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Behavior and bioavailability of antibiotics introduced in agricultural soils through the recycling of exogenous organic matter

Anais Goulas

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THESE DE DOCTORAT
DE
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L'INSTITUT DES SCIENCES ET INDUSTRIES DU VIVANT ET DE
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ECOLE DOCTORALE N°581
Agriculture, alimentation, biologie, environnement et santé (ABIÉS)

Spécialité du doctorat : Sciences de l'Environnement

Par

Anaïs GOULAS

Devenir et biodisponibilité des antibiotiques entrant dans les sols agricoles
lors du recyclage des matières fertilisantes d'origine résiduaire

Thèse présentée et soutenue à Paris, le 16 décembre 2016 :

Composition du Jury :

Mme VULLIET Emmanuelle	CR, HDR, CNRS, ISA, Lyon	Rapporteur
Mme CASELLAS Magali	MC, HDR, ENSIL, Limoges	Rapporteur
Mme PONS Marie-Noëlle	DR, CNRS, LRGP, Nancy	Examinatrice
Mr BAVEYE Philippe	Professeur, AgroParisTech, ECOSYS, Grignon	Président du jury
Mme MOREL Marie-Christine	MC, CNAM, Paris, LTHE, Grenoble	Invitée
Mme HAUDIN Claire-Sophie	MC, AgroParisTech, ECOSYS, Grignon	Co-encadrante de thèse
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Table des matières

Introduction générale	- 1 -
Chapitre I : Analyse bibliographique	- 5 -
I.1. Les antibiotiques dans l'environnement	- 5 -
I.1.1. Utilisation des antibiotiques.....	- 5 -
I.1.2. Contexte agronomique et contamination des sols.....	- 6 -
I.1.3. Devenir des antibiotiques dans les sols.....	- 11 -
I.1.4. Effets des antibiotiques sur les micro-organismes du sol.....	- 17 -
I.2. La biodisponibilité	- 22 -
I.2.1. Définition du concept de biodisponibilité.....	- 22 -
I.2.2. Disponibilité environnementale et biodisponibilité des contaminants.....	- 24 -
I.2.3. Méthodes chimiques pour estimer la (bio)disponibilité des antibiotiques.....	- 32 -
I.3. Hypothèses et objectifs	- 33 -
Chapter II: A new extraction method to assess the environmental availability of ciprofloxacin in agricultural soils amended with exogenous organic matter	- 39 -
II.1. Introduction	- 40 -
II.2. Materials and methods	- 42 -
II.2.1. Ciprofloxacin.....	- 42 -
II.2.2. Soil and exogenous organic matter.....	- 43 -
II.2.3. Preselection of centrifuge tube material.....	- 43 -
II.2.4. Spiking of soil/EOM mixture and soft extraction method.....	- 44 -
II.2.5. Incubation experiments.....	- 45 -
II.2.6. Analytical determinations.....	- 46 -
II.2.7. Statistical analyses.....	- 47 -
II.3. Results and Discussion	- 47 -
II.3.1. Selection of the centrifuge tube material.....	- 47 -
II.3.2. Selection of the soft extracting solutions to assess the environmental availability in soil/EOM mixtures.....	- 48 -
II.3.3. Behavior of ¹⁴ C-ciprofloxacin during the incubation of soil/EOM mixtures.....	- 51 -
II.3.4. About soft extraction methodology to target the environmental availability.....	- 58 -
II.4. Conclusion	- 59 -
II. Supplementary material	- 60 -
Chapter III: Assessing the environmental availability of sulfamethoxazole and its acetylated metabolite in agricultural soils amended with compost and manure: an experimental and modelling study	- 65 -
III.1. Introduction	- 66 -

III.2. Materials and methods	- 68 -
III.2.1. Chemicals and reagents	- 68 -
III.2.2. Exogenous organic matter and soils	- 69 -
III.2.3. Incubation experiment	- 69 -
III.2.4. Modelling of the behavior of SMX and AcSMX in EOM-amended soil	- 73 -
III.3. Results	- 78 -
III.3.1. Behavior of ¹⁴ C-SMX and ¹⁴ C-AcSMX in EOM-amended soils.....	- 78 -
III.3.2. Modelling of ¹⁴ C-SMX and ¹⁴ C-AcSMX in EOM-amended soils.....	- 82 -
III.4. Discussion	- 86 -
III.4.1. Effect of the exogenous organic matter	- 86 -
III.4.2. Comparison between SMX and AcSMX.....	- 89 -
III.4.3. Comparison between the soft extracting solutions	- 90 -
III.5. Conclusion	- 92 -
III. Supplementary material	- 94 -
Chapter IV: Investigating the effects of dissolved organic matter on the environmental availability assessment of sulfamethoxazole and transformation products in soils amended with sludge compost or manure by a new soft extraction method	- 107 -
IV.1. Introduction	- 108 -
IV.2. Materials and methods	- 111 -
IV.2.1. Chemicals and reagents	- 111 -
IV.2.2. Soil and exogenous organic matter (EOM)	- 112 -
IV.2.3. Aqueous extraction method	- 112 -
IV.2.4. Analytical conditions for online SPE-UHPLC-MS-MS.....	- 113 -
IV.2.5. Characterization of the method.....	- 115 -
IV.2.6. Effect of freezing storage of soil samples on the aqueous extractability of SMX.....	- 116 -
IV.2.7. Application of the aqueous extraction method to incubated soils	- 116 -
IV.2.8. Fluorescence spectroscopy analysis	- 117 -
IV.2.9. Statistical analyses.....	- 118 -
IV.3. Results and discussion	- 119 -
IV.3.1. Characterization of the analytical performances of the aqueous extracts.....	- 119 -
IV.3.2. Freeze-storage of soil samples and effect on the aqueous extractability of SMX.....	- 122 -
IV.3.3. Aqueous extractability of SMX and AcSMX.....	- 123 -
IV.3.4. Availability of SMX in compost- and manure-amended soil over a three-month incubation.....	- 125 -
IV.3.5. Characterization of co-extracted organic matter.....	- 128 -
IV.4. Conclusion	- 133 -
IV. Supplementary material	- 135 -
Chapter V: Influence of soil properties on the assessment of short-term environmental availability of sulfamethoxazole by soft extraction	- 141 -
V.1. Introduction	- 142 -

V.2. Material and methods	- 143 -
V.2.1. Chemicals	- 143 -
V.2.2. Soils	- 144 -
V.2.3. Soft extraction method.....	- 145 -
V.2.4. Analysis of dissolved organic carbon	- 146 -
V.3. Results and discussion	- 146 -
V.3.1. Availability of SMX in the different soils	- 146 -
V.3.2. SMX availability as a function of soil properties and extraction mechanisms	- 148 -
V.4. Conclusion	- 153 -
V. Supplementary material	- 154 -
Chapter VI: Environmental availability and biodegradation of ciprofloxacin, sulfamethazine and erythromycin in agricultural soils in a context of long-term exposure to antibiotics	- 161 -
VI.1. Introduction	- 162 -
VI.2. Materials and methods	- 164 -
VI.2.1. Chemicals and reagents	- 164 -
VI.2.2. Agricultural soils and manure.....	- 165 -
VI.2.3. Incubation experiment	- 167 -
VI.2.4. Statistical analyses.....	- 170 -
VI.3. Results and discussion	- 170 -
VI.3.1. Selection of the soft extractant from the preliminary study	- 170 -
VI.3.2. Environmental availability of antibiotics in soils long-term exposed to antibiotics.....	- 172 -
VI.3.3. Potential cross-enhancement of antibiotic biodegradation	- 177 -
VI.3.4. Biodegradation of sulfamethazine and erythromycin in manure-amended soils.....	- 177 -
VI.4. Conclusion	- 180 -
Chapter VII: Environmental availability of sulfamethoxazole and ciprofloxacin and effects on soil N-cycle microbial processes in agricultural soils	- 183 -
VII.1. Introduction	- 183 -
Part VII.A. Environmental availability of ¹⁴C-ciprofloxacin and ¹⁴C-sulfamethoxazole in soils amended with exogenous organic matter: impacts of ¹²C-antibiotics on microbial activities during one-month incubation	- 187 -
VII.A.2. Materials and methods	- 187 -
VII.A.2.1. Incubation experiments	- 187 -
VII.A.2.2. Chemical determinations	- 188 -
VII.A.2.3. Microbiological determinations	- 189 -
VII.A.2.4. Statistical analyses.....	- 189 -
VII.A.3. Results	- 190 -
VII.A.3.1. Fate of ¹⁴ C-SMX and ¹⁴ C-CIP in soil/EOM mixtures	- 190 -
VII.A.3.2. Effects of SMX and CIP on potential nitrification activity (PNA)	- 192 -
VII.A.4. Discussion	- 193 -
VII.A.4.1. Effects of SMX and CIP on soil microbial communities	- 193 -

VII.A.4.2. SMX and CIP bioavailability for soil microbial communities	- 194 -
VII.A.5. Conclusion	- 196 -
Part VII.B. Dose-response relationships between extracted SMX fractions and impacts on microbial activity during a three-month incubation of compost-amended soils	- 197 -
VII.B.2. Materials and methods	- 197 -
VII.B.2.1. Incubation experiments.....	- 197 -
VII.B.2.2. Chemical analyses	- 198 -
VII.B.2.3. Microbiological determinations.....	- 199 -
VII.B.2.4. Data analysis and statistics	- 199 -
VII.B.3. Results	- 201 -
VII.B.3.1. SMX quantification by online SPE-UHPLC-MS-MS	- 201 -
VII.B.3.2. Impacts of SMX on potential nitrification activity (PNA) in soil/compost mixtures..	- 201 -
VII.B.4. Discussion	- 203 -
VII.B.4.1. SMX toxicity on potential nitrification activity.....	- 203 -
VII.B.4.2. Selection of the chemical method suitable to assess the SMX bioavailability	- 204 -
VII.5. Conclusion	- 208 -
VII. Supplementary material.....	- 210 -
VIII. Discussion générale et perspectives	- 213 -
Références bibliographiques.....	- 231 -

Table des Figures

Figure I.1. Voies principales de contamination des sols agricoles par les antibiotiques et leurs produits de transformation.....	7 -
Figure I.2. Schéma du devenir des antibiotiques (et des produits de transformation) après un épandage de MAFOR sur les sols agricoles.....	11 -
Figure I.3. Résumé des effets possibles des antibiotiques sur la structure et la fonction des communautés microbiennes du sol	18 -
Figure I.4. Description des processus de biodisponibilité d'un contaminant dans le sol jusqu'à son effet biologique au sein d'un organisme.....	24 -
Figure I.5. Représentation schématique de la disponibilité environnementale et de la biodisponibilité des molécules dans le sol et types de mesures pour les estimer à un temps donné	28 -
Figure I.6. Questions principales abordées au cours de ce travail en lien avec l'estimation de la biodisponibilité des antibiotiques dans les sols amendés par les MAFOR.....	34 -
Figure II.1. Partitioning of ¹⁴ C-ciprofloxacin residues among the different fractions (% initial ¹⁴ C-activity) in function of the incubation time (d) of A] soil/compost and B] soil/manure mixtures (n = 3).....	52 -
Figure II.2. Key interaction sites of ciprofloxacin adsorption on EOM and soil in function of the pH and the chemical ionic form.....	54 -
Figure II.3. Partitioning of the ¹⁴ C-activity (% maximum extractable, n = 3) in fractions obtained with the solvent-based extraction, depending on the pH of the acetonitrile/buffer mixture for A] soil/compost and B] soil/manure mixtures.....	57 -
Figure II.S1. Cumulative mineralization of the organic matter (% of the carbon initially added by the EOM, n = 3) in soil amended with manure or compost, with or without ¹⁴ C-ciprofloxacin.....	60 -
Figure II.S2. Color of Borax, Na ₂ EDTA and 2-HP-b-CD extracts. The color depended on the amount of co-extracted organic matter: Borax < 2-HP-b-CD <<< Na ₂ EDTA.....	60 -
Figure III.1. General scheme of the COP-Soil model coupling the organic carbon module with the organic pollutant module.....	72 -
Figure III.2. Repartition of the ¹⁴ C-activity (% initial ¹⁴ C-activity, n = 3) in the different fractions for ¹⁴ C-AcSMX and ¹⁴ C-SMX in function of the incubation time (d) in A] soil/compost mixtures and in B] soil/manure mixtures.....	80 -
Figure III.3. Soil/compost mixtures: Simulations of ¹⁴ C-SMX concentrations in available (OP _{AV}), weakly adsorbed (OP _{WS} + OP _{EOM}), non-extractable (OP _{Ss} + OP _{Bs}) and mineralized (OP-CO ₂) fractions.....	84 -
Figure III.4. Comparison of simulations between soil compost/mixtures and soil/manure mixtures for OC mineralization and ¹⁴ C-SMX concentrations in available (OP _{AV}), weakly adsorbed (OP _{WS} + OP _{EOM}), non-extractable (OP _{Ss} + OP _{Bs}) and mineralized (OP-CO ₂) fractions (CaCl ₂ modality).....	88 -
Figure III.S1. Available and non-extractable fractions (% initial ¹⁴ C-activity) of ¹⁴ C-AcSMX (n = 3, missing data for non-extractable fractions, not measured) in soil/EOM mixtures	96 -
Figure III.S2. Partitioning of the ¹⁴ C-activity (% solvent-extractable; average, n = 3) in hydro-organic phase (pH 9), organic phase (pH 2) and aqueous phase (pH 2), for ¹⁴ C-AcSMX or ¹⁴ C-SMX in soil/compost and soil/manure mixtures. CaCl ₂ , EDTA and HPCD referred to the soft extractant used before the solvent-based extraction.....	100 -
Figure III.S3. Cumulative mineralization of organic carbon (OC) of compost or manure in soil during the incubation experiments (1) with ¹⁴ C-AcSMX and (2) with ¹⁴ C-SMX.....	101 -
Figure III.S4. Soil/compost mixtures: Simulations of ¹⁴ C-AcSMX concentrations in available (OP _{AV}), weakly adsorbed (OP _{WS} + OP _{EOM}), non-extractable (OP _{Ss} + OP _{Bs}) and mineralized (OP-CO ₂) fractions.....	101 -
Figure III.S5. Soil/manure mixtures: Simulations of ¹⁴ C-SMX concentrations in available (OP _{AV}), weakly adsorbed (OP _{WS} + OP _{EOM}), non-extractable (OP _{Ss} + OP _{Bs}) and mineralized (OP-CO ₂) fractions.....	102 -

Figure III.S6. Soil/manure mixtures: Simulations of ^{14}C -AcSMX concentrations in available (OP_{AV}), weakly adsorbed ($\text{OP}_{\text{WS}} + \text{OP}_{\text{EOM}}$), non-extractable ($\text{OP}_{\text{Ss}} + \text{OP}_{\text{Bs}}$) and mineralized (OP-CO_2) fractions.....	- 103 -
Figure IV.1. 3D-fluorescence spectra.....	- 118 -
Figure IV.2. Matrix effects (% , n = 2) for the compounds in A] CaCl_2 extracts and B] EDTA extracts without modification (pH 7) or with modification (pH 2-3 and 9) of the extract pH.....	- 120 -
Figure IV.3. QuEChERS recoveries (%) for the compounds in HPCD extracts (n = 3), with and without modification of pH.	- 121 -
Figure IV.4. Concentrations of available SMX ($\mu\text{g kg}^{-1}$ of dry mass) quantified in CaCl_2 , EDTA and HPCD extracts after the freezing of soil/manure samples in function of the concentrations quantified in fresh soil/manure samples (n = 27).	- 123 -
Figure IV.5. Available fractions (%) of SMX (n = 4) and AcSMX (n = 3) in soil/manure mixtures in function of the initial concentration and the aqueous extractant. A 30-60 min contact was let between the molecule and manure before the incorporation of manure into soil and the extraction. The lowest concentration level (x1) corresponded to 22 $\mu\text{g SMX}$ or AcSMX kg^{-1} dry soil and the highest (x60) to 1.32 mg SMX or AcSMX kg^{-1} dry soil.	- 125 -
Figure IV.6. Available fractions of SMX (% initial concentration, n = 4) in A] soil/compost mixtures and B] soil/manure samples, extracted with CaCl_2 , EDTA and HPCD solutions over the incubation time.	- 126 -
Figure IV.7. 3D-fluorescence spectra obtained for CaCl_2 , EDTA and HPCD extracts diluted to 1/100 or 1/50 from A] soil/compost mixtures and B] soil/manure mixtures, at day 0, for $[\text{SMX}] \times 3$	- 130 -
Figure IV.8. Principal component analysis (PCA): SMX concentration measured at day 0 in CaCl_2 , EDTA and HPCD extracts in function of the fluorescence zones (Z1 to Z7) and the complexity index, in both soil/compost and soil/manure mixtures, initially spiked with the concentration $[\text{SMX}] \times 3$	- 130 -
Figure IV.9. 3D-fluorescence spectra obtained for CaCl_2 extracts diluted to 1/50, 1/20 or 1/10 from A] soil/compost mixtures and B] soil/manure mixtures, after 0, 28 and 84 days of incubation for $[\text{SMX}] \times 3$	- 132 -
Figure IV.10. Evolution of the complexity index in CaCl_2 extracts from soil/compost and soil/manure mixtures over the incubation time, for $[\text{SMX}] \times 3$ and $[\text{SMX}] \times 100$ concentration levels.....	- 132 -
Figure IV.S1. Fluorescence (%) measured in the different zones in CaCl_2 , EDTA and HPCD extracts of A] soil/compost mixtures and B] soil/manure mixtures at day 0.....	- 135 -
Figure IV.S2. Fluorescence (%) measured in the different zones in CaCl_2 extracts of A] soil/compost mixtures and B] soil/manure mixtures after day 0, 28 and 84 days of incubation.	- 136 -
Figure IV.S3. Principal component analysis (PCA): SMX concentration measured over the incubation time (0, 28 and 84 days) in CaCl_2 extracts in function of the fluorescence zones (Z1 to Z7) and the complexity index, in A] soil/compost mixtures and B] soil/manure mixtures, initially spiked with the lowest SMX concentration..	- 137 -
Figure V.1. SMX availability in soils from Epoisses (France), Feucherolles (France) and London (Canada), in function of the initial SMX concentration and the aqueous extractant used (n = 3).	- 147 -
Figure V.2. Speciation of sulfamethoxazole (SMX) according to the pH value	- 148 -
Figure V.3. Principal component analysis (PCA) of available fractions of SMX (%) in function of selected soil properties: % sand/silt/clay, CEC, pH and % organic carbon (OC) in soils from Feucherolles, London and Epoisses.	- 152 -
Figure V.S1. Graphical representation of SMX, AcSMX, SMX-D4 surface areas in filtered and unfiltered extracts.....	- 154 -
Figure VI.1. Speciation diagrams of antibiotics A) sulfamethazine (SMZ), B) ciprofloxacin (CIP) and C) erythromycin (ERY) as a function of pH..	- 166 -

Figure VI.2. Fractions of ¹⁴ C-antibiotics (% initial ¹⁴ C-activity) extracted from soils with aqueous solutions of Borax or EDTA, after 30-60 min of contact (n = 3).....	- 172 -
Figure VI.3. Distribution of the ¹⁴ C-activity in fractions (% total recovered) in soils spiked with ¹⁴ C-antibiotics. Soil samples were taken from plots receiving 0 (control) or 10 mg antibiotics per kg of dry soil... ..	- 173 -
Figure VI.4. Distribution of ¹⁴ C-activity (% total recovered, n = 2) in fractions for A) ¹⁴ C-SMZ and B) ¹⁴ C-ERY in soils. Soils were taken from plots receiving 0 (control), 10 mg kg ⁻¹ of mixture of sulfamethazine, tylosin and chlortetracycline (STC) or mixture of macrolides (MC).	- 176 -
Figure VI.5. Distribution of ¹⁴ C-activity (% total recovered) in fractions for A) ¹⁴ C-SMZ and B) ¹⁴ C-ERY in soil supplemented with the antibiotic directly, or added via supplemented manure. Soils were taken from plots receiving 0 (control) and 10 mg antibiotics kg ⁻¹ soil.	- 178 -
Figure VII.1. Available fractions of ¹⁴ C-SMX (44 µg kg ⁻¹ dry soil) and ¹⁴ C-CIP (146 µg kg ⁻¹ dry soil) in soil/compost (solid line) and soil/manure mixtures (dashed line) assessed by CaCl ₂ -, EDTA-, Borax- or HPCD-based extraction.....	- 191 -
Figure VII.2. Response of potential nitrification activities (PNA) in microcosms containing: a) soil/compost mixtures; or b) soil/manure mixtures, contaminated by SMX and CIP, after 7 and 28 days of incubation.....	- 193 -
Figure VII.3. Concentrations of available and solvent-extractable SMX (µg kg ⁻¹ dry soil, n = 4; log ₁₀ scale) in soil/compost mixtures in function of the initial SMX concentrations and the incubation time.	- 200 -
Figure VII.4. Effect of SMX on the potential nitrification activity (PNA) in function of the theoretical initial SMX concentrations, in the microcosms containing soil/compost mixtures, at days 8, 28 and 84.....	- 202 -
Figure VII.5. Dose-response curves of PNA inhibition (% control) in function of SMX concentration (in µg kg ⁻¹ dry soil; in A, B, C) or SMX fraction (% the initial theoretical SMX concentration; in D)..	- 205 -
Figure VII.6. Linear regression between the inhibition of PNA (% control; y axis) and fractions of SMX (% initial concentration; x axis).	- 207 -
Figure VIII.1. Structure chimique des sulfonamides, types d'interactions impliquées dans l'adsorption, mécanismes d'extraction des solutions aqueuses.	- 216 -
Figure VIII.2. Structure chimique des fluoroquinolones, types d'interactions impliquées dans l'adsorption, mécanismes d'extraction des solutions aqueuses.	- 217 -

