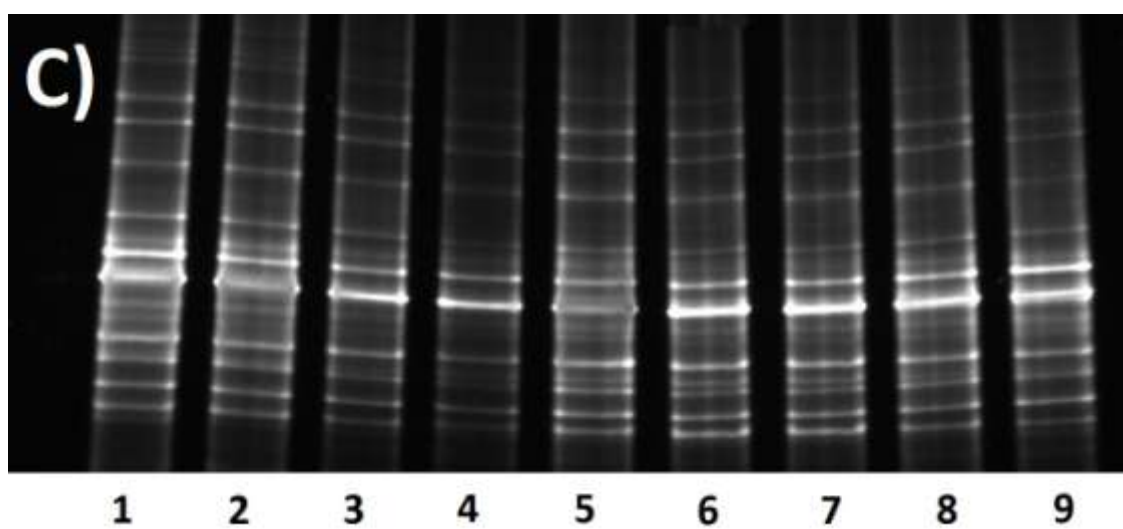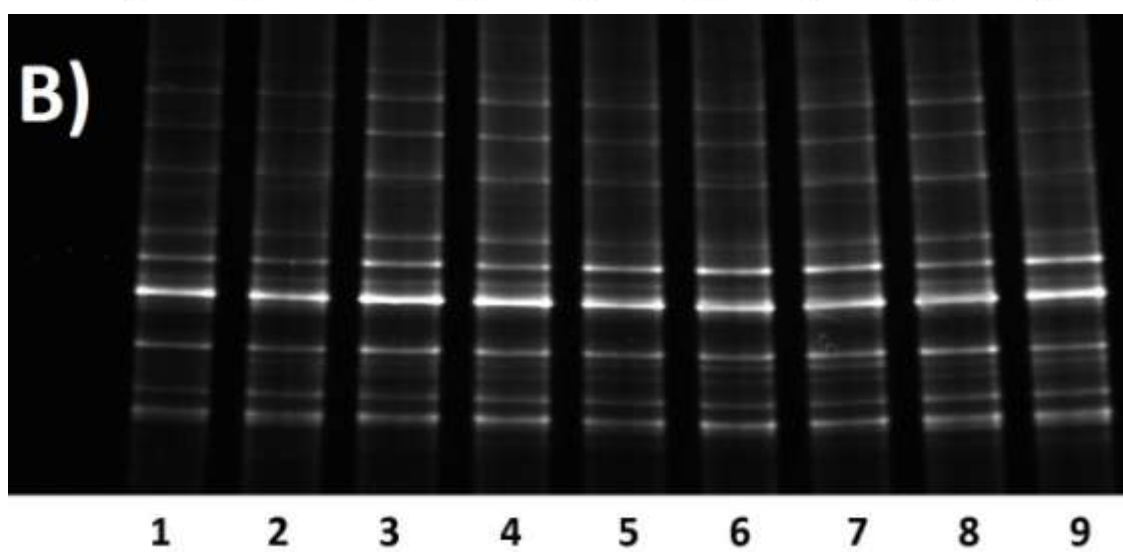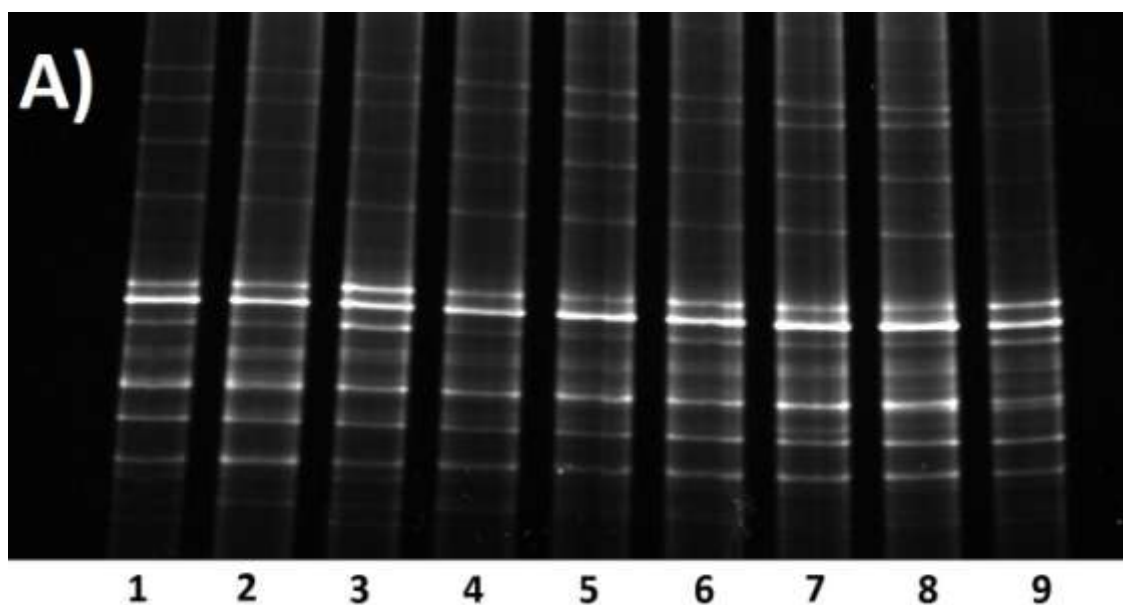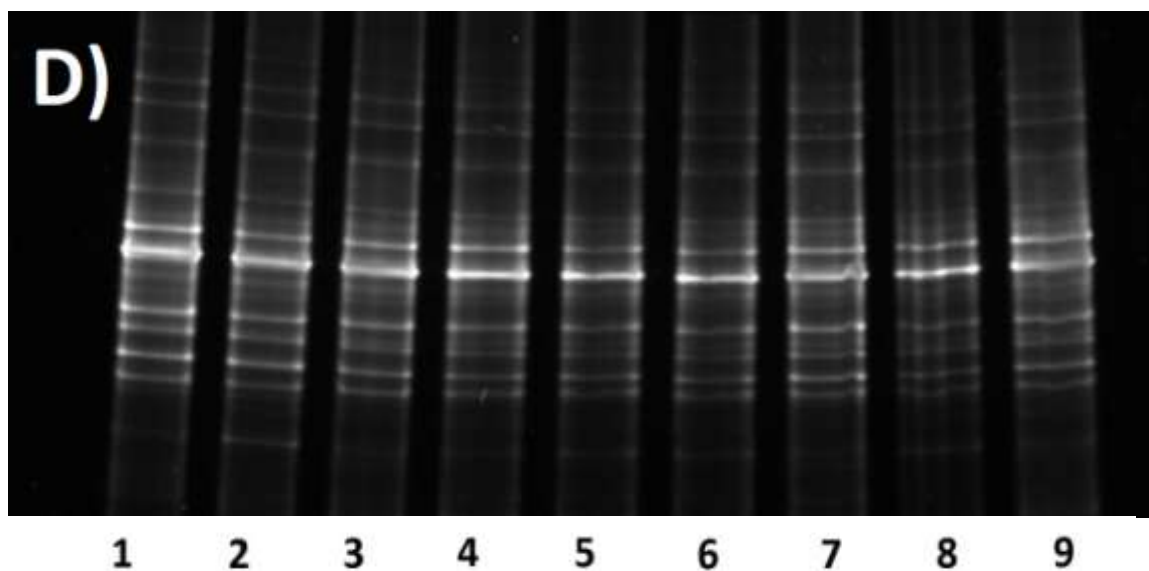
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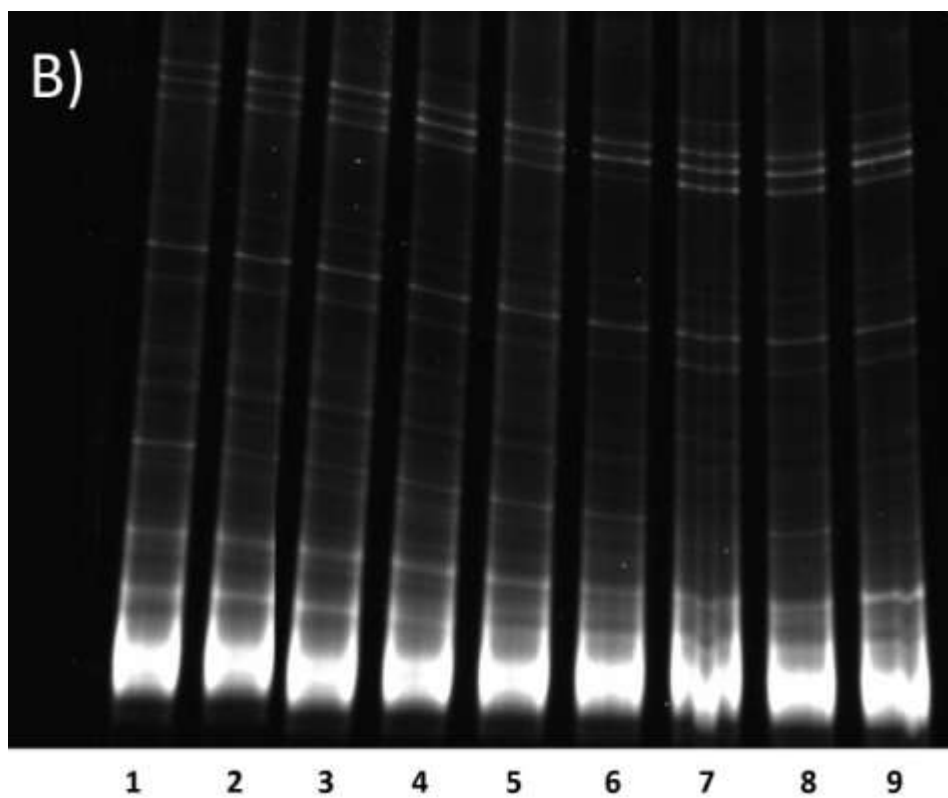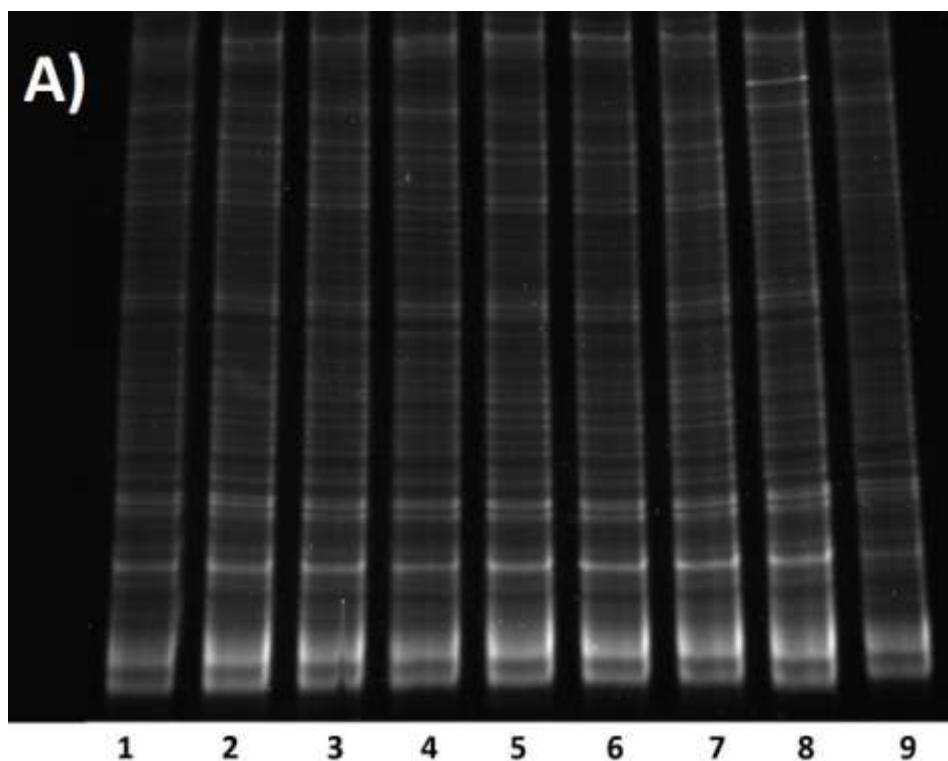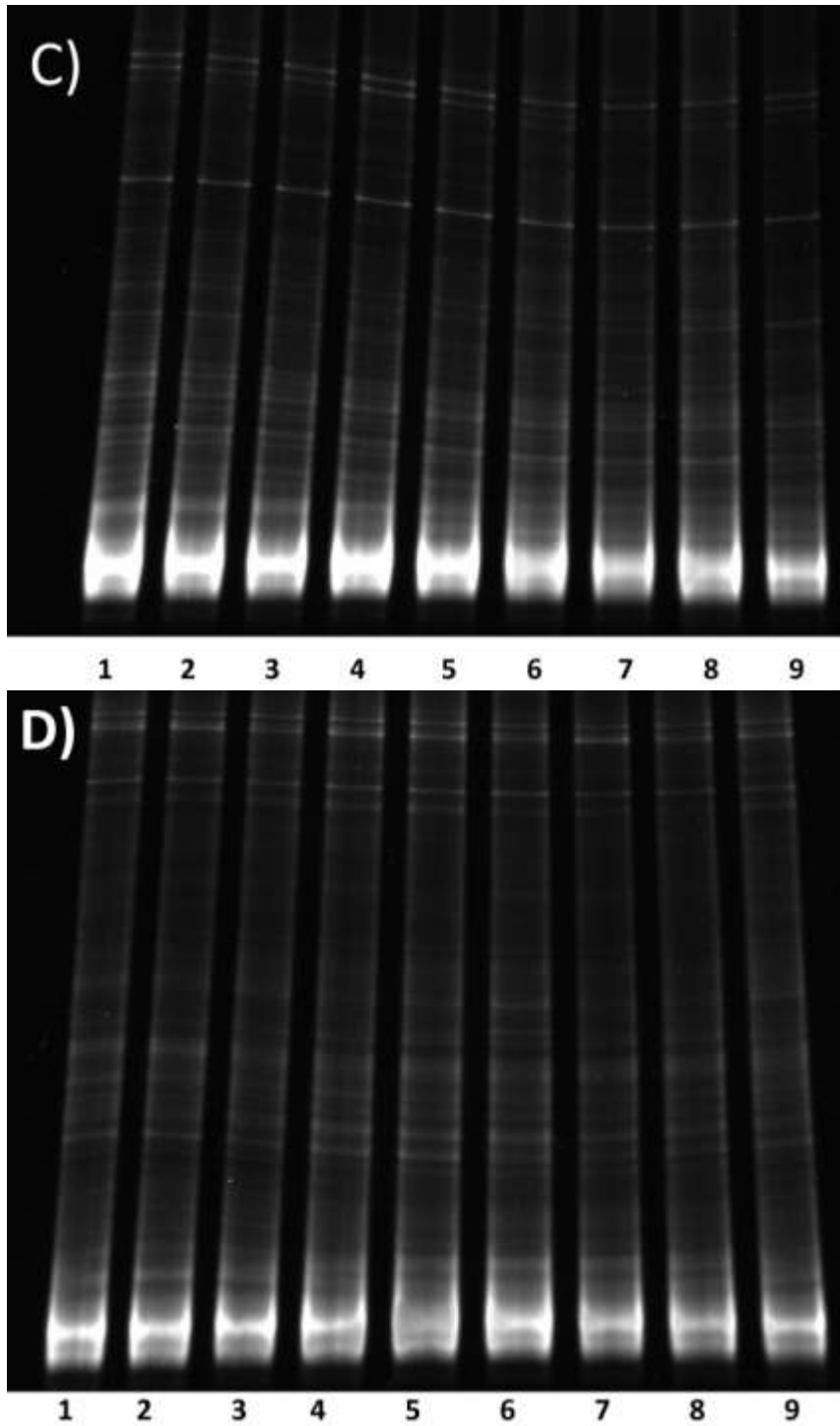