Table des Tableaux

Tableau I.1. Familles d'antibiotiques et exemples de molécules : structures, propriétés physicochimiques des molécules et paramètres pouvant caractériser leur adsorption dans le sol.....	8 -
Tableau I.2. Concentrations des antibiotiques dans les MAFOR et les sols amendés, en μg ou mg par kg de matière sèche (MS).....	10 -
Tableau I.3. Principaux types d'interactions impliqués dans les mécanismes d'adsorption des antibiotiques sur les surfaces adsorbantes du sol	15 -
Table II.1. Losses of ^{14}C -ciprofloxacin (% initial ^{14}C -activity, average \pm standard deviation) by adsorption on tube after the shaking in A) Milli-Q water ($39 \mu\text{g L}^{-1}$) in different materials ($n = 3$); B) the extracting solutions ($46 \mu\text{g L}^{-1}$) in Flacon [®] tubes ($n = 2$).....	48 -
Table II.2. Mechanisms, targeted fractions and example of compounds extracted by the pre-selected soft extractants (Reid et al., 2000a; Harmsen, 2007; Ortega-Calvo et al., 2015).....	50 -
Table II.3. Available and non-extractable fractions (% initial ^{14}C -activity) of ^{14}C -ciprofloxacin ($n = 3$, mean \pm standard deviation) in soil/EOM mixtures (24 h-contact, $\sim 20^\circ\text{C}$).....	50 -
Table II.S1. Characteristics and compositions of the compost of sewage sludge/green waste and the bovine manure ($n = 3$, average \pm standard deviation).....	61 -
Table II.S2. Aqueous solutions tested for the extraction of the available fraction of ^{14}C -ciprofloxacin....	62 -
Table III.1. Physicochemical properties of sulfamethoxazole (SMX) and N-acetylsulfamethoxazole (AcSMX), with *: radiolabeling on the molecule structures.....	68 -
Table III.2. Variables (A) and parameters (B) presented in the COP-Soil model.....	74 -
Table III.3. Parameter values (in d^{-1}) optimized in the simulations using the COP-Soil model for AcSMX and SMX in soil/compost mixtures.....	83 -
Table III.S1. Characteristics and compositions of the compost of sewage sludge/green waste and the bovine manure ($n = 3$, average \pm standard deviation).....	94 -
Table III.S2. Counted ^{14}C -activity (% initial ^{14}C -activity, average \pm standard deviation) obtained after the shaking of ^{14}C -acetylsulfamethoxazole in the extracting solution ($6.8 \mu\text{g L}^{-1}$, $n = 2$).....	95 -
Table III.S3. Parameter values of the organic matter module for compost and manure.....	97 -
Table III.S4. Statistical evaluation of model efficiency in the simulations of the carbon mineralization (5 observations) using the OC module alone.....	98 -
Table III.S5. Statistical evaluation of model efficiency in the scenarios retained using the COP-Soil model for A) ^{14}C -AcSMX and B) ^{14}C -SMX in soil/compost mixtures.....	98 -
Table III.S6. Statistical evaluation of model efficiency in the simulations obtained using the COP-Soil model for A) ^{14}C -AcSMX and B) ^{14}C -SMX in soil/manure mixtures.....	99 -
Table IV.1. Chemical structures and physicochemical properties of sulfamethoxazole and the three selected transformation products.....	112 -
Table IV.2. MS-MS parameters of the different molecules (electrospray ionization in positive mode): cone voltage, quantification and confirmation transitions with respective collision energies.....	114 -
Table IV.3. Limits of detection and quantification (LOD / LOQ, $\mu\text{g kg}^{-1}$ dry soil) of ANI, 3A5MI, SMX and AcSMX extracted by CaCl_2 , EDTA or HPCD solutions.....	122 -
Table V.1. Key properties of the three different soils.....	145 -
Table V.S1. Significant results of correlation tests (Pearson) between SMX availability and soil properties selected from the principal component analysis.....	155 -

Table VI.1. Structures and physicochemical properties of antibiotics. The asterisk on the molecule structures indicates the location of ^{14}C atoms.....- 165 -

Table VII.S1. Distribution of ^{14}C -activity (% initial ^{14}C -activity) during the incubation of soil/compost and soil/manure mixtures with ^{14}C -SMX or ^{14}C -CIP (n=3), firstly extracted by aqueous solutions (EDTA, HPCD, CaCl_2 or Borax). - 210 -

Introduction générale

Les déchets organiques tels que les boues de station d'épuration générées au cours du traitement des eaux usées et les effluents d'élevage sont riches en matière organique et en éléments nutritifs. Ces matières fertilisantes d'origine résiduaire (MAFOR) sont valorisées en agriculture pour améliorer la fertilité des sols en favorisant par exemple, la disponibilité des nutriments, la structure du sol et les activités biologiques (García-Gil et al., 2000 ; Diacono et Montemurro, 2010 ; Annabi et al., 2011). En France, environ 70% des boues de station d'épuration sont recyclées en agriculture (Kelessidis et Stasinakis, 2012) et les effluents d'élevage sont totalement valorisés. Toutefois, les MAFOR contiennent aussi des contaminants minéraux et organiques, qui sont par conséquence introduits dans les sols au moment de l'épandage (Houot et al., 2016). Les produits pharmaceutiques et plus particulièrement les antibiotiques, retrouvés dans les boues et les effluents d'élevage, suscitent un intérêt particulier (Song et Guo, 2014 ; Verlicchi et Zambello, 2015). En effet, des apports répétés d'antibiotiques, à des doses plus ou moins fortes, peuvent : i) contribuer à l'émergence et à la dissémination de bactéries résistantes et de gènes de résistance aux antibiotiques, ce qui représente un risque pour la santé humaine et animale sur le long terme (Wright, 2010) ; ii) affecter les fonctions microbiennes dans les sols et les services écosystémiques associés (Rapport d'expertise collective MAFOR, 2014).

Les teneurs des principales familles d'antibiotiques présentes dans les MAFOR (sulfonamides, fluoroquinolones, tétracyclines, macrolides) sont très variables (Rapport d'expertise collective MAFOR, 2014), avec des concentrations en antibiotiques s'échelonnant de 0,01 jusque 872 mg kg⁻¹ matière sèche dans les effluents d'élevage (Song et Guo, 2014) et de 0,1 µg kg⁻¹ jusque 4,8 mg kg⁻¹ matière sèche dans les boues de station d'épuration (Zhang et Li, 2011). Les teneurs en antibiotiques dépendent de nombreux paramètres, tels que le niveau de prescription, les taux de métabolisation et d'excrétion, l'affinité des molécules pour les MAFOR, mais aussi des traitements opérés sur ces MAFOR. Par exemple, le compostage, très utilisé en Europe, est un procédé aérobie de stabilisation par dégradation des fractions labiles et humification de la matière organique résiduelle. Il peut contribuer à la dissipation des contaminants organiques suivant deux processus : i) l'accélération de la transformation (biotique, abiotique) des contaminants grâce à des activités microbiennes et sous l'effet de la température ; ii) l'adsorption et la formation de résidus liés plus ou moins stabilisés, en relation avec la transformation de la matière organique (Kelessidis et Stasinakis, 2012 ; Martín et al., 2015). La part relative de ces deux processus reste mal connue et très variable pour les antibiotiques (Kim et al., 2012). En réalité, la dissipation des antibiotiques,

c'est-à-dire la disparition des molécules mesurée à partir d'extractions chimiques qui se veulent exhaustives, varie d'un composé à l'autre mais aussi pour un même composé, d'un type de MAFOR à un autre, en fonction de l'origine du procédé. Par exemple, le compostage de fumiers a pu conduire à une réduction de plus de 95% de la teneur en tétracyclines extractibles et de 54 à 76% de la teneur en tylosine extractible (Arikan et al., 2007 ; Dolliver et al., 2008 ; Arikan et al., 2009). Cependant, la dégradation de la sulfaméthazine n'a pas été observée lors du même traitement (Dolliver et al., 2008), ni celle de la sulfadiazine et la difloxacin (Lamshöft et al., 2010). Les prétraitements (digestion anaérobie) et le procédé du compostage des boues semblent influencer sur le taux de dégradation des fluoroquinolones et des sulfonamides (Lillenberg et al., 2010). De façon générale, ces processus de dégradation et d'adsorption déterminent les formes chimiques des résidus et la nature des liaisons engagées avec les constituants organiques et minéraux des MAFOR. Ces processus jouent donc un rôle déterminant dans le devenir des antibiotiques dans les sols agricoles après épandage.

Le devenir des antibiotiques dans les sols est principalement lié à leur dégradation (processus abiotiques et/ou microbiens) et à leur rétention, en fonction des conditions pédoclimatiques, des activités biologiques des sols et des propriétés physico-chimiques des antibiotiques. En particulier, la capacité des antibiotiques à s'adsorber plus ou moins fortement dépend de mécanismes d'adsorption qui peuvent différer d'une famille chimique à une autre (Tolls, 2001 ; Figueroa-Diva et al., 2010). Par exemple, l'adsorption des sulfonamides sur le sol est plus faible que celle des tétracyclines ou encore des fluoroquinolones. Par conséquent, la persistance et la biodégradation des antibiotiques dans les sols, influencées par leur adsorption (Kemper, 2008) et leur possible désorption, sont variables d'une famille d'antibiotiques à une autre. De la même façon, l'exposition des micro-organismes du sol aux antibiotiques va dépendre des processus d'adsorption et de désorption des molécules qui régissent en grande partie la biodisponibilité des antibiotiques vis-à-vis des micro-organismes. Seuls les antibiotiques biodisponibles pourront être dégradés par les micro-organismes et/ou pourront impacter ces derniers (Harmsen, 2007). De plus, la sensibilité des communautés microbiennes exposées (fonctions, activités) peut dépendre de la fréquence des apports, des propriétés des MAFOR et des teneurs dans les sols (Rapport d'expertise collective MAFOR, 2014).

Les sites expérimentaux de longue durée sont particulièrement adaptés pour suivre le devenir et la biodisponibilité des antibiotiques dans les sols amendés par les MAFOR, en fonction des conditions pédoclimatiques, de l'historique des pratiques culturales et de la fréquence des épandages. De plus, les facteurs biologiques sont également suivis, ce qui permet d'évaluer la

résilience des sols ou non par rapport à l'introduction répétée d'antibiotiques (Topp et al., 2013 ; Rapport d'expertise collective MAFOR, 2014).

L'étude du devenir et de la biodisponibilité des antibiotiques dans les sols amendés par les MAFOR repose essentiellement sur la possibilité d'extraire et de quantifier les antibiotiques dans les matrices environnementales complexes telles que les sols et les MAFOR. De plus, compte tenu des teneurs en antibiotiques dans les sols (de l'ordre du μg par kg de sol sec ; DeVries et Zhang, 2016), la quantification des molécules dans des extraits aqueux représente un défi analytique. L'utilisation successive de solutions d'extraction aqueuse dite « douce » vers une extraction plus exhaustive permet d'extraire les molécules dans le sol avec une efficacité croissante. Ces extractions séquentielles peuvent être utilisées pour cibler des fractions d'antibiotiques de plus en plus retenues, et donc de moins en moins disponibles dans les matrices solides (Förster et al., 2009).

Dans le cadre du projet ANR-CESA CEMABS (2013-2017, piloté par Sylvie Nazaret, LEM, Lyon), les impacts sur les processus microbiens incluant la dynamique de l'antibiorésistance sont étudiés dans un contexte d'exposition chronique aux antibiotiques et aux métaux dans les sols. Cette thèse s'inscrit dans ce projet, et l'objectif majeur est de développer une méthode chimique par extraction douce afin d'estimer la biodisponibilité des antibiotiques dans les sols agricoles amendés par des MAFOR. Pour la majorité des travaux de thèse, deux antibiotiques ont été sélectionnés : la ciprofloxacine (fluoroquinolone) et le sulfaméthoxazole (sulfonamide). Ces deux antibiotiques sont fréquemment retrouvés dans les MAFOR (Chapitre I, Tableau I.2) et présentent des propriétés physico-chimiques différentes responsables de différents mécanismes d'adsorption sur les sols (Chapitre I, Tableau I.1). Sur le site QualiAgro à Feucherolles (Yvelines, France), des MAFOR telles qu'un fumier de ferme (élevage bovin laitier) et une boue compostée avec des déchets verts sont apportées tous les deux ans depuis 1998. La caractérisation physico-chimique des MAFOR appliquées est réalisée (par exemple, pH, matière organique, teneurs en contaminants). Les deux antibiotiques étudiés n'ont été détectés ni dans le fumier, ni dans le sol de l'essai QualiAgro. En revanche, la concentration en ciprofloxacine était de $309 \mu\text{g kg}^{-1}$ de matière sèche dans le compost de boue et de $8.7 \mu\text{g kg}^{-1}$ dans le sol un mois après l'épandage du compost en 2013 ; le sulfaméthoxazole n'a pas été détecté dans le compost de boue et la concentration dans le sol était inférieure à la limite de quantification (Ferhri et al., 2016). Les effets des apports répétés de MAFOR sont évalués sur les différents compartiments de l'agrosystème (sol, plante, eau et air), notamment l'évolution des propriétés physico-chimiques des sols comme par exemple les teneurs en matière organique et en nutriments, et des indicateurs d'activité microbienne et d'abondance de bactéries et de gènes de résistance aux antibiotiques (Obriot et al., 2016).

Ce manuscrit est constitué de sept chapitres, les chapitres II à VII étant présentés sous forme d'articles. Le chapitre I présente tout d'abord une analyse bibliographique de l'état des connaissances sur, d'une part, la présence des antibiotiques dans les sols agricoles, et d'autre part, la biodisponibilité des contaminants et les méthodes existantes pour l'évaluer. A la fin de ce chapitre, les hypothèses et les objectifs du travail de recherche sont soulignés au regard des limites des connaissances sur l'estimation de la biodisponibilité des antibiotiques dans les sols amendés par des MAFOR.

Les chapitres II et III présentent les résultats expérimentaux de l'estimation de la disponibilité d'antibiotiques radiomarqués au carbone 14 apportés aux sols via des MAFOR dans des incubations en microcosmes. Le chapitre II est consacré à la ciprofloxacine tandis que le chapitre III porte sur le sulfaméthoxazole et son métabolite acétylé. Les résultats expérimentaux obtenus dans le chapitre III ont été utilisés par la suite pour simuler l'évolution de la disponibilité environnementale des deux molécules à l'aide d'un modèle couplant l'évolution de la matière organique de la MAFOR à l'évolution de fractions caractérisant le devenir d'un polluant organique.

Le chapitre IV présente le développement de la méthode d'extraction et d'analyse par extraction sur phase solide en ligne de la chromatographie liquide ultrahaute pression couplée à la détection par la spectrométrie de masse en tandem (online SPE-UHPLC-MS-MS) pour évaluer la disponibilité environnementale du sulfaméthoxazole et trois de ses produits de transformations dans les sols amendés par les MAFOR. Au cours d'une incubation de trois mois, la méthode a ensuite été appliquée et la matière organique co-extraite par les solutions aqueuses a été caractérisée par la technique de fluorimétrie en trois dimensions (en collaboration avec le Laboratoire de Biotechnologie de l'Environnement, INRA, Narbonne). Le chapitre V est consacré à l'application de la méthode d'estimation de la disponibilité environnementale du sulfaméthoxazole dans des sols d'origines et de propriétés physicochimiques différentes.

Enfin, pour déterminer si les méthodes d'extraction douce testées au cours de ce travail de recherche répondent à l'objectif d'évaluer la biodisponibilité des antibiotiques, le chapitre VI présente les résultats de l'estimation de la disponibilité environnementale d'antibiotiques de différentes familles au regard de leur biodégradation dans les sols agricoles dans un contexte d'exposition de longue durée (en collaboration avec Agriculture and AgriFood, Canada). Le chapitre VII présente les effets de la ciprofloxacine et du sulfaméthoxazole sur les activités microbiennes dans les sols amendés par les MAFOR au regard de leurs fractions disponibles.

Une synthèse générale reprend les différentes conclusions de ce travail de recherche pour tenter de discuter de leur caractère plus ou moins générique et propose différentes perspectives à la fin de ce manuscrit.

Chapitre I : Analyse bibliographique

I.1. Les antibiotiques dans l'environnement

I.1.1. Utilisation des antibiotiques

Les antibiotiques sont des produits pharmaceutiques actifs destinés à éliminer (effet bactéricide) ou réduire (effet bactériostatique) le développement de bactéries pathogènes. Plusieurs familles d'antibiotiques sont décrites en fonction de la structure chimique commune qui leur confère un mécanisme d'action antibactérien propre. Par exemple, les β -lactames, les sulfonamides, les fluoroquinolones, les macrolides et les tétracyclines sont des familles d'antibiotiques distinctes. Au sein de chaque famille existent de nombreuses molécules aux propriétés physicochimiques très proches (exemples dans le Tableau I.1).

Les antibiotiques sont utilisés en traitement curatif ou en prophylaxie en médecine humaine et vétérinaire. En France, la famille la plus prescrite en médecine humaine est celle des β -lactames (pénicillines) puis celles des macrolides ou des fluoroquinolones selon le lieu de prescription (hôpital ou en ville ; Rapport ANSM, 2014). En médecine vétérinaire, les tétracyclines sont la première famille d'antibiotiques prescrite suivie par celle des sulfonamides (Rapport ANSES, 2015). Les antibiotiques sont donnés aux animaux d'élevage en traitement mais aussi en métaphylaxie pour prévenir l'apparition de maladie bactérienne dans l'ensemble du troupeau suite à l'apparition d'un cas de maladie. De plus, la mise en stabulation des animaux peut conduire à une utilisation systématique des antibiotiques en prophylaxie. Ces produits pharmaceutiques sont également utilisés en aquaculture mais ce contexte ne sera pas développé dans ce travail. Certains antibiotiques sont utilisés en tant que promoteurs de croissance et sont mélangés dans l'alimentation des animaux d'élevage (Sarmah et al., 2006) ; cette utilisation est interdite en Europe depuis 2006. En France, certains antibiotiques utilisés en traitement préventif en médecine vétérinaire ont été classés comme substances d'importance critique et leur utilisation est restreinte voire interdite depuis avril 2016. Ces antibiotiques critiques sont les molécules particulièrement importantes en médecine (céphalosporines de 3^e et 4^e générations, fluoroquinolones) car seules alternatives dans le traitement de certaines maladies infectieuses chez l'Homme (décret n°2016-317 du 16 mars 2016).

Après absorption par l'organisme humain ou animal, les antibiotiques sont plus ou moins métabolisés selon les molécules, puis excrétés sous forme inchangée et/ou sous forme de métabolites. Par exemple chez l'homme, le taux d'excrétion urinaire sous forme inchangée est de 5% pour l'érythromycine, 14% pour le sulfaméthoxazole, contre 58% pour la tétracycline et 65% pour la ciprofloxacine (Benet et al., 2011). Ainsi, de nombreux résidus d'antibiotiques (composés parents et métabolites) sont rejetés dans les eaux usées et les effluents d'élevage (Figure I.1). Le traitement des eaux usées en station d'épuration permet d'éliminer partiellement les contaminants de l'eau traitée ; les contaminants sont dégradés et/ou adsorbés par les boues d'épuration produites quand ils présentent une forte affinité pour les matières solides (Lachassagne et al., 2015). Les boues de station d'épuration ainsi que les effluents d'élevage peuvent subir une durée plus ou moins longue de stockage et/ou un traitement tel que le séchage, le chaulage (pour les boues), la digestion (aérobie ou anaérobie) ou encore le compostage. Lors du stockage ou du traitement, les concentrations totales des molécules peuvent diminuer par dégradation (abiotique ou biotique), par effet de dilution lors du compostage avec d'autres déchets par exemple, ou encore par diminution de leur extractibilité suite à la formation de résidus liés aux matières solides (Lillenberg et al., 2010 ; Kästner et al., 2014 ; Geng et al., 2016). Des produits de transformation issus de la dégradation (abiotique ou biotique) des antibiotiques peuvent être formés.

Les déchets organiques sont ensuite recyclés en agriculture pour leur valeur agronomique, en tant qu'amendement et/ou fertilisant en fonction du type et de la qualité de la matière organique. L'apport de ces matières fertilisantes d'origine résiduaire (MAFOR) aux sols agricoles conduit également à leur contamination par les métaux et d'autres polluants contenus dans ces MAFOR (Houot et al., 2016). Cette pratique agricole est l'une des voies d'introduction des antibiotiques dans les sols, sous formes inchangées et/ou transformées (Figure I.1). Les sols peuvent aussi être contaminés directement par les fèces des animaux traités par les antibiotiques (Kemper, 2008). L'environnement à proximité des fermes peut ainsi être contaminé par les résidus d'antibiotiques ; de nombreuses études ont montré leur présence dans les eaux de surface, les eaux souterraines, les sédiments et les sols (Hirsch et al., 1999 ; Hu et al., 2010 ; Kim et Carlson, 2007 ; Kemper, 2008 ; Kümmerer, 2009). Des concentrations dans différentes matrices environnementales sont indiquées dans le Tableau I.2 pour les antibiotiques pris en exemple dans le Tableau I.1. De plus, l'épandage des MAFOR représente un risque biologique en raison de leur contenu possible en germes pathogènes. Plus particulièrement dans le cas des antibiotiques, certaines MAFOR peuvent contenir des germes résistants et des gènes de résistance qui peuvent aussi être transférés dans les sols, ce qui

peut contribuer à la dissémination des antibiotés (Sengeløv et al., 2003 ; Heuer et al., 2011a).

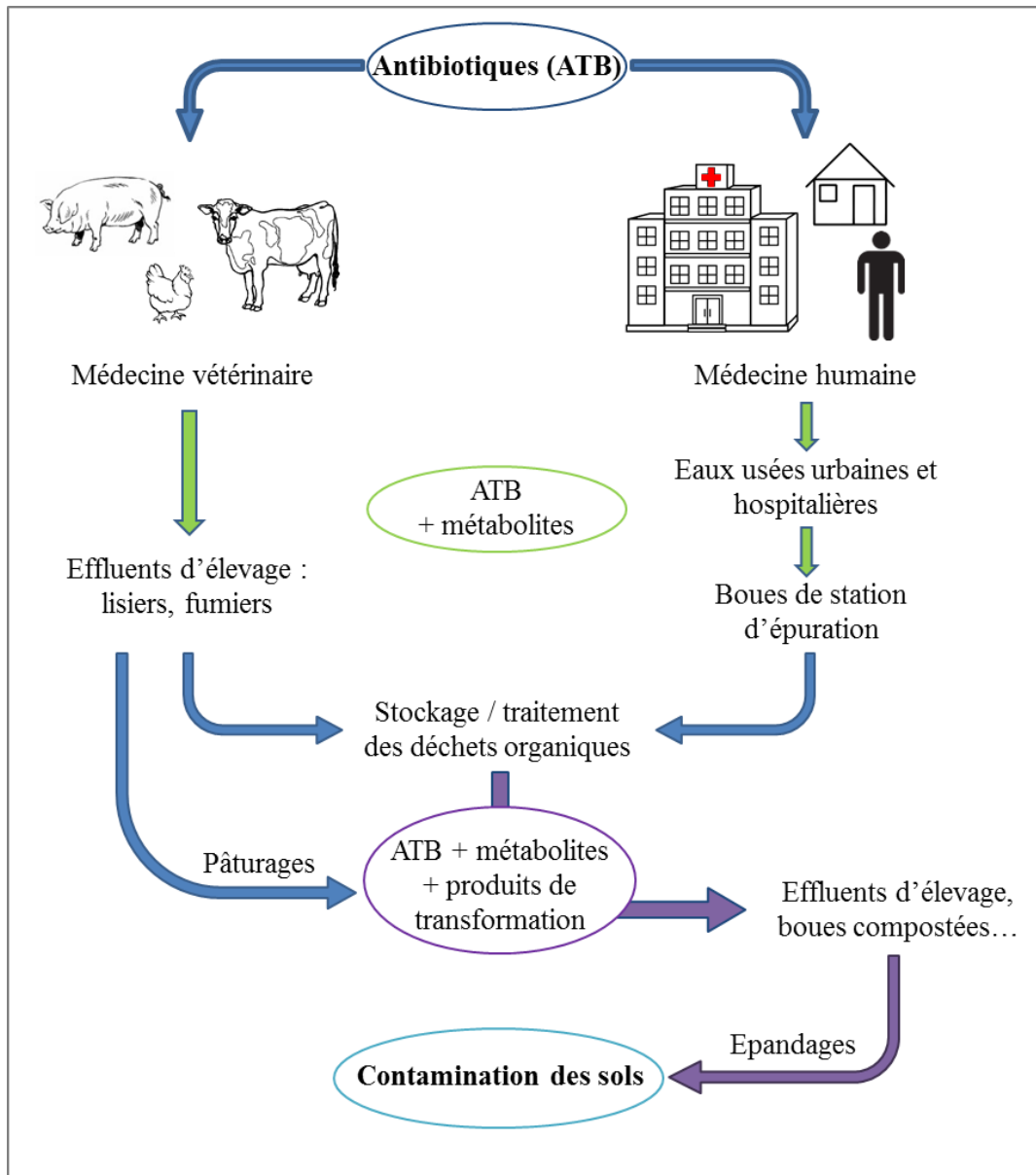


Figure I.1. Voies principales de contamination des sols agricoles par les antibiotés et leurs produits de transformation.

Tableau I.1

Familles d'antibiotiques et exemples de molécules : structures, propriétés physicochimiques des molécules et paramètres pouvant caractériser leur adsorption dans le sol.

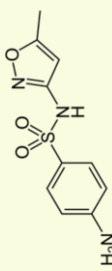
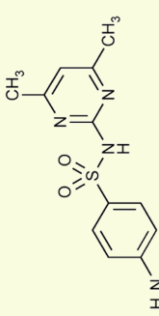
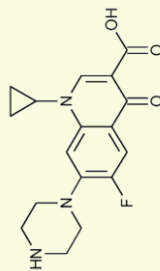
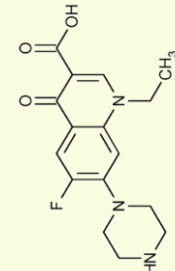
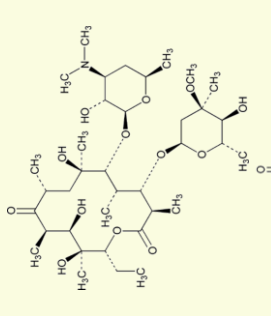
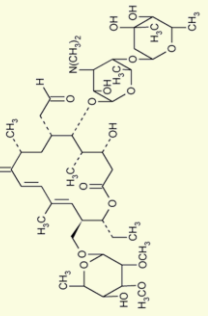
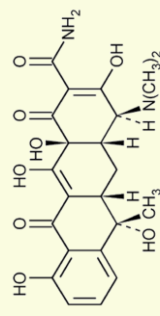
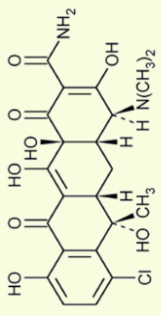
Familles et exemples d'antibiotiques	Principal usage	Structure	Propriétés physicochimiques			Comportement dans le sol*			
			Masse molaire (g mol ⁻¹)	pK _a ^a	log K _{ow} ^g	Solubilité dans l'eau (g L ⁻¹)	K _d (L kg ⁻¹)	K _{oc} (L kg ⁻¹)	DT ₅₀ (jours)
Sulfonamides									
Sulfaméthoxazole	Humain et vétérinaire		253,3	1,8-5,6	0,89 ^b	0,6 ^e			
Sulfaméthazine	Vétérinaire		278,3	2,1-7,45	0,76 ^c	1,5 ^d			
Fluoroquinolones									
Ciprofloxacine	Humain et vétérinaire (métabolite de l'énrofloxacine)		331,3	3,0-6,1-8,7-10,6	0,28 ^b	30 ^e			
Norfloxacine	Humain		319,3	3,1-6,1-8,6-10,6	-1,03 ^c	178 ^e	427-591 ^(c, i)	2,9-4,8 ^(c, d)	25-2300 ^(b, l, n)

Tableau I.1 (suite)

Macrolides		Macrolides							
Erythromycine	Humain et vétérinaire		733,9	8,9	3,06 ^b	5,2x10 ⁻⁴ ^e	8-130 (c, j)	2,2-3,9 (c, j)	8-89->1000 (b, k)
Tylosine	Vétérinaire		917,1	3,3-7,5	3,4 ^d	5 ^f			
Tétracyclines		Tétracyclines							
Tétracycline	Humain et vétérinaire		443,4	3,3-7,8-9,6	-1,3 ^c	1,7 ^d			
Chlortétracycline	Humain et vétérinaire		478,9	3,3-7,6-9,3	-0,36 ^b	0,6 ^d	400-2386 (b, c, d)	1,5-3,14 (c, e)	30-180 (b, l)

* Les paramètres K_d (coefficient d'adsorption dans le sol), K_{oc} (K_d normalisé par la teneur en carbone organique) et DT_{50} (temps de demi-vie de l'antibiotique dans le sol) sont donnés à titre d'exemple puisqu'ils dépendent des propriétés physicochimiques, de la composition des sols et du pédoclimat pour un même antibiotique.

^a Valeurs de pK_a (constantes de dissociation acide) déterminées par Qiang et Adams (2004); ^b DeVries et Zhang, 2016; ^c Pan et Chu, 2016; ^d Sarmah et al., 2006; ^e Valeurs estimées par Ma et al. (2015); ^f Kay et al., 2004; ^g $\log K_{ow}$: Coefficient de partage octanol/eau; ^h Höltge et Kreuzig, 2007; ⁱ Nowara et al., 1997; ^j Rabølle et Spliid, 2000; ^k Kodešová et al., 2016; ^l Thiele-Bruhn, 2003; ^m Lin et Gan, 2011; ⁿ Girardi et al., 2011.

Tableau I.2

Concentrations des antibiotiques dans les MAFOR et les sols amendés, en μg ou mg par kg de matière sèche (MS).

Les valeurs dans les boues et les effluents d'élevage sont issues du Rapport d'expertise collective MAFOR (2014), sauf si une autre référence est spécifiée. Les valeurs indiquées pour les teneurs dans les MAFOR représentent les valeurs minimum - moyenne - maximum retrouvées.

Antibiotiques	Boues de station d'épuration mg kg^{-1} MS (sauf si autre unité spécifiée)	Effluents d'élevage mg kg^{-1} MS (sauf si autre unité spécifiée)	Sols amendés (MAFOR apportée) $\mu\text{g kg}^{-1}$ MS
Sulfaméthoxazole	0,03 $\mu\text{g kg}^{-1}$ - 1,5 - 68	1 $\mu\text{g kg}^{-1}$ - 0,9 - 5,7	6,2 (fumier de vaches ; Watanabe et al., 2010) 0,03-0,9 (lisier ; Hu et al., 2010) 50-100 (fumiers ; Karci et Bacioglu, 2009) 19,3-54,5 (DeVries et Zhang, 2016)
Sulfaméthazine	0,74 - 40 - 360 $\mu\text{g kg}^{-1}$	0,14 - 4,0 - 20	$\leq 10,4$ (effluents de bétail ; Aust et al., 2008) ≤ 2 (lisiers de porcs ; Hamscher et al., 2005) 3,2-74 (DeVries et Zhang, 2016)
Ciprofloxacine	0,004 $\mu\text{g kg}^{-1}$ - 2,6 - 11,7	2,2 $\mu\text{g kg}^{-1}$ - 0,9 - 4,3	0,8-30 (lisier ; Hu et al., 2010) 101-5600 (DeVries et Zhang, 2016)
Norfloxacin	21 $\mu\text{g kg}^{-1}$ - 2,9 - 11,1	0,85 - 2,2 - 4,6	21,5-2160 (DeVries et Zhang, 2016)
Ciprofloxacine Norfloxacine	3 (Golet et al., 2003)		290-400 (boues ; Golet et al., 2002) 370 (lisier/fumiers ; Martínez-Carballo et al., 2007)
Erythromycine			4,4-7,2 (DeVries et Zhang, 2016)
Tylosine		0,05-116 (Song et Guo, 2014)	2,4-55 (lisiers ; Jacobsen et al., 2004) 23-679 (DeVries et Zhang, 2016)
Tétracyclines (en mélange)			86-199 (lisiers ; Hamscher et al., 2002) 8,8-105 (fumier de vaches ; Watanabe et al., 2010)
Chlortétracycline	6,2 $\mu\text{g kg}^{-1}$ - 0,65 - 0,35	1,3 $\mu\text{g kg}^{-1}$ - 27,4 - 879	≤ 87 (effluents de bétail ; Aust et al., 2008) 10-15 (lisiers ; Jacobsen et al., 2004) 102,3-12900 (DeVries et Zhang, 2016)
Tétracycline	0,6 $\mu\text{g kg}^{-1}$ - 0,4 - 1,9	1,2 $\mu\text{g kg}^{-1}$ - 6,5 - 43,5	105-1010 (DeVries et Zhang, 2016)

I.1.3. Devenir des antibiotiques dans les sols

Le devenir des antibiotiques (et des produits de transformation) dans les sols dépend de nombreux facteurs : tout d'abord de la molécule, de ses propriétés physicochimiques et de l'état d'ionisation dans lequel se présente l'antibiotique dans la MAFOR lors de l'épandage. Le devenir de l'antibiotique dépend aussi du type de MAFOR, de sa composition et de ses propriétés physicochimiques, et enfin, des caractéristiques du sol et des conditions environnementales qui influencent les processus physicochimiques dans le sol et les activités des organismes vivants dans le sol (Thiele-Bruhn, 2003). Après épandage des MAFOR, en tenant compte des facteurs évoqués ci-dessus, les antibiotiques peuvent soit rester liés aux constituants de la MAFOR, soit être transférés dans la phase liquide ou la phase solide du sol (Figure I.2). Tout comme les pesticides, le devenir des antibiotiques dans les sols dépend de leurs groupes fonctionnels chimiques puisque ceux-ci déterminent les propriétés physicochimiques des molécules, elles-mêmes en relation avec les processus de transferts (eaux souterraines, plantes), de rétention à la surface des matrices solides, de dégradation et des effets biologiques dans les sols (Calvet et al., 2005). Deux processus principaux régissent le devenir des antibiotiques dans les sols, la dégradation (section I.1.3.1) et la rétention (section I.1.3.2), qui sont à l'origine de l'adsorption (K_d , K_{oc}) et de la persistance plus ou moins longue des antibiotiques dans les sols (DT_{50} , temps de demi-vie dans les sols ; Tableau I.1).

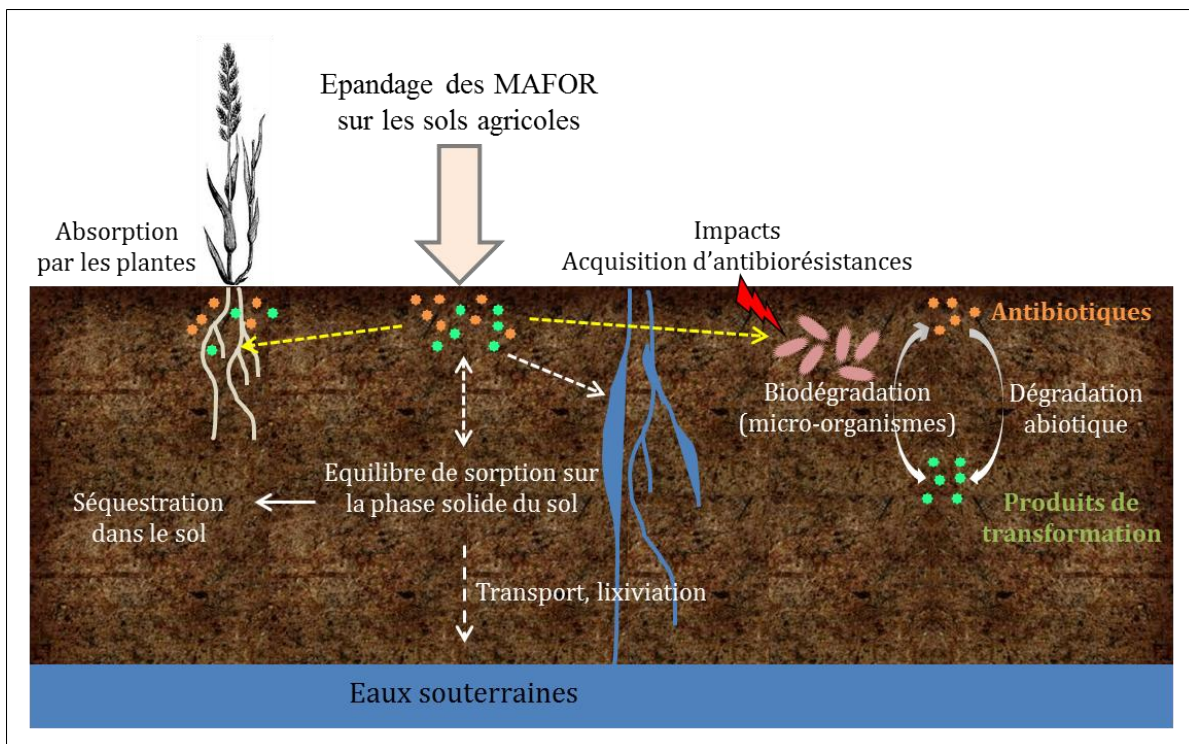


Figure I.2. Schéma du devenir des antibiotiques (et des produits de transformation) après un épandage de MAFOR sur les sols agricoles (inspiré de Jechalke et al., 2014).

1.1.3.1. Dégradation des antibiotiques dans les sols

Dans le sol, les antibiotiques peuvent subir une dégradation d'origine abiotique et/ou d'origine biotique. Les réactions de dégradation peuvent être influencées par les facteurs environnementaux (humidité, température) et la composition du sol (Stoob et al., 2007).

Les réactions de dégradation d'origine abiotique regroupent les réactions de photodégradation, d'oxydation, de réduction et d'hydrolyse. Par exemple, le sulfaméthoxazole (sulfonamide) peut subir une transformation dans le sol en réagissant avec les ions ferreux Fe^{2+} issus de la réduction microbienne du fer (Mohatt et al., 2011). La photodégradation est possible pour certains antibiotiques photosensibles, par exemple les fluoroquinolones, mais reste une réaction plutôt limitée dans le sol notamment en raison de la faible pénétration de la lumière dans le sol (Ozaki et al., 2011) et de la protection des composés de la lumière lorsque ceux-ci sont adsorbés sur la matière organique des MAFOR (Kemper, 2008).

Les micro-organismes du sol contribuent aux réactions de dégradation biotique. Les antibiotiques peuvent être transformés par des bactéries et des champignons (Ingerslev et Halling-Sørensen, 2001 ; Marengo et al., 2001) à condition que les molécules soient biodisponibles (Hatzinger et Alexander, 1997 ; section I.2). Des réactions enzymatiques, parfois réversibles, sont essentiellement responsables de la transformation des antibiotiques par hydroxylation ou encore par décarboxylation. La biodégradation des antibiotiques peut se faire en conditions aérobies, principalement à la surface du sol, par exemple, dans l'horizon de labour, où la présence d'oxygène est plus importante qu'en profondeur du sol ; ou en conditions anaérobies en cas d'anoxie, lorsque le sol est saturé en eau par exemple. Etant donné que les antibiotiques sont retrouvés en plus grande quantité à la surface du sol, la biodégradation se fait majoritairement dans l'horizon superficiel et certains produits de dégradation peuvent être retrouvés en concentration plus importante que le composé parent (Solliec et al., 2016).

La biotransformation peut parfois résulter de la transformation de métabolites en composés parents ; cette réaction a notamment été montrée dans le cas des sulfonamides, pour lesquels les métabolites *N*-acétylés sont retransformés en molécule mère dans le fumier et dans les sols amendés (Höltge et Kreuzig, 2007 ; Förster et al., 2009 ; Zarfl et al., 2009).

L'apport de MAFOR peut impliquer une plus grande activité microbienne et faciliter la biodégradation des antibiotiques dans le sol (Ingerslev et Halling-Sørensen, 2001 ; Wang et al., 2006) ; il s'agit alors de co-métabolisme, c'est-à-dire que les molécules d'antibiotiques sont

transformées de façon non-spécifique par les micro-organismes, par exemple lors de la dégradation de la matière organique.