Appendix 1. DGGE images using 16S rDNA primers at D0 (A), D7 (B), D30 (C) and fall (D) after field pharmaceutical addition. Line 1 represents the control, lines 2-5 represent 0.1 mg/kg treatments, and lines 6-9 represent 10 mg/kg treatments. Treatments are: 2 , 6 = ivermectin;, 3, 7 = monensin; 4, 8 = zinc bacitracin; 5, 10 = mixture.

Appendix 2. DGGE image using 16S rDNA primers and soils treated with pharmaceuticals at 100 mg/kg. Images represent soils at D7 (lines 1-5) or D30 (lines 6-10). Treatments are as follows: 1, 6 = control; 2, 7 = ivermectin; 3, 8 = monensin; 4, 9 = zinc bacitracin; 5, 10 = mixture.

Appendix 3. DGGE images obtained using archaeal *amoA* primers at D0 (A), D7 (B), D30 (C) and fall (D) after field pharmaceutical addition. Line 1 represents the control, lines 2-5 represent 0.1 mg/kg treatments, and lines 6-9 represent 10 mg/kg treatments. Treatments are: 2, 6 = ivermectin; 3, 7 = monensin; 4, 8 = zinc bacitracin; 5, 10 = mixture.
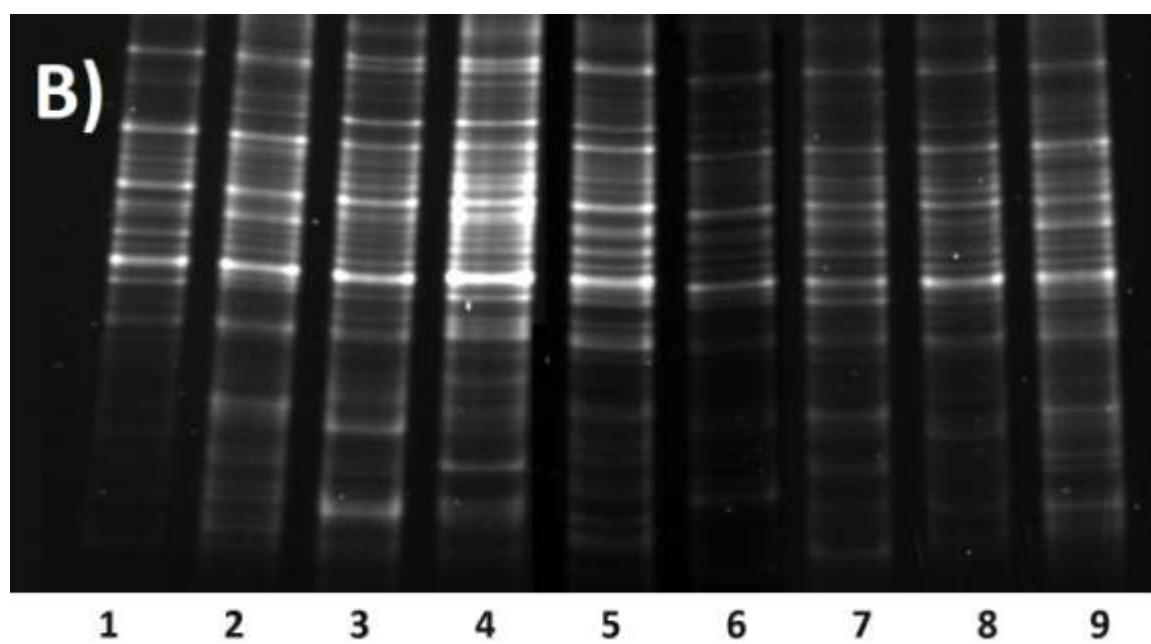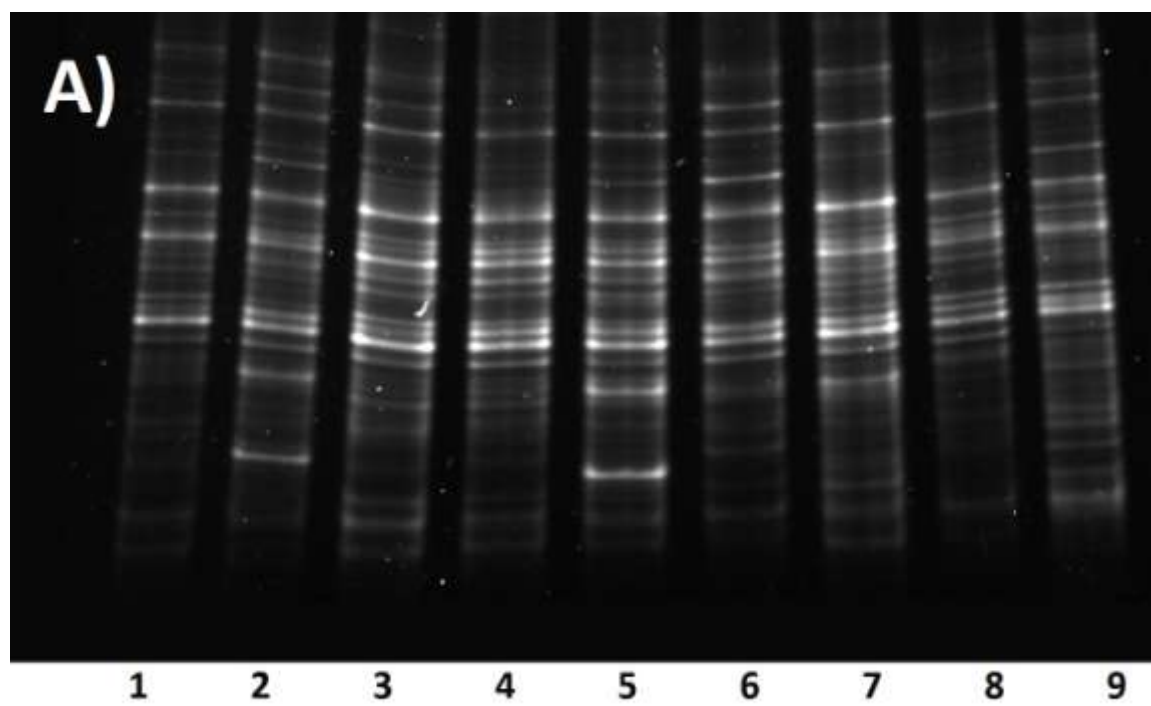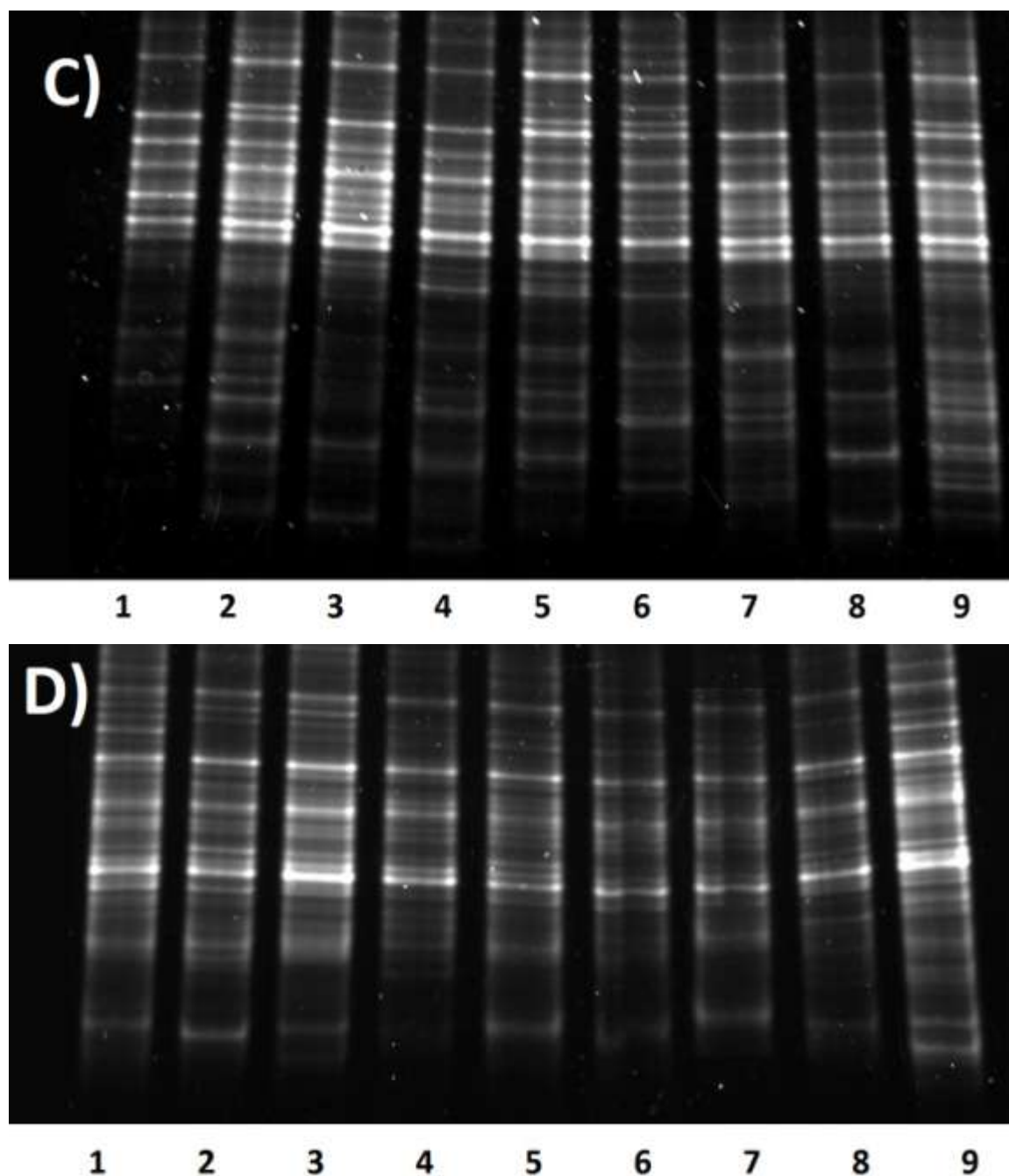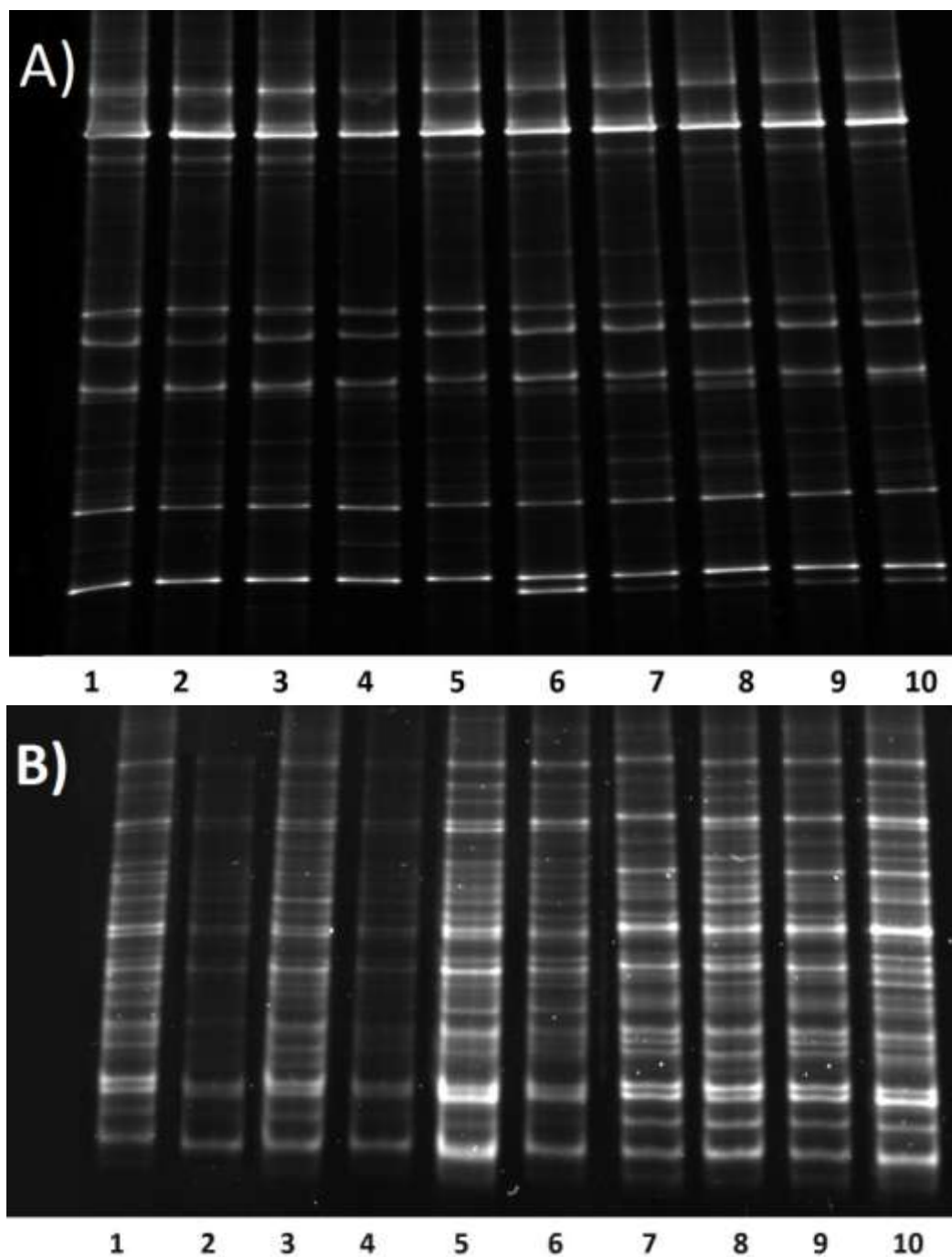
Appendix 4. DGGE images obtained using bacterial *amoA* primers at D0 (A), D7 (B), D30 (C) and fall (D) after field pharmaceutical addition. Line 1 represents the control, lines 2-5 represent 0.1 mg/kg treatments, and lines 6-9 represent 10 mg/kg treatments. Treatments are: 2, 6 = ivermectin; 3, 7 = monensin; 4, 8 = zinc bacitracin; 5, 10 = mixture.