D'autre part, il peut exister un métabolisme spécifique, c'est-à-dire que les micro-organismes utilisent de façon directe la molécule d'antibiotique comme source de carbone et de nutriment (azote). La biodégradation spécifique a notamment été mise en évidence dans les sols exposés de façon chronique aux antibiotiques et des bactéries (*Microbacterium sp.*) responsables de la biodégradation de la sulfaméthazine et de la sulfadiazine ont été isolées (Tappe et al., 2013 ; Topp et al., 2013).

Tous les processus de biotransformation de l'antibiotique, par co-métabolisme ou par métabolisme spécifique, conduisent à la formation de produits de transformation et peuvent aboutir à la minéralisation de l'antibiotique (dégagement de CO₂). Cependant, la rétention possible des antibiotiques au niveau des matrices solides des MAFOR et/ou des sols peut limiter leur disponibilité pour la biodégradation dans ces matrices (Al-Ahmad et al., 1999 ; Kemper, 2008).

1.1.3.2. Rétention des antibiotiques dans les sols

Notons que les mécanismes de rétention des antibiotiques décrits dans ce paragraphe pourront aussi s'appliquer aux MAFOR puisque ces dernières sont tout comme les sols, composées d'une phase organique et d'une phase minérale. Selon les familles d'antibiotiques, les molécules peuvent être plus ou moins retenues par les constituants minéraux et/ou organiques du sol. Parmi les processus de rétention, les antibiotiques peuvent s'associer à la phase solide du sol par adsorption. Le partage entre les phases liquide et solide est défini par la distribution du contaminant entre ces deux phases selon une loi type action de masse, décrite macroscopiquement par une constante de distribution ou de partage entre la phase solide et la phase liquide (K_d). Des exemples de coefficients d'adsorption et de coefficients d'adsorption normalisés par rapport à la teneur en carbone organique (K_{oc}) sont donnés dans le Tableau I.1 pour différentes familles d'antibiotiques. Les valeurs indiquées ne sont pas exhaustives car ces paramètres dépendent des caractéristiques physicochimiques des sols (pH, matière organique, composition) et les valeurs sont très variables selon les études pour un même antibiotique (Rabølle et Spliid, 2000 ; Thiele-Bruhn, 2003 ; Wegst-Uhrich et al., 2014).

Des mécanismes d'adsorption sur la phase solide bien particuliers sont décrits en fonction des familles d'antibiotiques (Tableau I.3). Les mécanismes les plus décrits pour les antibiotiques

sont : l'échange ionique, la liaison de complexation, les liaisons hydrogènes, les interactions polaires et hydrophobes (Xing et al., 2015).

Les liaisons ioniques et les liaisons de complexation ont lieu entre certains sites réactifs de la phase minérale (cations sur les argiles, oxydes de fer) et/ou organique (formes cationiques ou anioniques) du sol et certains groupements fonctionnels de l'antibiotique. Les antibiotiques sont pour la majorité des molécules ionisables en fonction du pH de la matrice et des constantes de dissociation acide (pK_a) de l'antibiotique ; les échanges ioniques (cationiques en général) sont donc possibles pour tous les antibiotiques (Tolls, 2001 ; Figueroa-Diva et al., 2010). La complexation (complexation de surface et/ou pont cationique) est un mécanisme d'adsorption relativement spécifique des fluoroquinolones et des tétracyclines. En effet, lors de liaison par pont cationique, les cations divalents (par exemple, Ca^{2+}) vont être complexés par un groupe céto-carboxylique déprotoné de ces antibiotiques (Figueroa-Diva et al., 2010). Pour un même site de chélation, le type d'interaction peut varier selon le cation : complexe monodentate, complexe bidentate ou complexe bidentate pontant dans le cas de la ciprofloxacine (Gu et Karthikeyan, 2005 ; Trivedi et Vasudevan, 2007 ; Rakshit et al., 2013). Les fluoroquinolones et les tétracyclines vont alors être inclus dans les feuillets des minéraux argileux (Aristilde et Sposito, 2010 ; Aristilde et al., 2016). Par conséquent, pour ces deux familles d'antibiotiques, les valeurs des coefficients d'adsorption varient peu d'une molécule à une autre de la même famille, les substituants des molécules ayant peu d'influence sur les mécanismes d'adsorption (Figueroa-Diva et al., 2010).

Les interactions polaires sont possibles pour la plupart des antibiotiques. Les interactions hydrophobes sont beaucoup décrites pour les sulfonamides, qui interagissent principalement avec la matière organique, et les substituants des molécules influencent fortement l'adsorption d'un sulfonamide à un autre (Figueroa-Diva et al., 2010 ; Schwarz et al., 2012). Même si les interactions hydrophobes jouent un rôle majeur dans la sorption des sulfonamides (Leal et al., 2013), les interactions avec les oxydes/hydroxydes métalliques interviennent également dans leur rétention. Ainsi, en présence de cuivre Cu (II), les sulfonamides peuvent s'adsorber en formant des complexes avec le cuivre et la phase solide du sol (Morel et al., 2014 ; Xu et al., 2015a).

Les caractéristiques des sols les plus importantes dans la rétention des antibiotiques sont finalement la teneur en matière organique, le pH, la texture (teneurs en argiles, limons, sables) et la capacité d'échange cationique (CEC).

Tableau I.3

Principaux types d'interactions impliqués dans les mécanismes d'adsorption des antibiotiques sur les surfaces adsorbantes du sol (Calvet et al., 2005 ; Tolls, 2001).

Type d'interaction	Propriétés de la surface adsorbante du sol	Propriétés et exemples d'antibiotiques
Liaison ionique, échange d'ions	Charges électriques portées par les minéraux argileux, les oxydes et hydroxydes métalliques, les groupes chimiques ionisés (O ⁻) Charges électriques portées par les groupes chimiques polaires (RCOO ⁻ , RO ⁻ , RNH ₂ ⁺) de la matière organique	Charges électriques portées par les antibiotiques ionisés : la plupart des antibiotiques , en fonction de leurs pKa Formation de ponts cationiques : sulfonamides
Liaison de complexation, transfert d'électrons	Cations de la phase minérale	Présence d'atomes porteurs d'électrons libres (O, N, S, P) : Formation de complexes cationiques : tétracyclines, fluoroquinolones
Liaison hydrogène	Groupes chimiques porteurs de proton : - groupes OH dans la phase minérale - RCOOH, ROH, RNH et atomes possédant des électrons libres (O, N, S, P) dans la phase organique	Présence d'atomes porteurs d'électrons libres (O, N, S, P) : la plupart des antibiotiques
Interactions polaires		Molécules polaires : la plupart des antibiotiques
Interactions hydrophobes	Groupes chimiques apolaires aliphatiques ou aromatiques de la matière organique	Présence de groupes chimiques aliphatiques, aromatiques : formes neutres des antibiotiques : sulfonamides et macrolides

De nombreux facteurs influencent la rétention des antibiotiques dans les sols : propriétés physicochimiques de l'antibiotique et du sol, concentration de l'antibiotique, activité biologique, temps d'exposition, conditions environnementales (Ehlers et Loibner, 2006 ; Riding et al., 2013 ; Adedigba et Semple, 2015). La fréquence des épandages peut aussi modifier l'adsorption des antibiotiques dans les sols. Par exemple, la sorption du sulfaméthoxazole est réduite, mais pas celle de la ciprofloxacine, dans les sols irrigués par des eaux usées (Dalkmann et al., 2014). Parmi les conditions environnementales, l'adsorption des antibiotiques sur les MAFOR et/ou le sol dépend fortement du pH de la matrice, étant donné que les antibiotiques sont des molécules ionisables. Par exemple, les sulfonamides ont tendance à s'adsorber davantage dans le sol lorsque le pH de celui-ci diminue ; en effet, à des valeurs de pH en dessous de leur pKa (autour de 5), les sulfonamides chargés positivement interagissent par attraction électrostatique avec les charges négatives de la surface du sol (Lertpaitoonpan et al., 2009 ; Wegst-Uhrich et al., 2014). De la même façon,

l'adsorption de la ciprofloxacine et de la tylosine est plus forte lorsque le pH diminue (Vasudevan et al., 2009 ; Wegst-Uhrich et al., 2014). De plus, la solubilité des antibiotiques peut dépendre de leur état d'ionisation, et dépend donc du pH (Yu et al., 1994). La température et l'humidité sont également des facteurs essentiels qui peuvent modifier l'adsorption des antibiotiques dans le sol (Rosendahl et al., 2011 ; Radl et al., 2015).

Les propriétés physicochimiques de la MAFOR par laquelle sont apportés les antibiotiques peuvent également influencer leur rétention dans les sols (Riding et al., 2013). En particulier, l'association des antibiotiques avec la matière organique dissoute et/ou les colloïdes (diamètre de 1 nm à 10 µm) apportés par les MAFOR peut retarder ou au contraire, favoriser leur transport dans le sol (Xing et al., 2015). Les colloïdes peuvent être constitués de minéraux argileux, d'oxydes métalliques ou de macromolécules organiques (Xing et al., 2015). Par exemple, dans le cas d'association de sulfonamides avec des colloïdes organiques apportés par différents fumiers, un transport dans le sol a été facilité pour certains sulfonamides par des interactions essentiellement hydrophobes avec les colloïdes, tandis qu'un transport retardé a été montré pour d'autres sulfonamides, en fonction des substituants sur les molécules de sulfonamides et aussi des propriétés des fumiers, favorisant la mobilité ou non des colloïdes (Chabauty et al., 2016 ; Zhou et al., 2016). L'association des antibiotiques avec les colloïdes peut également protéger les molécules de la biodégradation (Xing et al., 2015).

L'adsorption des antibiotiques peut être réversible ou irréversible. Dans le cas d'une adsorption réversible, la possibilité de la désorption des antibiotiques doit aussi être considérée. En effet, les processus de sorption et de désorption peuvent être responsables de la mobilité des antibiotiques dans les sols et de leur transfert vers d'autres compartiments de l'environnement, soit par ruissellement en surface du sol, soit par transport facilité par les particules (Jechalke et al., 2014). Par exemple, les sulfonamides sont mobiles et peuvent être transférés dans les eaux souterraines contrairement aux tétracyclines, macrolides ou fluoroquinolones qui représentent moins de risques (Rabølle et Spliid, 2000 ; Topp et al., 2008 ; Blackwell et al., 2009 ; Pan et Chu, 2016). Certains antibiotiques peuvent également être transférés dans les plantes cultivées (Boxall et al., 2006 ; Hu et al., 2010 ; Du et Liu, 2012).

Dans le cas d'une adsorption irréversible, la séquestration physique, chimique et/ou biologique des antibiotiques entraîne la formation de résidus liés ou non-extractibles (Kästner et al., 2014). La stabilisation physique des résidus peut avoir lieu notamment dans la structure du sol (nano-, microporosités). L'immobilisation instantanée (mais pas entièrement réversible) peut être le résultat d'interactions par complexation des antibiotiques (Vasudevan et al., 2009 ; Schwarz et al.,

2012 ; Ahmed et al., 2016). Les résidus liés peuvent au cours du temps être remobilisés par les végétaux, les micro-organismes ou par des modifications physico-chimiques des constituants du sol (Calvet et al., 2005 ; Kästner et al., 2014). Heise et al. (2006) ont montré que la remobilisation chimique et biologique des résidus non-extractibles des sulfonamides dans le sol restait très faible.

La sorption des antibiotiques ne contrôle pas seulement leur mobilité ou leur transfert dans les plantes, mais aussi leur disponibilité vis-à-vis des micro-organismes qui peuvent être impactés, s'adapter voire biodégrader les antibiotiques.

1.1.4. Effets des antibiotiques sur les micro-organismes du sol

La biodiversité microbienne des sols (bactéries, archées, champignons, protozoaires) assure une multitude de fonctions telles que la minéralisation de la matière organique et la libération de nutriments, la biodégradation des contaminants organiques, les symbioses. Ces fonctions contribuent notamment aux cycles biogéochimiques des éléments (par exemple, carbone, azote, phosphore), à la qualité physico-chimique des sols (nutriments, rôle de filtre) et des systèmes aquatiques connexes. Cette biodiversité microbienne peut se caractériser par ses aspects structuraux (composition et diversité taxonomique) et fonctionnels (activités métaboliques). D'une manière générale, les processus écosystémiques sont influencés en majeure partie par l'activité et les capacités métaboliques des microorganismes (Treseder et al., 2012). Les stressés environnementaux et chimiques peuvent affecter directement (effets toxiques) ou indirectement (modification de communautés) les activités métaboliques (Brandt et al., 2015). A l'échelle des communautés, les relations entre la diversité et le fonctionnement vont influencer les capacités de réponse aux contaminants.

En considérant leurs différents mécanismes d'action thérapeutique, les antibiotiques de la famille des sulfonamides et fluoroquinolones ont des effets sélectifs sur les bactéries, mais avec des spectres d'action plus ou moins larges. De plus, les sensibilités des micro-organismes varient selon les familles d'antibiotiques, notamment en raison de leurs propriétés physico-chimiques différentes influençant leurs devenir dans les sols (Liu et al., 2012). Un résumé des effets des antibiotiques sur les micro-organismes du sol est présenté dans la Figure I.3. Ceci va conditionner les effets à court terme ou à long terme, sur une partie plus ou moins grande de la biodiversité microbienne des sols.

Les antibiotiques introduits dans les sols peuvent induire différents effets sur les micro-organismes du sol, en fonction du temps et des niveaux d'exposition et de la fréquence d'apport.

Ainsi, deux grands types d'effets peuvent être distingués : les effets toxiques induits à court ou moyen terme (« toxicité aigüe »), et les effets induits par la présence à long terme ou la récurrence de faibles doses (« toxicité chronique »).

I.1.4.1. Effets des antibiotiques à court ou moyen terme

Les antibiotiques dans les sols peuvent altérer l'activité des micro-organismes et affecter l'abondance, la structure et les fonctions des communautés bactériennes de manière transitoire ou durable (Jechalke et al., 2014 ; Figure I.3). En fait, les antibiotiques d'action bactériostatique (sulfonamides, fluoroquinolones, tétracyclines, macrolides) vont essentiellement affecter les populations bactériennes en croissance et affecter leur activité surtout en stoppant leur multiplication ou leur synthèse *de novo* d'enzymes (Backhaus et Grimme, 1999 ; Thiele-Bruhn et Beck, 2005).

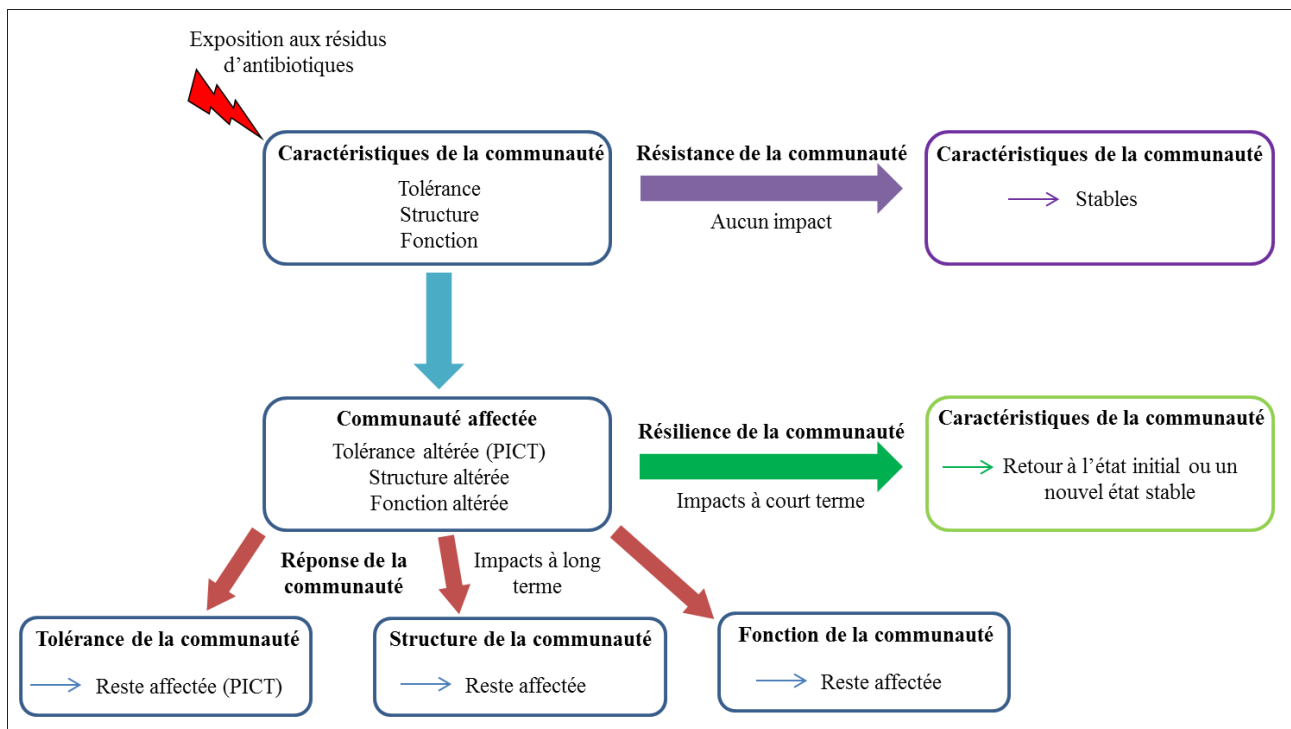


Figure I.3. Résumé des effets possibles des antibiotiques sur la structure et la fonction des communautés microbiennes du sol ainsi que sur la tolérance d'une communauté qui peut être vue comme une réponse à moyen ou long terme (d'après Brandt et al., 2015).

Les activités microbiennes dans les sols amendés sont affectées par les antibiotiques contenus dans les MAFOR. Généralement, il y a moins d'activité dans ces sols par rapport aux sols amendés par des MAFOR non contaminées, mais plus d'activité par rapport aux sols non amendés (Hammesfahr et al., 2011a). Par ailleurs, le stockage du fumier contaminé peut réduire les impacts de la sulfadiazine (sulfonamide) sur les communautés microbiennes du sol amendé (Hammesfahr et al., 2011b).

Parmi les activités microbiennes, des impacts sur les activités enzymatiques, sur la respiration du sol ou encore sur la minéralisation du carbone soluble ont été parfois observés, mais à des doses d'antibiotiques très élevées et irréalistes ($> 50 \text{ mg kg}^{-1}$ sol sec ; Liu et al., 2009). Les activités impliquées dans le cycle de l'azote (par exemple, nitrification et dénitrification) ou dans la réduction du fer sont plus sensibles (Kotzerke et al., 2008, 2011a, 2011b ; Schauss et al., 2009a ; Toth et al., 2011 ; Hammesfahr et al., 2011a, 2011b ; Ollivier et al., 2013). Les différents niveaux de sensibilité des activités viennent du fait que ces dernières activités sont assurées par un faible nombre de groupes microbiens (surtout bactériens) spécifiques dans les sols, et que la redondance et la complémentarité fonctionnelle sont faibles au sein de ces groupes, alors que la dégradation de la matière organique et les activités enzymatiques sont réalisées par une très grande part de la biodiversité microbienne (bactéries, archées et champignons) qui est donc plus tolérante aux stressés toxiques.

Par ailleurs, même si des antibiotiques adsorbés sur le sol peuvent conserver une activité antimicrobienne (Chander et al., 2005 ; Peng et al., 2014, Peng et al., 2015), la dissipation des antibiotiques par dégradation et/ou adsorption contribuent à diminuer progressivement l'exposition des microorganismes aux antibiotiques (Liu et al., 2012). Ainsi, les effets précédemment décrits suite à l'épandage de MAFOR contaminées sont transitoires et les communautés microbiennes peuvent résilier, c'est-à-dire revenir à un état initial d'activité voire de composition (Figure I.3 ; Schauss et al., 2009a ; Ding et He, 2010 ; Kotzerke et al., 2011b ; Hammesfahr et al., 2011a).

Les antibiotiques peuvent affecter plus durablement la structure des communautés à des doses élevées ($> 10 \text{ mg sulfonamide kg}^{-1}$ sol ; Ding et al., 2014) en induisant l'émergence de populations non sensibles (par exemple, les champignons ou les archées), ou tolérantes aux dépens des populations sensibles (Schauss et al., 2009a ; Ollivier et al., 2013 ; Jechalke et al., 2014). Ces changements de structures des communautés peuvent induire des effets durables sur les fonctions écologiques des sols comme la transformation de nutriments ou encore la dégradation des polluants. Cependant, dans des cas où les populations tolérantes ou résistantes sont fonctionnellement redondantes par rapport aux populations sensibles, ces changements de communautés peuvent ne

pas induire d'effet fonctionnel (Hammesfahr et al., 2011a) et même contribuer à la résilience des activités microbiennes initialement impactées (Figure I.3 ; Brandt et al., 2015).

1.1.4.2. Effets des antibiotiques à long terme

A long terme ou suite à la récurrence de l'exposition, les micro-organismes peuvent développer une réponse adaptative à la présence des antibiotiques dans les sols, qui induisent des pressions de sélection. Des mécanismes de défense vis-à-vis des antibiotiques peuvent être acquis par les bactéries (gènes de résistance). L'apport chronique des antibiotiques dans les sols, suite à la récurrence des amendements, peut entraîner l'acquisition et le maintien d'antibiorésistances dans les sols (Heuer et al., 2011b) et contribuer à la dissémination de celles-ci dans l'environnement, ce qui représente un risque pour la santé humaine et animale (Wright, 2010). De plus, les gènes de résistances, portés par des bactéries contenues dans les MAFOR, peuvent aussi être introduits dans les sols lors des épandages et favoriser la dissémination des résistances. Les gènes de résistance ont ainsi eux-mêmes été considérés comme des polluants (Martinez, 2009). La présence d'autres polluants tels que les métaux dans les MAFOR peut conduire à l'augmentation de l'abondance des résistances en exerçant une pression de co-sélection (Hölzel et al., 2012). Les effets des antibiotiques apportés seuls ou en mélanges peuvent être différents sur le développement de souches résistantes (Ding et He, 2010).

Au travers des changements structuraux, ces résistances à l'échelle individuelle vont être préférentiellement sélectionnées dans les communautés au profit des populations sensibles et ainsi conférer une tolérance communautaire acquise. Cette tolérance d'une communauté induite par un contaminant peut expliquer la résilience observée à moyen terme sur les activités et le fonctionnement des communautés (Figure I.3). Cette tolérance va ainsi varier selon les niveaux d'exposition passé et présent des micro-organismes dans les sols, et plus les pressions de sélections seront fortes, plus la tolérance sera élevée (Ding et He, 2010 ; Brandt et al., 2015). Cette tolérance acquise est spécifique de l'antibiotique induisant les pressions de sélection.

Certaines adaptations, pouvant conférer une résistance, se traduisent par le développement de capacités de biodégradation des antibiotiques de façon spécifique, qui sont alors pris comme source d'énergie ou de nutriments. Ce cas particulier de biodégradation dite accélérée avait été observée pour certains pesticides comme l'atrazine (Topp et al., 2000), mais a également été rapporté pour des antibiotiques (Topp et al., 2013 ; section I.1.3.1).

1.1.4.3. Limites des connaissances sur les effets des antibiotiques

De nombreuses études apportent des connaissances sur l'occurrence, le devenir et le transport des résidus d'antibiotiques dans les sols, ainsi que sur les effets des antibiotiques sur les micro-organismes et la dynamique de l'antibiorésistance dans les sols. Cependant, nous pouvons constater qu'il existe très souvent une divergence entre les quantifications chimiques des molécules dans les sols et les doses expérimentalement testées sur le fonctionnement microbien des sols. Très peu d'études incluent des concentrations dites « environnementales » d'antibiotiques, qui sont au maximum de quelques centaines de $\mu\text{g kg}^{-1}$ de sol pour les sulfonamides et fluoroquinolones (Tableau I.2 ; Rosendahl et al., 2011 ; Rosendahl et al., 2012). Il est pourtant primordial de se placer à ce niveau de concentrations afin de réaliser des études réalistes (Lindberg et al., 2007 ; Martinez, 2009 ; Brandt et al., 2015). En effet à très fortes doses, les antibiotiques peuvent induire des effets de stress oxydant ; ces effets ne sont pas représentatifs des effets bactériostatiques des antibiotiques à faibles doses dans les sols. Dans la plupart des travaux cités auparavant, les doses d'antibiotiques testées sont comprises entre quelques mg à plusieurs centaines de mg kg^{-1} de sol, les effets étant souvent aléatoires ou non détectés à des doses plus faibles ($< 1 \text{ mg kg}^{-1}$) avec les indicateurs utilisés. L'absence d'effets est parfois due au faible niveau d'activité des communautés étudiées (sols non amendés), sur lesquelles les effets bactériostatiques des antibiotiques n'ont alors pas lieu.

Lors des expérimentations au laboratoire, il est aussi primordial de respecter le plus possible les conditions au champ, c'est-à-dire l'introduction des antibiotiques dans les sols par l'apport de MAFOR (Boxall et al., 2012). A notre connaissance, seuls quelques auteurs ont respecté ces conditions dans leurs études, avec des concentrations en antibiotiques de 25-200 $\mu\text{g sulfonamides kg}^{-1}$ et de 0,3-30 $\mu\text{g tétracyclines kg}^{-1}$ sol (Toth et al., 2011) ; de 600 $\mu\text{g fluoroquinolones kg}^{-1}$ sol (Rosendahl et al., 2012) et de 200-600 $\mu\text{g sulfonamides kg}^{-1}$ sol (Ollivier et al., 2013). Les conditions d'apport de MAFOR contaminées au sol ont aussi été respectées dans d'autres études (Kotzerke et al., 2008, 2011a, 2011b ; Hammesfahr et al. 2008, 2011a), mais les concentrations des antibiotiques appliquées étaient plus élevées (de 10 à 100 mg kg^{-1} sol) que celles retrouvées dans l'environnement. L'étude de Konopka et al. (2015) était réaliste par rapport aux applications sur des parcelles expérimentales au champ, mais l'application de MAFOR n'a pas été prise en compte et les antibiotiques étaient apportés directement en solution. Un autre aspect essentiel est la présence de mélanges d'antibiotiques et les effets mélanges associés (Backhaus et al., 2000), mais cela ne relève pas du travail réalisé durant cette thèse.

Etant donné que les effets des antibiotiques sur les micro-organismes sont influencés par le temps d'exposition, la concentration en antibiotiques, le type de matrice environnementale et le nombre d'antibiotiques ou d'autres contaminants, les relations entre devenir et effets des antibiotiques restent mal comprises (Sarmah et al., 2006 ; Martinez, 2009 ; Brandt et al., 2015). La rétention des antibiotiques par adsorption sur les phases solides des sols et/ou des MAFOR peut influencer leur disponibilité et donc leur toxicité et pressions de sélection. Il est donc primordial de développer des méthodes afin d'estimer la biodisponibilité des antibiotiques dans les sols amendés par les MAFOR.

I.2. La biodisponibilité

I.2.1. Définition du concept de biodisponibilité

Dans ce travail, nous nous focaliserons sur le concept de biodisponibilité utilisé pour l'environnement terrestre, et les définitions de la biodisponibilité utilisées en médecine ne seront pas abordées. Le terme de biodisponibilité est apparu dès le 19^e siècle lorsque Mulder (1860) indique que les besoins en engrais des plantes cultivées ne doivent pas être déduits des analyses chimiques des sols, la disponibilité des nutriments ne pouvant pas être établie à l'avance. Dès lors, des méthodes chimiques ont été développées pour prédire la biodisponibilité des nutriments nécessaires aux plantes. La définition de la biodisponibilité a largement évolué au cours du temps selon les auteurs, notamment en fonction de la discipline environnementale et des organismes vivants ciblés (Naidu et al., 2008). De nombreuses définitions sont basées sur des flux ou des taux de transfert, tandis que d'autres définitions sont basées sur des teneurs. En théorie, ces deux groupes de définitions sont liés, les teneurs résultant de l'intégration des flux ou taux de transferts sur une période choisie (ou les flux/taux estimés en divisant les teneurs mesurées par le temps d'exposition) (Harmsen et al., 2005). En 2004, Semple et al. ont défini le concept de biodisponibilité par la fraction de molécules dans une matrice environnementale disponibles pour interagir ou être absorbées par un organisme vivant à un temps donné. Cette définition plutôt large a été l'une des premières définitions qui ait semblé faire consensus et qui a été reprise par les comités d'experts ISO 11074 (2005) et ISO/DIS 17402 (2008) comme suit : « la biodisponibilité est le degré pour lequel des molécules chimiques présentes dans la matrice sol peuvent être absorbées ou métabolisées par des récepteurs humains ou écologiques ou disponibles pour interagir avec des systèmes biologiques » (Harmsen et al., 2005). Il n'existe cependant pas de définition générique de

la biodisponibilité car celle-ci dépend des propriétés physico-chimiques de la molécule, de celles de la matrice environnementale, du temps d'exposition, des organismes cibles et des conditions environnementales, dont dépendent les activités des organismes (Harmsen et al., 2005 ; Harmsen, 2007). La définition dépend également de l'objectif de l'auteur qui la formule. Ainsi, actuellement, le concept de biodisponibilité est intégré dans l'évaluation des risques liés à la contamination des sols et des sédiments, même si celle-ci utilise encore beaucoup les concentrations totales des contaminants (Ortega-Calvo et al., 2015).

Il semblerait préférable de discuter des processus impliqués dans le passage d'une molécule dans un organisme vivant (Figure I.4). Ehlers et Luthy (2003) distinguent les interactions des molécules avec le sol, responsables de la répartition entre une fraction dite associée au sol et une fraction dite dissoute dans le sol, et le transport des molécules contenues dans ces deux fractions jusqu'à la membrane cellulaire d'un organisme. La fraction biodisponible est la fraction de molécules interagissant avec l'organisme, à un temps donné, tandis que la bioaccessibilité comprend ce qui est biodisponible immédiatement *plus* ce qui est potentiellement biodisponible (Ehlers et Luthy, 2003 ; Semple et al., 2004 ; Riding et al., 2013). La fraction potentiellement biodisponible peut correspondre, par exemple, aux molécules isolées qui peuvent devenir disponibles dans le temps, au cours de modifications de la composition de la solution au contact de la phase solide ou au cours de modifications de la phase solide consécutive à des processus de transformations chimiques biotiques ou abiotiques (Calvet et al., 2005 ; Riding et al., 2013).

Les processus dynamiques impliqués dans la biodisponibilité peuvent être décrits par trois étapes successives (Figure I.4) : la disponibilité environnementale, relative à l'accessibilité de la molécule dans le sol, en particulier par son passage en solution ; la biodisponibilité environnementale, correspondant à l'absorption de la molécule disponible dans le sol par l'organisme ; et la biodisponibilité toxicologique, relative à la réponse biologique induite par la molécule au sein de l'organisme (Lanno et al., 2004 ; Harmsen, 2007).

La bioaccumulation des molécules au sein de l'organisme peut représenter un intermédiaire entre biodisponibilité environnementale et biodisponibilité toxicologique, tant que la bioaccumulation de la molécule n'induit pas d'effets biologiques, c'est-à-dire tant que la concentration en résidus dans l'organisme ne dépasse pas la concentration corporelle critique (CBR, critical body residues ; McCarty et Mackay, 1993 ; Lanno et al., 2004).

En dehors d'effets biologiques induits par la molécule chimique dans l'organisme (biodisponibilité toxicologique), la biodisponibilité d'une molécule peut être évaluée à l'aide de deux autres marqueurs : 1) la fraction disponible pour être bioaccumulée, métabolisée et/ou excrétée par des organismes supérieurs ; 2) la fraction disponible pour être transformée voire minéralisée par des micro-organismes (« disponibilité biodégradable » selon Ehlers et Loibner, 2006).

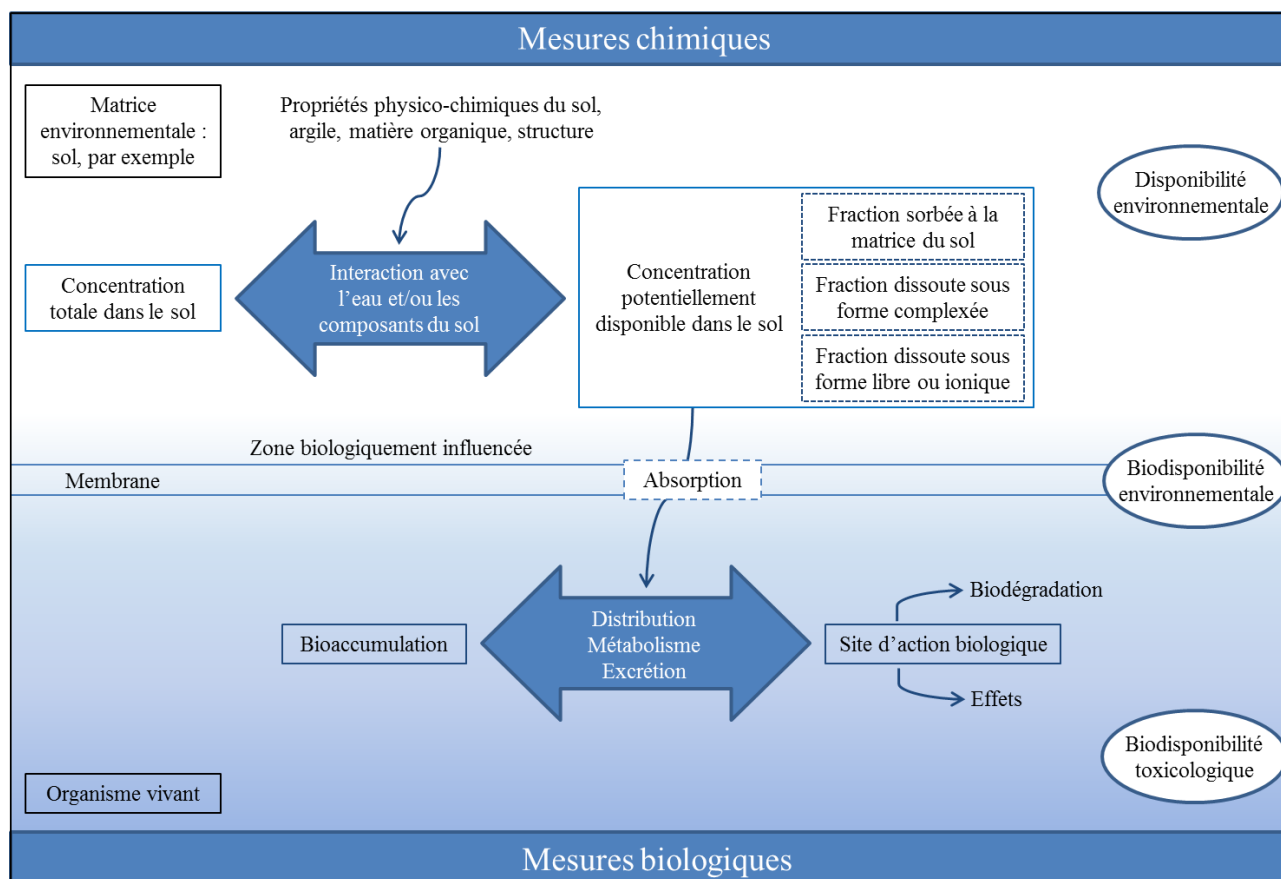


Figure I.4. Description des processus de biodisponibilité d'un contaminant dans le sol jusqu'à son effet biologique au sein d'un organisme (d'après Lanno et al., 2004 ; Harmsen, 2007).

I.2.2. Disponibilité environnementale et biodisponibilité des contaminants

De nombreuses méthodes sont développées afin d'estimer la biodisponibilité d'un contaminant dans le sol et de comprendre les effets biologiques associés. Les méthodes sont répertoriées en deux principales catégories : les méthodes chimiques (section I.2.2.1) et les méthodes biologiques (section I.2.2.2).

La mesure chimique permet d'estimer la disponibilité environnementale alors que la mesure biologique cherche à quantifier la biodisponibilité environnementale ou toxicologique (Figures I.4

et I.5). Les mesures chimiques doivent donc être corrélées à des mesures biologiques pour estimer la biodisponibilité d'un contaminant pour un organisme donné (Harmsen, 2007). Aucune méthode n'est universelle, et le choix des méthodes dépend en grande partie des propriétés physicochimiques des contaminants. Les deux types de méthodes étant complémentaires, des exemples de mesures biologiques associées aux mesures chimiques seront donnés à titre d'exemple dans la section suivante.

1.2.2.1. Méthodes chimiques

Au sein des méthodes chimiques d'estimation de la (bio)disponibilité des contaminants, nous pouvons distinguer différentes techniques d'extractions, utilisées seules ou successivement, selon les propriétés d'adsorption des contaminants. Le choix de la méthode et des conditions d'extraction est important et l'objectif est de garder un mécanisme d'extraction dit biomimétique (Ehlers et Loibner, 2006) afin de mesurer la fraction facilement extractible et/ou active et donc potentiellement biodisponible (Stokes et al., 2006).

a) Extraction de la solution du sol

Le principe de cette technique est basé sur l'hypothèse que toutes les molécules dissoutes dans la solution du sol sont (bio)disponibles (Figure I.5). L'extraction de la solution du sol par simple centrifugation ou par l'eau permet d'extraire les molécules dissoutes et les molécules non ionisées faiblement adsorbées sur les constituants organiques et/ou minéraux du sol. De plus, l'utilisation de solutions salines (par exemple, CaCl_2) pour jouer sur la force ionique de la solution d'extraction permet d'extraire les molécules ionisées (cations) par échange cationique. Les molécules sont dosées dans les extraits à l'équilibre. Ces méthodes d'extraction sont dites douces ou non-exhaustives (Rauret et al., 1998 ; Reid et al., 2000a ; Reid et al., 2000b).

Cette technique a été très utilisée pour les pesticides pour estimer leur (bio)disponibilité afin de connaître la concentration toxique pour plantes et déterminer la quantité à appliquer. La corrélation entre les concentrations mesurées disponibles dans la solution du sol et les effets toxiques a permis une bonne estimation de la biodisponibilité. (Harmsen et al., 2005 ; Calvet et al., 2005). Folberth et al. (2009) ont comparé l'extraction de la solution du sol par centrifugation ou par l'eau et ont conclu que la centrifugation suffisait pour estimer la quantité biodisponible d'isoproturon (pesticide), la minéralisation du contaminant servant de référence pour la disponibilité microbienne. De la même façon, cette technique est utilisée pour les contaminants inorganiques tels

que les métaux au regard de leur toxicité pour les micro-organismes ou les plantes (Chaudri et al., 2000 ; Harmsen et al., 2005). L'extraction avec un agent chélatant en solution aqueuse (par exemple, EDTA : acide éthylène diamine tétraacétique) est aussi utilisée pour estimer la fraction de métaux (bio)disponibles pour les plantes (Harmsen, 2007). Pour des polluants organiques hydrophobes, tels que les hydrocarbures aromatiques polycycliques (HAP), l'extraction aqueuse ne semble pas être un bon compromis pour estimer leur (bio)disponibilité (Harmsen et al., 2005).

De la même façon que pour les pesticides, la technique d'extraction de la solution du sol par une solution de CaCl_2 a beaucoup été utilisée pour les antibiotiques (Hammesfahr et al., 2011a ; Kotzerke et al., 2011a, 2011b ; Rosendahl et al., 2012). Les fractions disponibles de sulfadiazine (sulfonamide) ou de difloxacin (fluoroquinolone) ont été utilisées en tant qu'indicateur d'exposition des micro-organismes du sol sur lesquels les antibiotiques avaient des effets (communautés et activités microbiennes).

b) Extraction par des solvants organiques

L'utilisation de solvants organiques à froid et à pression atmosphérique (par exemple, méthanol) permet d'extraire les contaminants sous forme non ionisée et adsorbés. Une partie des molécules extraites est probablement (bio)disponible (Figure I.5), selon leur facilité de désorption (Calvet et al., 2005). L'objectif est d'utiliser un procédé intermédiaire entre l'extraction aqueuse et l'extraction exhaustive car cette dernière ne permet pas d'estimer la (bio)disponibilité. L'extraction « totale » a en effet pour objectif d'extraire le maximum de contaminant dans le sol (molécules non ionisées adsorbées et fortement retenues par la matière organique), ce qui ne reflète pas sa biodisponibilité et peut conduire à une surestimation de l'exposition (Alexander, 2000). Il est donc nécessaire de bien sélectionner les conditions pour diminuer le pouvoir extractant des solvants, par exemple en réduisant leur caractère hydrophobe (pesticides : Barriuso et al., 2004 ; Yu et al. 2005), en diminuant la durée d'extraction ou en ajustant les conditions d'extraction (température, pression), par exemple dans le cas des extractions par liquide pressurisé ou par fluide supercritique (HAP : Hawthorne et Grabanski, 2000).

Des protocoles d'extraction séquentielle avec une puissance d'extraction croissante peuvent être utilisés afin d'extraire les fractions de molécules de moins en moins disponibles dans le sol. La composition des solutions d'extraction varient en fonction des propriétés physico-chimiques des contaminants. Ainsi, les extractants dits « moyens », constitués de solvants, de mélanges solvant/eau ou d'acides organiques, ont largement été utilisés pour évaluer la biodisponibilité de

contaminants organiques hydrophobes comme les HAP ou certains pesticides (Stokes et al., 2006 ; Cui et al., 2013). L'utilisation de ces solvants a permis de cibler la fraction labile des contaminants hydrophobes, c'est-à-dire les molécules rapidement désorbables et une part des molécules lentement désorbables, et elle a permis ainsi d'exclure en grande partie voire totalement la fraction très lentement désorbable (Riding et al., 2013). De plus, les concentrations en contaminants mesurées ont été corrélées à des effets biologiques tels que la bioaccumulation ou la biodégradation (Kelsey et al., 1997 ; Barriuso et al., 2004 ; Cui et al., 2013).