Appendix 5. DGGE image obtained using archaeal (A) and bacterial (B) *amoA*, and soils treated with 100 mg/kg of pharmaceuticals. Lines 1-5 represent D7 soil, and lines 6-10 represent D30 soil. 1, 6 = control, 2, 7 = ivermectin, 3, 8 = monensin, 4, 9 = zinc bacitracin, 5, 10 = mixture.
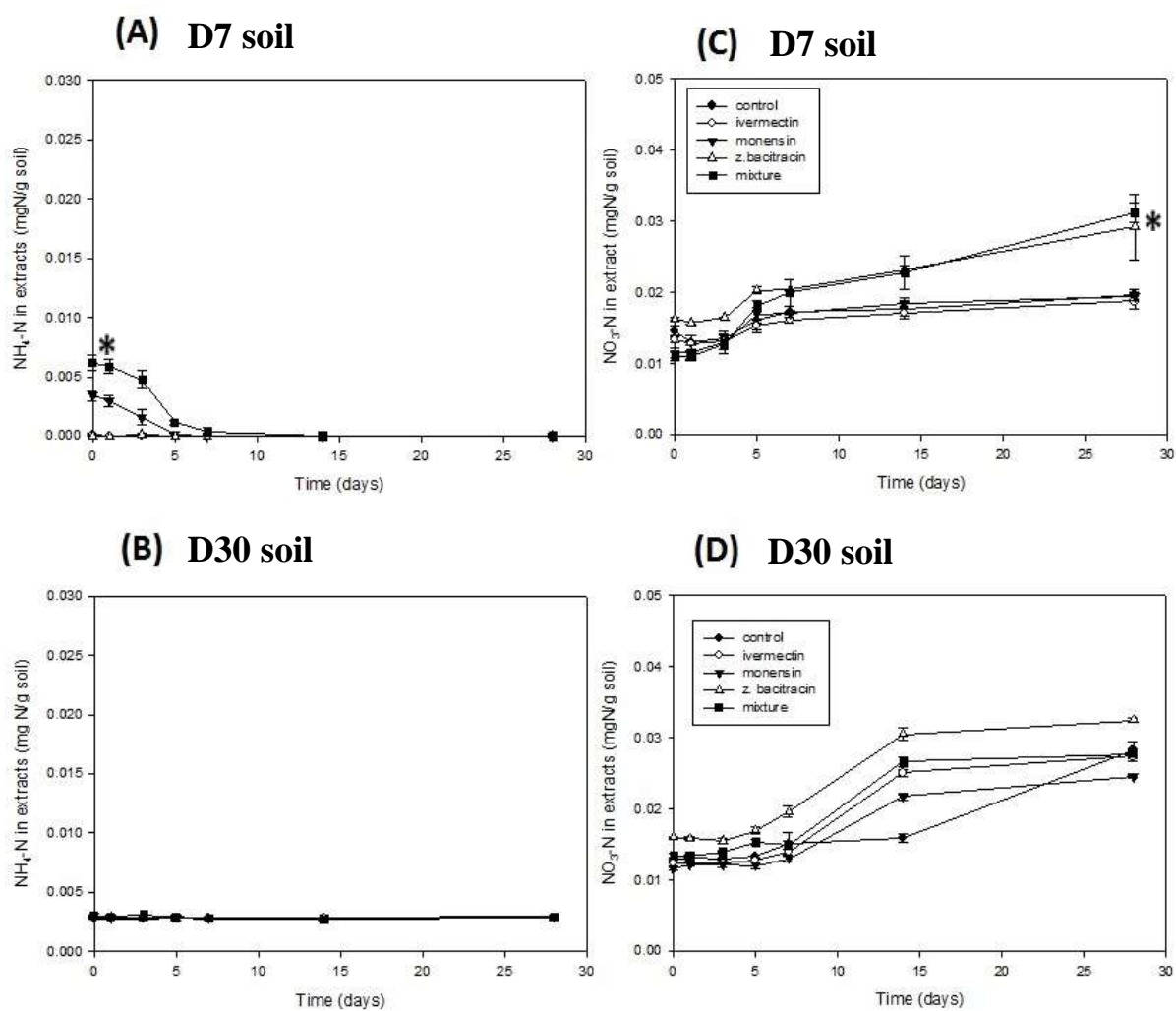
Appendix 6. Nitrification potential experiments with soils treated with 100 mg/kg of pharmaceuticals. Panels A and B show ammonia utilization, and panels C and D show nitrate accumulation.