Concernant les antibiotiques, molécules polaires, un solvant organique (le moins apolaire) tel que le méthanol a été classiquement utilisé dans les protocoles d'extractions successives après l'extraction par une solution de CaCl₂ (Hammesfahr et al., 2008 ; Hammesfahr et al., 2011a, 2011b ; Kotzerke et al., 2011a ; Rosendahl et al., 2012).

c) Extraction par une phase adsorbante

La volonté de mettre en œuvre une extraction chimique sans solvant organique afin de ne pas modifier les propriétés du sol a entraîné l'utilisation d'autres méthodes d'extraction. Les techniques d'extractions font appel à une « troisième phase » afin d'extraire les contaminants (bio)disponibles qui se trouvent en équilibre entre la phase liquide et la phase solide du sol (Figure I.5). Cette troisième phase, qui piège les contaminants sans risquer de se saturer, induit un déplacement de l'équilibre au fur et à mesure de la désorption des contaminants sous l'effet d'un gradient de concentration entre la phase solide et la phase aqueuse. Elle est constituée d'un adsorbant qui permet le piégeage des contaminants, soit sur des phases solides (par exemple, C₁₈, Tenax[®]), soit dans des molécules cages (cyclodextrines) ou des micelles de surfactants.

Les échantillonneurs passifs permettent de retenir les contaminants libres dissous en solution (Ehlers et Loibner, 2006). Par exemple, les disques C₁₈ sont constitués d'une phase adsorbante (silice C₁₈) entre deux membranes semi-perméables. Les disques C8 ont par exemple été utilisés pour estimer la biodisponibilité de l'antibiotique érythromycine dans les eaux et sédiments (Jessick, 2010). Les échantillonneurs POCIS (Polar Organic Chemical Integrative Samplers) sont utilisés pour les composés polaires tels que les molécules pharmaceutiques (Morin et al., 2013). La phase adsorbante peut être remplacée par une phase de substitution (par exemple, polydiméthylsiloxane, Tenax[®] TA : copolymère de styrène-divinylbenzene) dans des échantillonneurs utilisés pour des composés plus apolaires tels que certains pesticides ou les HAP (Harwood et al., 2013 ; Bernhardt et al., 2013).

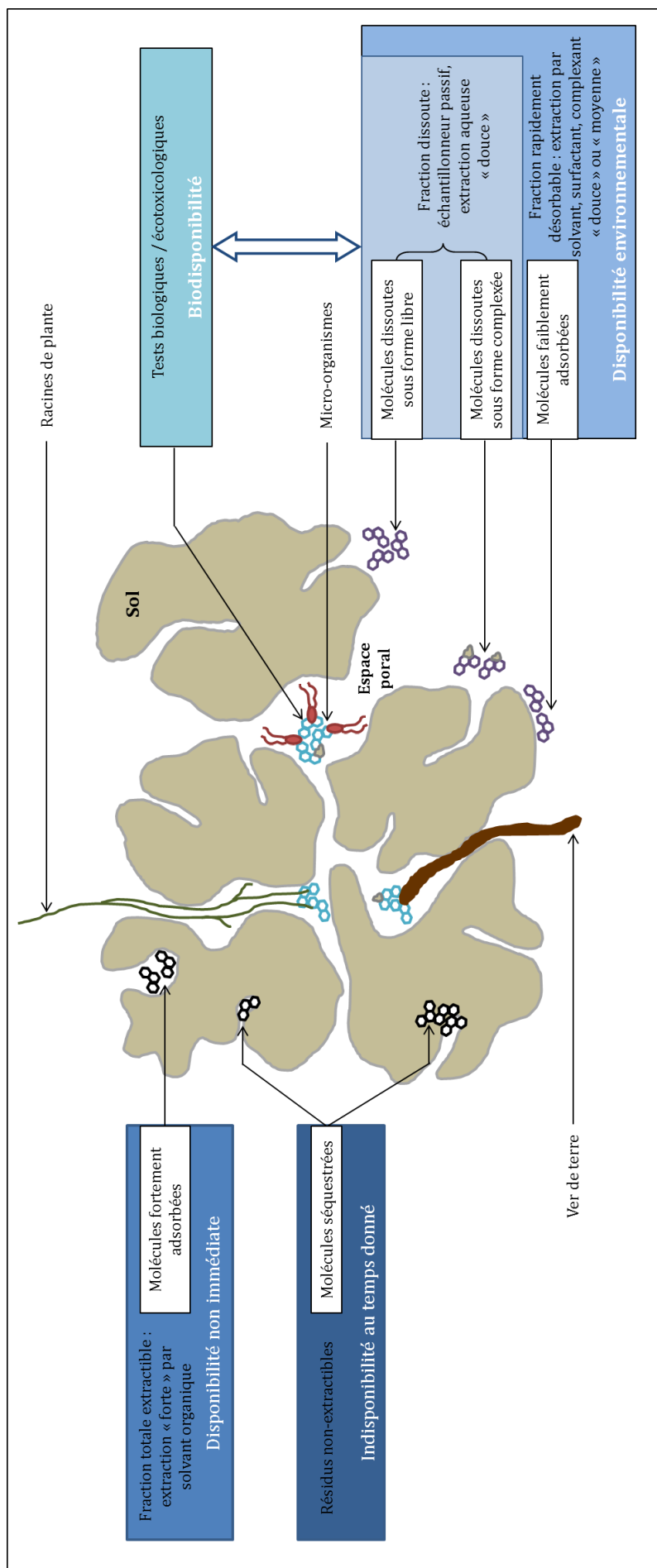


Figure I.5. Représentation schématique de la disponibilité environnementale et de la biodisponibilité des molécules dans le sol et types de mesures pour les estimer à un temps donné (adapté de Semple et al., 2004 ; Ehlers et Loibner, 2006 ; Harmsen, 2007 ; Ortega-Calvo et al., 2015).

Le principe est basé sur l'extraction sur phase solide. Il existe également des fibres, contenant de la silice coiffée d'une fine couche de polymère qui sont utilisées pour réaliser des micro-extractions sur phase solide (SPME, solid-phase micro-extraction), mesurer la concentration de contaminants libres dissous dans la solution et ainsi évaluer l'accumulation de contaminants dans un biote (Ehlers et Loibner, 2006). Néanmoins l'utilisation des fibres SPME ou des disques C₁₈ peut conduire à une mauvaise estimation de la biodisponibilité du contaminant vis-à-vis de l'organisme étudié, en particulier si celui-ci n'absorbe pas exclusivement l'eau poreuse lors de son itinéraire dans le sol (Sijm et al., 2000). Certains auteurs ont aussi utilisé le Tenax[®] lors de l'extraction par fluide supercritique pour évaluer la biodisponibilité des hydrocarbures dans les sols contaminés (Cuypers et al., 2001) après avoir adapté les conditions d'extraction (Stokes et al., 2006).

Les surfactants ont la propriété de former des micelles en solution lorsque leur concentration est proche de leur concentration micellaire critique ; tandis que les agents solubilisants, comme les cyclodextrines, sont des oligosaccharides cycliques dont l'extérieur est hydrophile et la cavité interne hydrophobe. Le mécanisme d'extraction repose sur la solubilisation des polluants organiques par leur inclusion dans les micelles ou dans les cavités des cyclodextrines. L'utilisation de tels extractants, notamment la solution de 2-hydroxypropyl- β -cyclodextrine, a permis d'évaluer la bioaccessibilité de polluants organiques (HAP, pesticides) en mesurant leurs concentrations dans la phase poreuse du sol et/ou la fraction facilement désorbable du sol (Reid et al., 2000b ; Adedigba et Semple, 2015). La mesure de la fraction minéralisée des HAP peut être une technique pour évaluer leur biodisponibilité microbienne au regard de l'extraction par les cyclodextrines (Doick et al., 2005 ; Semple et al., 2006). De la même façon, les fractions (bio)disponibles mesurées ont été corrélées à la bioaccumulation des pesticides dans les vers de terre (Hartnik et al., 2008 ; Néliu et al., 2016). En général, le potentiel de bioaccumulation des contaminants organiques est lié à leur hydrophobicité. Les antibiotiques sont des molécules plutôt polaires, mais des études ont tout de même montré la bioaccumulation de certains antibiotiques (les moins polaires, par exemple, les fluoroquinolones) dans des organismes aquatiques (Puckowski et al., 2016).

d) Autres méthodes chimiques

L'oxydation partielle de la matière organique permet d'accéder aux contaminants organiques faiblement adsorbés à sa surface et donc (bio)disponibles, sans atteindre les molécules adsorbées sur la phase complexe de la matière organique. En particulier cette technique a été utilisée pour estimer la fraction (bio)disponible de HAP pour les micro-organismes dégradants (Harmsen, 2007).

La désorption thermique (pyrolyse) est une technique basée sur l'hypothèse que les contaminants les plus séquestrés dans le sol nécessitent plus de temps et d'énergie pour se désorber (Calvet et al., 2005). Ainsi, les molécules non extractibles et fortement retenues par la matière organique sont accessibles. En réalité, ces molécules pourraient être (bio)disponibles seulement en cas de dégradation de la matière organique.

1.2.3.2. Méthodes biologiques

Des méthodes biologiques directes permettent d'estimer la biodisponibilité en mesurant la concentration (bioaccumulation ou flux et efflux) du contaminant dans l'organisme d'intérêt. Cette méthode prend en compte les facteurs biotiques (métabolisme) et également les facteurs abiotiques (pH, matière organique du sol) pouvant jouer sur la biodisponibilité du contaminant. Cependant, la présence du contaminant dans l'organisme n'induit pas forcément d'effet toxique ou de réponse de l'organisme (section I.2.1); il s'agit dans ce cas de l'estimation de la biodisponibilité environnementale d'un contaminant (Lanno et al., 2004).

La biodisponibilité toxicologique, quant à elle, peut être estimée à l'aide de bioessais et de biomarqueurs, permettant de quantifier un effet sur l'organisme, induit par l'exposition et/ou l'absorption du contaminant dans l'organisme. Il s'agit de méthodes biologiques indirectes. Lors de l'utilisation de ces méthodes, il est possible qu'une réponse de l'organisme soit observée sans avoir connaissance du ou des contaminant(s) responsable(s) de cet effet (Hamsen, 2007). Par conséquent, il est essentiel de coupler aux bioindicateurs susceptibles de répondre aux contaminants, une méthode chimique permettant de quantifier la fraction disponible du ou des contaminant(s) recherché(s), caractérisant l'exposition de l'organisme.

L'écotoxicité d'extraits chimiques ou de lixiviats de sols ou de sédiments contaminés par des antibiotiques a souvent été étudiée avec l'utilisation de tests normalisés classiques utilisés par diverses réglementations. Ces tests peuvent être par exemple, les tests Microtox[®] reposant sur la bactérie modèle *Vibrio fischeri*, ou les diverses variantes des tests microalgues et daphnies (Harmsen, 2007). Cependant, ce type d'approche manque de pertinence pour aborder la question de la biodisponibilité ou d'exposition des micro-organismes aux contaminants dans les matrices complexes telles que le sol. En effet, ce type de test tend à répondre à la question relative à la toxicité d'extraits (et effets matrices associés), avec l'estimation des formes bioactives des résidus d'antibiotiques, sans distinction entre les molécules mères et les produits de transformation, en

complément de mesures réalisées en chimie analytique pour quantifier les molécules recherchées (Quack et al., 2005). Par ailleurs, il a été démontré que le test normalisé Microtox[®] (recommandé par la norme pour les tests avec des métaux ou des contaminants organiques) manque de pertinence pour évaluer la toxicité d'antibiotiques du fait d'un temps d'exposition préconisé (30 min) beaucoup trop court. Il est donc recommandé d'allonger les temps d'exposition (24 h) lors de la réalisation du test écotoxicologique avec des antibiotiques, en particulier pour les antibiotiques bactériostatiques (Backhaus et al., 1997).

Dans le contexte d'étude de la biodisponibilité des contaminants dans un milieu, ces mesures devraient préférentiellement se faire dans la matrice environnementale étudiée (sol, sédiment, eau) et non pas à partir d'extraits chimiques. Dans le cas d'organismes supérieurs (par exemple, plante, annélides, arthropodes) et pour des contaminants tels que les métaux, HAP, les pesticides et parfois même les antibiotiques (Jessick, 2010), les paramètres mesurés peuvent être la reproduction, la mortalité, la croissance, et le comportement mais aussi des marqueurs biochimiques (protéines) ou moléculaires (expressions de gènes) (Harmsen, 2007 ; Beaumelle et al., 2016). Cependant, les métazoaires ne présentent pas les cibles moléculaires des antibiotiques et ces modèles biologiques manquent de pertinence pour l'étude de la toxicité des antibiotiques dans les sols, en dehors des mesures de bioaccumulation.

Les antibiotiques affectent en effet principalement les métabolismes bactériens. Les micro-organismes et plus particulièrement les bactéries sont donc des modèles pertinents. Les paramètres mesurés peuvent être des métabolismes, comme ceux des cycles du carbone (par exemple, minéralisation du carbone, respiration) ou des activités intracellulaires comme celles du cycle de l'azote (par exemple, nitrification, dénitrification), les activités enzymatiques (par exemple, déshydrogénase) (Harmsen, 2007).

De telles mesures ont été réalisées dans les études précédemment citées (section I.1.4), mais l'objectif de leurs études n'était pas de comprendre la biodisponibilité des antibiotiques. En revanche, les relations entre adsorption, disponibilité environnementale et biodégradation ont été beaucoup mieux décrites pour d'autres catégories de contaminants organiques (pesticides : Barriuso et al., 2004 ; HAP : Semple et al., 2006).

Les pressions de sélection exercées à moyen et long termes par les antibiotiques entraînent l'acquisition d'antibiorésistances au sein de communautés microbiennes (Heuer et Smalla, 2007 ; Heuer et al., 2011b). Divers outils de biologie moléculaire existent pour quantifier les gènes de résistance propres à certaines familles d'antibiotiques (Knapp et al., 2010 ; Marti et al., 2013). Ainsi, les bactéries résistantes (approche par culture) et/ou les gènes de résistance aux antibiotiques

(biologie moléculaire) pourraient être respectivement sélectionnés en tant que bioindicateurs et biomarqueurs pour évaluer indirectement l'exposition des micro-organismes, et donc la biodisponibilité des antibiotiques dans les sols.

1.2.3. Méthodes chimiques pour estimer la (bio)disponibilité des antibiotiques

Dans un premier temps, la disponibilité environnementale des antibiotiques dans les sols pourrait être estimée en sélectionnant une méthode chimique d'extraction. Etant donné que les antibiotiques sont des molécules relativement polaires et ionisables en fonction du pH du sol (Tableau I.1), les méthodes d'extraction mises en œuvre doivent cibler les mécanismes d'adsorption des composés (Tableau I.3).

Parmi les techniques d'extraction « douce », l'extraction de la solution du sol par une solution saline (par exemple, CaCl_2) est envisageable pour les antibiotiques, molécules relativement solubles dans l'eau. L'extraction va donc extraire les molécules présentes sous forme dissoute dans la solution du sol et une autre partie des molécules faiblement adsorbées sur la phase solide. Cette technique a d'ailleurs été utilisée par plusieurs auteurs (Hammesfahr et al., 2008 ; Kotzerke et al., 2011a, 2011b ; Rosendahl et al., 2012). Etant donné que certains antibiotiques (fluoroquinolones et tétracyclines) peuvent complexer les cations divalents (Tableau I.3), il serait préférable de remplacer le sel CaCl_2 par un autre sel composé d'un cation monovalent (par exemple, KCl).

En considérant l'adsorption des antibiotiques par échange ionique sur la phase solide du sol, un agent chélatant de type EDTA peut entrer en compétition avec les cations de la phase organominérale du sol et permettre d'extraire les antibiotiques adsorbés sur cette phase.

De plus, l'état d'ionisation des antibiotiques dépendant du pH de la matrice dans laquelle il se trouve, les extractions doivent être testées en faisant varier les pH des solutions d'extraction.

Parmi les techniques d'extraction, les solvants ne sont pas un choix prioritaire car leur principe d'extraction ne présente pas une base mécaniste/physiologique en lien avec la biodisponibilité (ISO/DIS 17402, 2008). De plus, les antibiotiques sont relativement solubles dans l'eau et leur ionisation possible en fonction du pH. En revanche, l'utilisation de cyclodextrines ou de surfactants (par exemple, tétraborate de sodium, sodium dodécylsulfate) peut être envisagée pour extraire les antibiotiques grâce à leur possibilité de former des liaisons hydrophobes (Tableau I.3),

et ainsi d'être inclus dans les cavités hydrophobes des molécules cages (cyclodextrines) ou des micelles (surfactants).

Dans un deuxième temps, les fractions disponibles des antibiotiques mesurées doivent être mises au regard de mesures biologiques qui visent les micro-organismes du sol. La biodisponibilité environnementale peut être estimée en suivant la biodégradation des antibiotiques ; et la biodisponibilité toxicologique peut être estimée en étudiant les effets des antibiotiques sur les communautés et les activités microbiennes.

I.3. Hypothèses et objectifs

Le devenir et la biodisponibilité des antibiotiques introduits dans les sols agricoles lors de l'épandage des MAFOR peuvent dépendre de l'origine et de la nature de la MAFOR ainsi que des propriétés des sols. En fonction de la biodisponibilité des antibiotiques, les micro-organismes peuvent être impactés, tant au niveau des structures et des fonctions des communautés bactériennes que par leur adaptation aux antibiotiques. Dans ce contexte, l'objectif de ce travail est de développer une méthode chimique afin d'estimer la disponibilité environnementale voire la biodisponibilité des antibiotiques dans sols amendés par les MAFOR. L'approche de l'estimation de la disponibilité par des extractions douces a été sélectionnée afin d'évaluer la disponibilité du sulfaméthoxazole et de la ciprofloxacine, dans les sols amendés soit par un compost de boue soit par un fumier de ferme. Plusieurs questions apparaissent et seront traitées dans les chapitres suivant (Figure I.6) :

1) Quelles sont les fractions d'antibiotiques extraites en fonction de leurs mécanismes d'adsorption et des mécanismes d'extraction mis en jeu ?

2) En quoi la nature des MAFOR, telle que la composition chimique et la qualité de la matière organique, influence-t-elle, initialement et dans le temps, la disponibilité des antibiotiques dans les sols amendés ?

3) La matière organique des MAFOR influence-t-elle la disponibilité et le devenir des antibiotiques dans les sols ? Un modèle simulant les évolutions conjointes de la matière organique et d'un polluant organique dans le sol peut-il être utilisé dans le cas des antibiotiques ?

4) Etant donné l'adsorption possible des antibiotiques dans les matrices complexes telles que les MAFOR et les sols, et l'extraction aqueuse n'étant pas exhaustive : la présence de matière organique extraite par les différentes extractions aqueuses peut-elle expliquer les interactions entre les antibiotiques et les matrices solides, qui peuvent limiter la disponibilité ? Par ailleurs, ces

matières organiques dissoutes peuvent-elles expliquer certains effets matrices au moment de la quantification ?

5) En quoi la composition et les propriétés physico-chimiques des sols influencent-elles la disponibilité des antibiotiques ?

6) Les antibiotiques sont-ils dégradés dans les sols après application des MAFOR ?

7) La méthode chimique d'extraction permet-elle d'estimer la biodisponibilité des antibiotiques vis-à-vis des micro-organismes du sol ? Deux types d'effets seront considérés pour répondre à cette question : 7a) la biodégradation des antibiotiques par des micro-organismes adaptés ; 7b) les effets toxiques des antibiotiques sur l'activité des communautés nitrifiantes du sol.