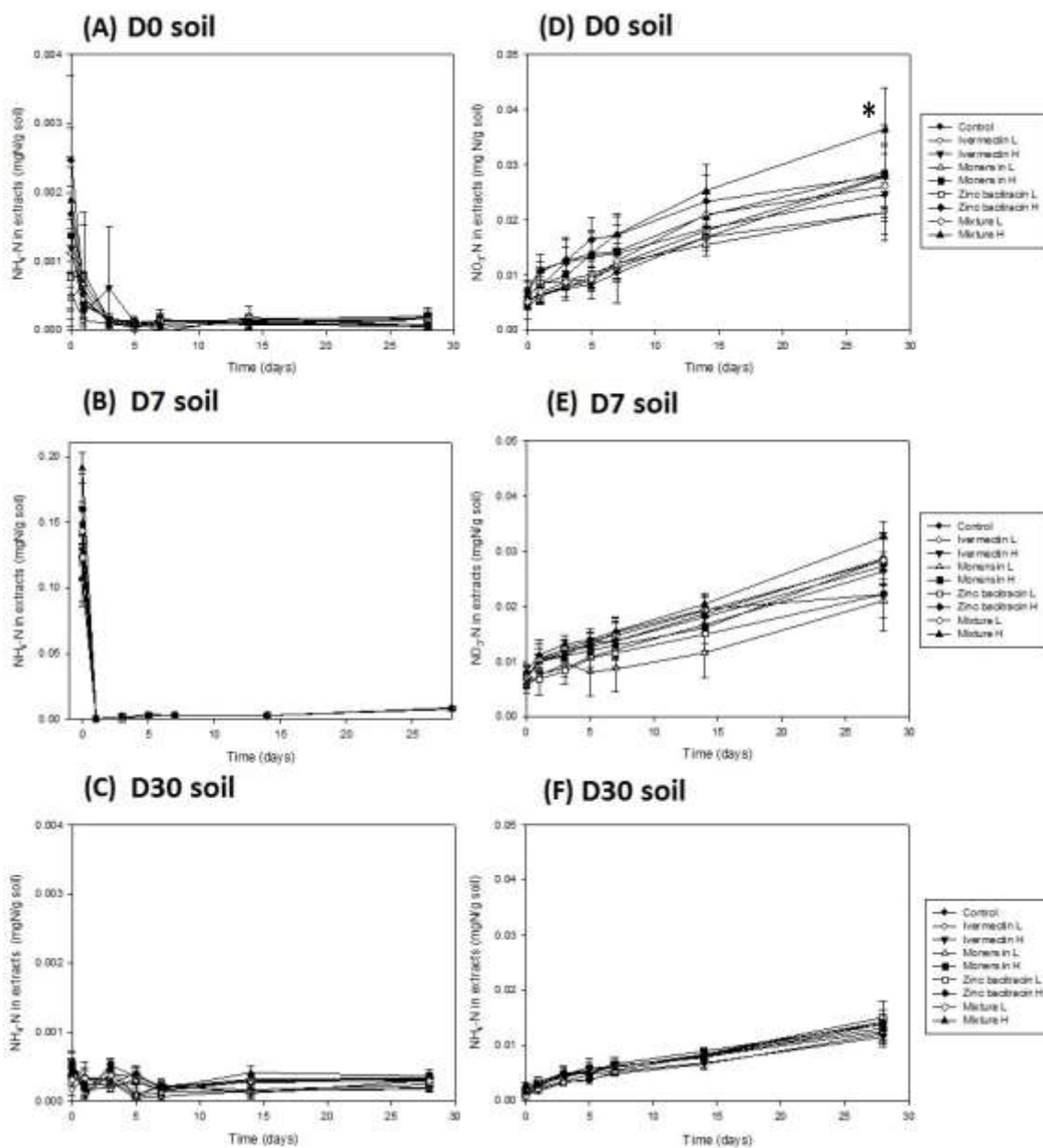
Appendix 7. Nitrification potential experiments in soil treated with 0.1 mg/kg ("L") or 10 mg/kg ("H") of pharmaceuticals. Panels A, B and C represent ammonia utilization, while panels D, E and F represent nitrate accumulation.

Appendix 8. Summary of performed statistics.

			ANOVA				Kruskal-Wallis ANOVA on ranks			
	experiment type	sampling time	F stat	df	P value	Holm-Sidak	H stat	df	p value	Dunn's Method
field	mineralization	fall	1.79	8, 29	0.12	ns				
		D0	1.05	8, 30	0.42	ns				
		D7					11.76	8	0.16	ns
		D30	0.26	8, 29	0.97	ns				
	bait lamina	fall	1.59	8, 31	0.17	ns				
	nitrification	fall					3.52	8	0.90	ns
		D0	3.38	8, 31	0.01	*				
		D7					8.28	8	0.41	ns
		D30					12.09	8	0.15	ns
	Ammonia, initial concentration	fall					15.07	8	0.06	ns
		D0	2.88	8, 29	0.02	ns				
		D7					22.75	8	<0.05	*
		D30					10.48	8	0.23	ns
	archaeal amoA abundance	fall	1.85	8, 31	0.11	ns				
		D0					14.79	8	0.06	ns
		D7	1.14	8, 31	0.37	ns				
		D30	11.10	8, 30	0.02	*				
	bacterial amoA abundance	fall	1.23	8, 30	0.32	ns				
		D0	1.11	8, 28	0.39	ns				
		D7	20.33	8, 30	<0.001	*				
		D30	8.62	8, 30	<0.001	*				
laboratory	mineralization	D7	0.84	4, 10	0.53	ns				
		D30	2.74	4, 10	0.09	ns				
	nitrification	D7	186.92	4, 10	<0.001	*				
		D30					18.90	4	0.03	*
	Ammonia, initial concentration	D7	176.10	4, 10	<0.001	*				
		D30	1.78	4, 10	0.21	ns				
	archaeal amoA abundance	D7	4.71	4, 10	<0.001	*				
		D30	5.62	4, 10	0.01	*				
	bacterial amoA abundance	D7	0.69	4,10	0.62	ns				
		D30	3.56	4, 10	0.05	ns				

Note. Asterisks represent significant differences (P<0.05) of the treatment compared to the control soil; ns means there was no significant differences between treatment and controls.

Curriculum Vitae

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Publications:

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