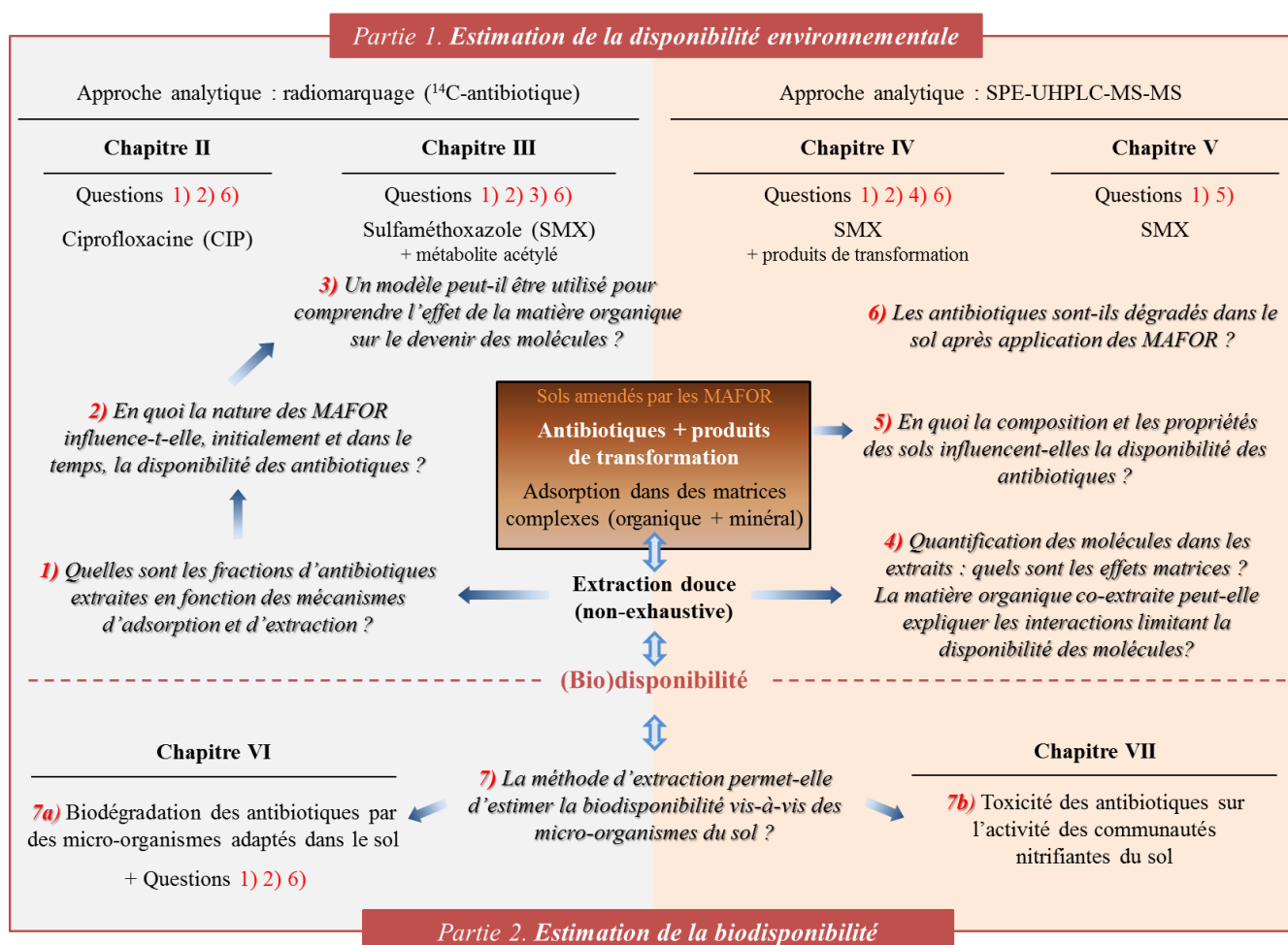


Figure I.6. Questions principales abordées au cours de ce travail en lien avec l'estimation de la biodisponibilité des antibiotiques dans les sols amendés par les MAFOR.

Partie 1. Estimation de la disponibilité environnementale

L'analyse bibliographique a permis d'identifier les limites de connaissances quant aux méthodes d'évaluation de la biodisponibilité des antibiotiques dans les sols amendés par les MAFOR. L'adsorption des antibiotiques sur les matrices solides telles que le sol et les MAFOR dépend de leurs structures chimiques et conditionne leur biodisponibilité dans les sols. La biodisponibilité peut être estimée par une méthode chimique d'extraction aqueuse au regard d'effets des contaminants sur des organismes vivants. En premier lieu, la méthode d'extraction vise à estimer la disponibilité environnementale des antibiotiques, c'est-à-dire la fraction des molécules présentes dans la solution du sol voire celles facilement désorbées de la matrice solide. Ces molécules sont alors potentiellement disponibles à la fois pour leur transfert dans le sol vers les eaux souterraines et pour interagir les micro-organismes du sol.

Les propriétés physico-chimiques des fluoroquinolones et différents mécanismes d'adsorption (interactions électrostatiques, complexation) sont responsables de la forte adsorption de ces antibiotiques sur les matrices solides. Par conséquent, plusieurs questions se posent :

- 1) Quelle méthode chimique d'extraction peut être utilisée pour estimer leur fraction disponible ?
- 2) Quelle est leur disponibilité environnementale dans les sols amendés par les MAFOR ?
- 3) Le type de MAFOR influence-t-il leur disponibilité dans les sols ?

Dans le Chapitre II, la ciprofloxacine marquée au carbone 14 a été sélectionnée en tant que fluoroquinolone pour répondre à ces questions, le radiomarquage permettant un suivi total de la distribution de la molécule dans différentes fractions (disponible, extractible, non-extractible, minéralisée) au cours du temps. L'antibiotique a été apporté sur les MAFOR avant leur incorporation dans les sols, et de façon à obtenir une concentration proche des concentrations environnementales.

Approche analytique : radiomarquage (^{14}C -antibiotique)

Chapitre II

Sols amendés par les MAFOR
**Antibiotiques + produits
de transformation**
Adsorption dans des matrices
complexes (organique + minéral)



Extractions aqueuses



Disponibilité de la ciprofloxacin

Principaux mécanismes d'adsorption des fluoroquinolones :
interactions ioniques, complexation



- 1) Quelle méthode chimique d'extraction?
- 2) Quelle disponibilité environnementale dans les sols amendés par les MAFOR?
- 3) Disponibilité influencée par le type de MAFOR?

Chapter II: A new extraction method to assess the environmental availability of ciprofloxacin in agricultural soils amended with exogenous organic matter

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Abstract

Fluoroquinolone antibiotics such as ciprofloxacin can be found in soils receiving exogenous organic matter (EOM). Their long-term behavior in EOM-amended soils and their level of biodegradability are not well understood partly due to a lack of methods to estimate their environmental availability. We performed different aqueous extractions to quantify the available fraction of ^{14}C -ciprofloxacin in soils amended with a compost of sewage sludge and green wastes or a bovine manure contaminated at relevant environmental concentrations.

After minimizing ^{14}C -ciprofloxacin losses by adsorption on laboratory vessel tubes, three out of eleven different aqueous solutions were selected, i.e., Borax, Na_2EDTA and 2-hydroxypropyl- β -cyclodextrin. During 28 d of incubation, the non-extractable fractions were very high in all samples, i.e., 57-67% of the initial ^{14}C -activity, and the availability of the antibiotic was very low, explaining its low biodegradation. A maximum of 6.3% of the initial ^{14}C -activity was extracted from soil/compost mixtures with the Na_2EDTA solution, and 7.2% from soil/manure mixtures with the Borax solution. The available fraction level was stable over the incubation in soil/compost mixtures but slightly varied in soil/manure mixtures following the organic matter decomposition.

The choice of different soft extractants highlighted different sorption mechanisms controlling the environmental availability of ciprofloxacin, where the pH and the quality of the applied EOM appeared to be determinant.

Keywords

Antibiotic; chemical extraction; sorption; organic matter; pH; ionization

II.1. Introduction

Organic wastes such as sewage sludge and manure are recycled as exogenous organic matter (EOM) in agriculture for their agronomic potential (Diacono and Montemurro, 2010). The application of organic amendments may lead to the chronic introduction into cultivated soils of pollutants present in the EOM, such as antibiotics (Kümmerer, 2003; Boxall et al., 2012). Given to humans and animals in prophylaxis and for treatment of bacterial diseases, the antibiotics include several families like fluoroquinolones, and raise significant concern for soil quality.

Antibiotics can be excreted as the parent molecule and/or as metabolites, as either bioactive or inactive forms, and are found in wastewaters and livestock effluents. Concentrations of fluoroquinolones can be particularly high in the EOM, e.g., around 47 mg kg⁻¹ dry mass in bovine manure (Zhao et al., 2010) and 11 mg kg⁻¹ dry mass in sewage sludge (McClellan and Halden, 2010). The storage and the treatment of organic wastes can modify antibiotic concentrations by dilution, degradation and/or formation of non-extractable residues (Lillenberg et al., 2010; Geng et al., 2016). The origin of non-extractable residues may be physical, e.g., by pollutant sequestration in soil or organic waste, chemical, e.g., by binding of the pollutant to soil or organic waste components, or biological, e.g., by microbial transformation of the pollutant and further incorporation into microbial biomass (Kästner et al., 2014). After EOM application to soils, total concentrations of fluoroquinolones can range from 0.37 mg kg⁻¹ in soils treated with manure (Martínez-Carballo et al., 2007) up to 9.8 mg kg⁻¹ in a soil sampled near a farm (Morales-Muñoz et al., 2004). A concentration of 0.40 mg ciprofloxacin per kg was reported in topsoil, eight months after the application of sewage sludge (Golet et al., 2002). Fluoroquinolones can persist in soils (Rusu et al., 2015) and they also sorb strongly on EOM-treated soils (Golet et al., 2002; Van Doorslaer et al., 2014). As fluoroquinolones exhibit several pKa values, their adsorption on solid matrix depends on pH (Gu and Karthikeyan, 2005; Kümmerer, 2009). Different mechanisms have been advocated to account for fluoroquinolone sorption on soils, such as cation exchange, cation bridging, complexation, and H-binding (Tolls, 2001; Vasudevan et al., 2009).

In addition to these interactions, antibiotic residues may have an impact on soil microorganisms, with effects on microbial communities and/or processes like enzymatic activities, nitrification and denitrification activities (Kotzerke et al., 2008; Cui et al., 2014). Soil microorganisms can also acquire antibiotic resistance, whose spread in soils may be facilitated by

the potential presence of antibiotic-resistant bacteria and resistance genes in the EOM (Heuer et al., 2011a; Andersson and Hughes, 2014; Jechalke et al., 2014). Resistance to fluoroquinolones was shown in swine-manured soils in China (Xu et al., 2015b).

In order to assess the exposure of living organisms to contaminants, the concept of bioavailability was defined as the contaminant fraction in environmental matrix available to living organisms (Alexander, 2000; Lanno et al., 2004; Harmsen et al., 2005). The total concentration of a contaminant may include dissolved forms in the soil solution, exchangeable, adsorbed and tightly-bound forms in the solid compartment. The total concentration is generally not representative of the bioavailable concentration (Alexander, 2000), since only a fraction of dissolved or rapidly dissolved chemical forms is available to cross the organism membrane. According to Lanno et al., (2004), the bioavailability of a contaminant may be described by three successive steps. Firstly, the environmental availability corresponds to the fraction of contaminant in soils that is not sequestered and that could be transferred in the environment or interact with living organisms. Secondly, the environmental bioavailability corresponds to the part of the contaminant that is taken up by the organism. Finally, the toxicological bioavailability corresponds to the fraction of absorbed contaminant that reaches a site of biological response.

The environmental availability can be approached through extraction methods, taking into account the properties of the contaminant and the environmental matrix. The methods are generally based on pore water analysis, on trapping or partitioning in a solid support, or on partial extraction by mild solvents or aqueous media (Folberth et al., 2009; Cui et al., 2013). The objective of these methods is to use a mechanistic or a physiological basis (Harmsen, 2007; ISO 17402, 2008) such as the adsorption on a competitive solid support, the encapsulation in cyclodextrin, the complexing, or the solubilizing by chemical agents in solution (ISO/DIS 16751, 2015). These chemical extraction methods can be called non-exhaustive or soft extractions (Rauret, 1998; Reid et al., 2000a). As an example, cyclodextrins are suitable extractants to simulate the transfer of the hydrophobic contaminants through the organism membrane thanks to their capacity to encapsulate organic contaminants into their hydrophobic cavity. Reid et al. (2000b) and Rhodes et al. (2008) highlighted the correlation between the cyclodextrin-extracted fraction of hydrocarbons and their biodegradation. Similarly, the available fraction of pesticides assessed by cyclodextrin-based extraction was correlated to the bioaccessibility for earthworms (Hartnik et al., 2008; Nélieu et al., 2016). Performances of this extraction method were demonstrated only for compounds more hydrophobic than fluoroquinolones, i.e., with $\text{Log } K_{ow} > 1$, higher than the $\text{Log } K_{ow}$ of ciprofloxacin, equal to 0.28 (Takács-Novák et al., 1992).

In spite of the high total concentrations of fluoroquinolones and their persistence in EOM-treated soils, measuring their environmental availability is challenging due to their strong sorptive properties and to multiple interactions depending on pH and solid matrix properties. Girardi et al. (2011) showed that ciprofloxacin sorption does not completely eliminate the effects of this compound on microbial processes. This suggests that a certain amount of ciprofloxacin should be environmentally-available and could contribute to microbial exposure in soil after EOM application. In this context, the aim of this study was to develop an indirect chemical method to assess, at relevant environmental concentrations (5 mg ciprofloxacin kg⁻¹ dry mass of EOM), the environmental availability of ciprofloxacin in agricultural soils after EOM application. After a preliminary study to minimize the ciprofloxacin sorption on laboratory material, several soft extraction media were tested to extract the environmentally-available fraction of the antibiotic from soil/EOM mixtures. Two EOM amendments were considered: a compost of sewage sludge and green wastes as an example of urban waste compost, and a solid bovine manure, as an example of farmyard manure. Moreover, the EOMs were selected considering their different compositions and pH. The different sorptive interactions of ciprofloxacin described in the literature mostly involve cations and pH as well as the zwitterionic properties of ciprofloxacin. Therefore, we considered different mechanisms of extraction such as ionic exchange by a salt solution (KCl), chelation of cations by a Na₂EDTA solution, and solubilization by a cyclodextrin or a surfactant solution. Moreover, different pH values of extracting solutions were engaged to cover the different ionization states of ciprofloxacin. Three different soft extractants were selected and used to quantify the environmental availability of ¹⁴C-labelled ciprofloxacin in soil/EOM mixtures during a one-month incubation.

II.2. Materials and methods

II.2.1. Ciprofloxacin

[2-¹⁴C]-ciprofloxacin was purchased from American Radiolabeled Chemicals Inc. (Saint Louis, MO, USA). The radiochemical purity and specific activity were 98.4% and 0.55 GBq mmol⁻¹, respectively. The stock ethanolic solution was diluted to adequate concentration with Milli-Q water (Millipore, Saint-Quentin-en-Yvelines, France). Non-labelled ciprofloxacin was purchased for HPLC analysis (Dr Ehrenstorfer GmbH, Aurburg, Germany). Ciprofloxacin presents four pKa:

3.0, 5.8-6.1, 8.7-8.8 and 10.6 and its main form at pH 7 is a zwitterion (Vázquez et al., 2001; Qiang and Adams, 2004).

II.2.2. Soil and exogenous organic matter

The 0-28 cm horizon of an Albeluvisol soil (IUSS Working Group WRB, 2015) located in Feucherolles (Yvelines, France) was sampled in plots amended with either a bovine manure or a compost of sewage sludge and green wastes (20:80 v/v %, respectively) two months after EOM application in 2013. Since 1998 these plots have received organic amendments equivalent to 4 t carbon/ha/application every two years in September (Cambier et al., 2014). Soil samples were sieved to 4 mm, dried to 20% moisture (water/dry mass) and stored at 4 °C. Their main characteristics were similar: clay 15%, silt 79%, sand 6% for both soils; pH 6.8, organic carbon 15.6 g kg⁻¹ dry mass, CEC 9.8 cmol₊ kg⁻¹ for the compost-amended soil; pH 7.2, organic carbon 14.9 g kg⁻¹, CEC 9.9 cmol₊ kg⁻¹ for the manure-amended soil.

The two EOMs spread on soils in September 2013 differed according to pH: 7.7 and 8.8, organic carbon content: 257 and 355 g kg⁻¹ and dry mass: 67 and 34%, respectively for compost and manure. Other information is detailed in Table II.S1 (Supplementary material). The compost was sieved to 5 mm and the manure was ground. Both EOMs were freeze-stored at -20 °C. Ciprofloxacin was neither detected in the manure nor in the manure-amended soil, whereas 309 ± 22 and 8.7 ± 0.6 µg ciprofloxacin kg⁻¹ dry matter were quantified in the compost and in soil amended with this compost two months earlier (Ferhi et al., 2016).

II.2.3. Preselection of centrifuge tube material

The extractions were performed directly in centrifuge tubes to limit the transfer of contaminated samples in different containers, which could increase antibiotic losses by adsorption on container walls. A preliminary study was necessary to select the material on which the ciprofloxacin sorption would be the lowest. Five different centrifuge tubes were tested: 1) Corex[®] in aluminosilicate glass (25 mL, Corning, NY, USA); 2) regular laboratory glassware in borosilicate glass (30 mL, Cloup, Champigny-sur-Marne, France); 3) Falcon[®] in polypropylene (50 mL, Corning); 4) Nalgene[®] in polycarbonate (30 mL, ThermoFisher Scientific, Villebon-sur-Yvette, France); 5) Nalgene[®] in polypropylene copolymer (30 mL, ThermoFisher Scientific). The portion

of the caps in contact with the sample was made of PTFE for tubes 1-2, polyethylene for tubes 3 and polypropylene for tubes 4-5.

Fifteen milliliters of ^{14}C -ciprofloxacin solution in Milli-Q water ($39 \mu\text{g L}^{-1}$) were shaken overnight in the dark in each material with an overhead shaker (~ 17 h, 16 rpm, Heidolph[®] Reax 20, Schwabach, Germany). The fraction of antibiotic adsorbed on the material was deduced from the ^{14}C -activity measured in 2 mL aliquots after the shaking period (section II.2.6.1).

Then, the shaking was repeated in the selected centrifuge tube material with the ^{14}C -ciprofloxacin in each extracting solution ($46 \mu\text{g L}^{-1}$, section II.2.4, Table II.S2). The ^{14}C -ciprofloxacin concentration in water and in the extracting solution was based on the theoretical concentration in the extracts of soil/EOM mixtures.

II.2.4. Spiking of soil/EOM mixture and soft extraction method

To select extracting solutions and to carry out incubation experiments, 5.0 g dry mass of soil was weighed in the selected centrifuge tube (section II.2.3) and the corresponding EOM (0.15 g dry mass) was added at the surface of the soil. Then, 100 μL of ^{14}C -molecule solution in Milli-Q water (12 kBq mL^{-1}) were added on the EOM before its incorporation to the soil. This step allowed limiting the contact between the ^{14}C -molecule and the container and reproducing field conditions, i.e., the antibiotic transfer to the soil by the application of contaminated EOM (Boxall et al., 2012). To be realistic, the soil/EOM ratio corresponded to the incorporation of EOM by ploughing in the 28 cm topsoil layer. The ^{14}C -spiked EOM was mixed with soil after ~ 1 h of contact. For the extraction tests, applied ^{14}C -ciprofloxacin concentration was 4.6 mg kg^{-1} EOM and a 24 h-contact time at ambient temperature ($\sim 20 \text{ }^\circ\text{C}$) was allowed before extraction.

To perform soft extractions, the extracting solutions were prepared by dissolution in Milli-Q water of chemical products with purity of 98-99% at minimum (Table II.S2): potassium chloride (KCl, Carlo Erba Reagents, Val-de-Reuil, France), sodium ethylenediaminetetraacetic acid (Na_2EDTA , Sigma-Aldrich, Saint-Quentin Fallavier, France), sodium dodecylsulfate (SDS, Sigma-Aldrich), sodium tetraborate (Borax, Normapur RP, Prolabo), and five different cyclodextrins (Sigma-Aldrich): β -cyclodextrin (β -CD), 2-hydroxypropyl- β -cyclodextrin (2-HP- β -CD), 2-hydroxypropyl- γ -cyclodextrin (2-HP- γ -CD), 2-hydroxyethyl- β -cyclodextrin (2-HE- β -CD), triacetyl- β -cyclodextrin (TA- β -CD).

All aqueous solutions were preselected while bearing in mind that a gradient of extraction strength could be involved from aqueous extraction (CaCl_2 , KCl) to cyclodextrin-based extraction in order to access to available contaminants in soil, from the dissolved fraction up to the rapidly

desorbed fraction (Ortega-Calvo et al., 2015). A volume of 15 mL of each aqueous solution was added to the soil/EOM mixture and the suspension was shaken overnight as described in section II.2.3. The extracts were obtained in triplicates after centrifugation at $11\ 000 \times g$ for 10 min (Sorvall[®] Evolution-RC, ThermoFisher Scientific). The ^{14}C -activity was measured in the extract and in the centrifuge pellets as described in section II.2.6.1.

II.2.5 Incubation experiments

Soil samples moistened at pF 2.5 (0.24 g water per g dry soil) were pre-incubated for 3 d at 28 °C in closed Falcon[®] centrifuge tubes. Then, four treatments were prepared in three replicates: 1) soil alone; 2) soil mixed with the corresponding EOM (compost or manure); 3) soil mixed with the corresponding EOM (soil/compost and soil/manure) initially spiked with ^{14}C -ciprofloxacin and 4) blanks without any soil, EOM or antibiotic. The soil and EOM masses were the same as in section II.2.4. The final concentration of ^{14}C -ciprofloxacin was equal to 5.1 mg kg⁻¹ dry mass of EOM.

Each individual tube was introduced in a hermetically-closed jar (1 L, Le Parfait, France). A first vial filled with water was added to maintain the moisture conditions constant, followed by a second one filled with NaOH solution (1 N, 6 mL) to trap the ^{12}C -CO₂ and the ^{14}C -CO₂. Jars were placed in the dark at 28 °C for 7 or 28 d, and were aerated upon renewal of NaOH traps. Thereby, the mineralization of the total organic carbon (^{12}C -CO₂) and the ^{14}C -antibiotic (^{14}C -CO₂) were measured over the 28 d. The ^{12}C -CO₂ was analyzed using a colorimetric method (Colorimetric Skalar Analyzer, Breda, Netherlands) and the ^{14}C -CO₂ was determined according to the procedure described in section II.2.6.1.

After 0, 7 or 28 d of incubation, in a first step, the soft extraction method with the three selected extractants was applied (according to section II.2.4) in order to quantify the environmentally-available fractions of ciprofloxacin. The pH was measured in the extract over the incubation using a pH-meter (InoLab pH7110, WTW, Weilheim, Germany). In a second step, a solvent-based extraction was performed on the centrifugation pellets in order to measure the maximum extractable fraction, according to Ferhi et al. (2016). We could assume that this step extracted ciprofloxacin that was more strongly sorbed to soil/EOM mixture and therefore not immediately available. Briefly, this method is based on two successive 15 min ultrasonic extractions, using first a 30/70 (v/v) mixture of McIlvaine buffer pH 9 / acetonitrile and then a 30/70 (v/v) mixture of McIlvaine buffer pH 2 containing 0.41 mol L⁻¹ Na₂EDTA / acetonitrile. The ^{14}C -activity was measured in each extract and in EOM/soil solid phase to quantify the non-extractable ^{14}C -residues with the procedures described in section II.2.6.1.

II.2.6. Analytical determinations

II.2.6.1. Radioactivity measurements

All ^{14}C -activity measurements were determined by liquid scintillation counting (Tri-Carb 2100 TR, Packard Instruments, Meriden, CT, USA) after dilution of the sample with Ultima Gold XR scintillation cocktail (Perkin Elmer, Waltham, MA, USA). This method was applied to an aliquot of each supernatant extract from soft extractions and from solvent-based extraction. Mass balances on radioactivity were completed by quantification of the non-extractable residues in soil/EOM mixtures. The samples were dried at 40 °C for 24 h, ground and combusted in an oxidizer (Biological Oxidizer OX 700, Zinsser Analytic, Frankfurt, Germany). The ^{14}C -CO₂ produced during the combustion was trapped in Oxysolve 400 (Zinsser Analytic) and measured by liquid scintillation counting. All ^{14}C -fractions were expressed as percentages of initial ^{14}C -activity added to the sample.

II.2.6.2. HPLC analyses

Extracts containing sufficient ^{14}C -activity were analyzed by high performance liquid chromatography (HPLC). The injection volume was adapted according to the radioactivity measured in the sample, which needed to be in the 125-250 Bq range. In order to reach this threshold, samples were evaporated to a droplet and diluted in 1.5 mL of mobile phase. HPLC analysis was performed on a Flexar system (Perkin Elmer, Villebon-sur-Yvette, France) equipped with a photodiode array detector and a flow scintillation detector (Radiomatic 150TR). Separation was achieved on a 250 x 4.6 mm 5 μm XBridge-C18 column (Waters, Milford, MA, USA), using a 1 mL min⁻¹ mobile phase constituted of A) Milli-Q water + 0.01% TFA (trifluoroacetic acid, Sigma-Aldrich) and B) acetonitrile + 0.01% TFA, with a gradient from 5 to 35% of B in 31 min and to 100% in 1 min held for 5 min. Absorbance was measured continuously in the range of 190-400 nm. The data were processed with the Chromera[®] software from Perkin Elmer. The UV spectra of detected peaks were compared to that of ciprofloxacin standard, which presented a maximum of absorption at 281 nm. The ciprofloxacin retention times were 19.8 min (UV detection) and 20.3 min (radioactivity detection).

II.2.7. Statistical analyses

Statistical analyses were realized with RStudio software (version 0.98.501, RStudio Inc.) to compare the concentrations ($\mu\text{g } ^{14}\text{C}$ -residues per kg of total dry mass) measured in the available, extractable, non-extractable and mineralized fractions during the incubation; and also to compare the pH measured in soft extracts. Differences according to the first-used extractant and the EOM type added to the soil were highlighted using linear mixed-effects models (ANOVA) and pairwise differences were determined.

II.3. Results and Discussion

II.3.1. Selection of the centrifuge tube material

Ciprofloxacin is known to adsorb to laboratory materials, in particular onto glassware via ion exchange at silicate and silanol groups (Baker and Kasprzyk-Hordern, 2011). When the solution contained only ^{14}C -ciprofloxacin in water (Table II.1-A), the adsorbed fraction was very high on glassware and also on polycarbonate and polypropylene copolymer in Nalgene[®] tubes (67-95%). The ciprofloxacin loss was minimized with the polypropylene material (Falcon[®]) with only 50% adsorbed. Consequently, Falcon[®] centrifuge tubes were selected in all subsequent experiments.

The presence of the soft extractant decreased the fraction adsorbed in Falcon[®] tubes, ranging from 6% of ^{14}C -activity for 2-HP- β -CD and Borax solutions to 23% for KCl solution (Table II.1-B). This may be due to a higher affinity of ciprofloxacin to chemicals in solution and/or a competition between the antibiotic and the chemicals in solution for the sorption sites of the tube walls and caps.

Table II.1

Losses of ^{14}C -ciprofloxacin (% initial ^{14}C -activity, average \pm standard deviation) by adsorption on tube after the shaking in A] Milli-Q water ($39 \mu\text{g L}^{-1}$) in different materials (n = 3); B] the extracting solutions ($46 \mu\text{g L}^{-1}$) in Flacon[®] tubes (n = 2).

A] ^{14}C -ciprofloxacin in water		B] ^{14}C -ciprofloxacin in extracting solution	
Centrifuge tubes	Adsorption loss %	in Falcon [®] tubes	Adsorption loss %
Aluminosilicate glass (Corex [®])	90 ± 3.0	KCl	23 ± 0.1
		Na ₂ EDTA 0.1 M pH 4.6	15 ± 1.0
Borosilicate glass	78 ± 7.0	Na ₂ EDTA 0.1 M pH 7	14 ± 1.3
		Na ₂ EDTA 0.2 M pH 7	13 ± 1.4
		β -CD	7 ± 1.3
Polypropylene (Falcon [®])	50 ± 1.0	2-HP- β -CD	6 ± 0.3
		2-HP- γ -CD	8 ± 0.6
Polycarbonate (Nalgene [®])	81 ± 14	2-HE- β -CD	7 ± 0.6
		TA- β -CD	9 ± 0.5
Polypropylene (Nalgene [®])	83 ± 11	SDS	11 ± 1.6
		Borax	6 ± 0.7

II.3.2. Selection of the soft extracting solutions to assess the environmental availability in soil/EOM mixtures

Different extracting solutions were preselected (Table II.2). By varying their pH (Table II.S2), the different ionic forms of ciprofloxacin were covered (pKa, section II.2.1). Calcium chloride (CaCl₂) classically used in sorption experiments was replaced by potassium chloride (KCl) since divalent cations can be chelated by fluoroquinolones (Carrasquillo et al., 2008). The EDTA organic ligand, as an efficient competitor for cations complexation, could promote in excess the disruption of ciprofloxacin-cation chelates and thus ciprofloxacin extraction. Consequently, we tested two different concentrations: 0.1 and 0.2 M. At the concentration of 0.1 M Na₂EDTA, two pHs were tested: 4.6 (initial pH) and 7 (soil pH). The evaluation of environmental availability by entrapment (e.g., encapsulation in cyclodextrin or inclusion in micelles; ISO 17402, 2008) is largely encouraged and mostly documented for hydrophobic compounds with 2-HP- β -CD as the main trapping extractant tested in solution. β -CD was shown to engage another quinolone (Iacovino et al., 2013). Both determinant for cyclodextrin hydrophobicity and ability to engage various types of interactions, we chose to vary 1) the cyclodextrin size with cyclooctaamylose for 2-HP- γ -CD vs cycloheptaamylose for all β -CD and 2) the functionalization of the inner cavity with hydroxyl, hydroxyethyl and triacetyl groups for β -CD, 2-HE- β -CD and TA- β -CD respectively. Cyclodextrin

solutions were used at their initial pH in order to avoid structure alteration. The concentration for 2-HP- β -CD, 2-HP- γ -CD and 2-HE- β -CD was based on the literature (Reid et al., 2000b; Rhodes et al., 2008) and adapted for β -CD and TA- β -CD according to their water solubility. The pH of the SDS solution was adjusted to 7 (soil pH) and the SDS concentration was close to the critical micelle concentration (CMC) to obtain a sufficient formation of micelles that could efficiently extract the contaminant (Bustamante et al., 2012). The Borax solution (0.02 M) was chosen for its properties of both surfactant and alkaline buffer (natural pH at 9, Table II.S2).

The efficiency of soft extracting solutions was very low in all conditions, with less than 7.2% of ^{14}C -ciprofloxacin extracted (Table II.3). The KCl solution extracted only 0.5 and 1.7% of ^{14}C -ciprofloxacin from soil/compost and soil/manure mixtures, respectively. The Na_2EDTA solutions extracted 1.1-4.9% of ^{14}C -ciprofloxacin, the cyclodextrin or SDS solutions 0.5-2.8%. The greatest amounts were obtained with the Borax solution, with 2.4% in soil/compost and 7.2% in soil/manure mixtures. The mass balances reached 84 ± 5.4 to $94 \pm 6.6\%$ of initial added ^{14}C -activity showing no important loss by adsorption on centrifuge tubes.

From these extraction tests, three extracting solutions were selected with respect to three different operational criteria: 1) facility to prepare the aqueous solution, 2) repeatability and 3) extraction recoveries. These soft extractants should be also representative of different extraction mechanisms involving the chemical properties of ciprofloxacin and/or the change in organic matter composition. First, the solution of Na_2EDTA (0.1 M, pH 7) was selected among the three Na_2EDTA solutions, considering the higher extractability of ^{14}C -ciprofloxacin in both soil/EOM mixtures. The Borax solution maximizing the ^{14}C -ciprofloxacin available fraction in soil/manure mixtures was also retained. Finally, one cyclodextrin extractant was also selected although ^{14}C -ciprofloxacin recoveries were very low and equivalent for the five cyclodextrin solutions in each EOM/soil mixture. The 2-HP- β -CD was selected considering both its easy solubilization in water and its extended usage to evaluate the environmental availability of organic contaminants. These three extracting solutions were used to evaluate the environmentally-available fraction of ^{14}C -ciprofloxacin during incubation experiments.

Table II.2

Mechanisms, targeted fractions and example of compounds extracted by the pre-selected soft extractants (Reid et al., 2000a; Harmsen, 2007; Ortega-Calvo et al., 2015).

Soft extractants	Mechanisms of extraction	Targeted fractions	Example of targeted contaminants
CaCl ₂ , KCl	Ionic exchange	Aqueous phase	Major and trace elements
Na ₂ EDTA	Chelating agent, competition	Dissolved organic matter-associated fraction, potentially soluble fraction	Metals
Cyclodextrins (β-CD, 2-HP-β-CD, 2-HP-γ-CD, 2-HE-β-CD, TA-β-CD) Surfactants (SDS, Borax)	Solubilizing agent, competitive adsorption	Solid surface-associated and pore-diffused adsorbed fractions	Organic pollutants (e.g., pesticides, PAHs)

Table II.3

Available and non-extractable fractions (% initial ¹⁴C-activity) of ¹⁴C-ciprofloxacin (n = 3, mean ± standard deviation) in soil/EOM mixtures (24 h-contact, ~ 20°C).

Extracting solution	Soil/compost mixtures		Soil/manure mixtures	
	% available	% non-extractable	% available	% non-extractable
KCl	0.5 ± 0.0	91.0 ± 1.5	1.7 ± 0.2	89.6 ± 2.4
Na ₂ EDTA 0.1 M pH 4.6	1.3 ± 0.1	89.4 ± 3.4	1.1 ± 0.0	86.1 ± 1.1
Na ₂ EDTA 0.1 M pH 7	4.8 ± 0.2	87.0 ± 0.5	3.3 ± 0.1	80.8 ± 5.3
Na ₂ EDTA 0.2 M pH 7	2.7 ± 0.8	86.8 ± 1.4	2.1 ± 0.1	83.9 ± 3.6
β-CD	0.5 ± 0.1	92.4 ± 1.6	2.2 ± 0.1	85.4 ± 1.1
2-HP-β-CD	0.6 ± 0.0	93.4 ± 6.6	2.3 ± 0.1	87.3 ± 5.2
2-HP-γ-CD	0.5 ± 0.1	86.4 ± 5.3	2.4 ± 0.1	83.5 ± 5.5
2-HE-β-CD	0.5 ± 0.0	92.1 ± 7.0	2.8 ± 0.1	86.2 ± 3.2
TA-β-CD	0.5 ± 0.0	89.3 ± 1.5	2.2 ± 0.2	82.2 ± 5.3
SDS	0.5 ± 0.0	92.3 ± 0.8	2.5 ± 0.3	87.0 ± 5.1
Borax	2.4 ± 0.1	90.8 ± 0.7	7.2 ± 0.3	84.3 ± 2.9

II.3.3. Behavior of ¹⁴C-ciprofloxacin during the incubation of soil/EOM mixtures

II.3.3.1. Partitioning of the ¹⁴C-activity among the fractions

Whatever the applied EOM, the environmental availability of the ¹⁴C-ciprofloxacin was initially very low (Figure II.1) with a maximum of 6.3% of ¹⁴C-activity in soil/compost mixtures (Na₂EDTA) and 7.2% in soil/manure mixtures (Borax). Though the extractions were done only ~1 h and not 24 h after the antibiotic addition, these results were consistent with those of the extraction tests (Table II.3). In soil/compost mixtures, the extractability with Borax and 2-HP-β-CD solutions decreased during incubation ($p < 0.05$) whereas it remained stable with Na₂EDTA solution. By contrast, in soil/manure mixtures, the extractability increased at day 7 with all extracting solutions ($p < 0.05$); it slightly decreased from 16% to 9.2% with Borax solution ($p < 0.05$) whereas it was stable with Na₂EDTA and 2-HP-β-CD solutions in the 7-28 d period.

Between 32 and 35% of the ¹⁴C-activity were initially recovered through the soft and the solvent-based extractions in soil/compost mixtures, and 29-38% in soil/manure mixtures. While this cumulated fraction slightly decreased to 23% in soil/compost mixtures in 28 d ($p < 0.05$), it remained more stable in soil/manure mixtures at 28%. The ratio between available fraction and maximum extractable fraction was globally constant over the incubation, for the three soft extractants. Given the amounts of radioactivity in the different extracts, HPLC analysis could only be performed in the organic phases from the solvent-based extraction at pH 2 after 0 and 7 d. The chromatograms showed a main peak at the retention time of the ¹⁴C-ciprofloxacin standard, suggesting that the extractable ¹⁴C-residues were non-transformed ciprofloxacin.

The formation of non-extractable residues in the samples was high from the initial contact time, reaching 57-67% of the initial ¹⁴C-activity. This fraction was fairly stable over the incubation in soil/manure mixtures ($p > 0.05$, 60-62% at day 28). In soil/compost mixtures, however, this fraction increased after 7 d of incubation ($p < 0.05$) and was then stable ($p > 0.05$). The non-extractable fraction in soil/compost mixtures after 28 d was significantly higher than in soil/manure mixtures ($p < 0.05$). Only 0.3% of the initial applied ¹⁴C-ciprofloxacin was mineralized after 28 d in both soil/EOM mixtures. The level of mineralized ¹⁴C after one month of incubation was similar in soil amended with stabilized sludge (~0.3%, Girardi et al., 2011). Given the purity of the ¹⁴C-labelled ciprofloxacin (98%), the mineralization of some impurities instead of the ciprofloxacin itself cannot be excluded. Although the presence of ciprofloxacin in the compost and compost-

amended soil but not in manure (Ferhi et al., 2016) could suggest the potential long-term exposure of soil microorganisms to ciprofloxacin, there was no effect of microbial adaptation on biodegradation.

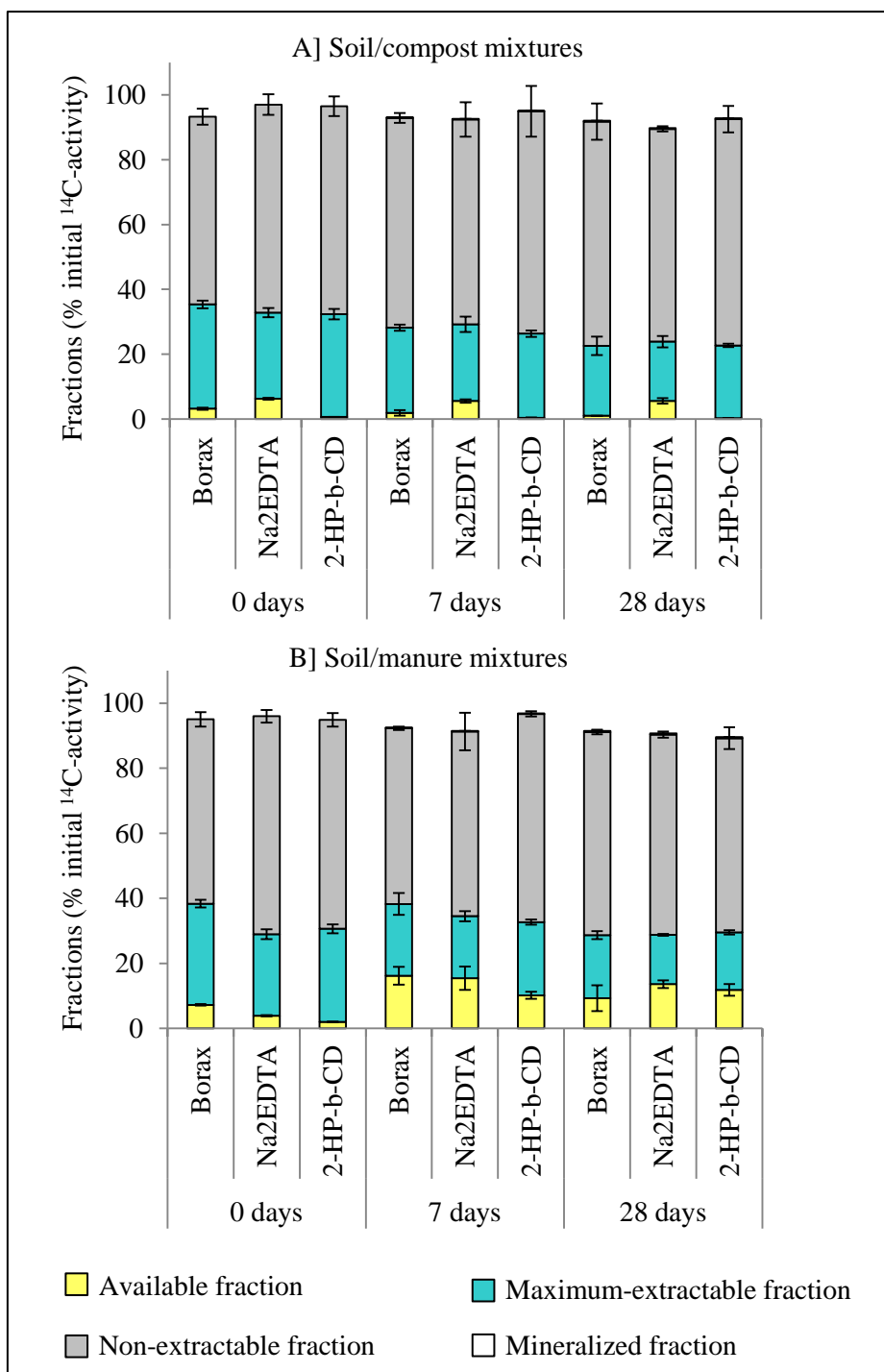


Figure II.1. Partitioning of ^{14}C -ciprofloxacin residues among the different fractions (% initial ^{14}C -activity) in function of the incubation time (d) of A) soil/compost and B) soil/manure mixtures (n = 3).

II.3.3.2. Mechanisms of ciprofloxacin adsorption

The very low extractability and the large non-extractability of ^{14}C -ciprofloxacin, from the beginning and over the incubation, were consistent with those of Girardi et al. (2011) and Mougin et al. (2013). All results together confirmed the strong sorption of ciprofloxacin on soil (Uslu et al., 2008). The ciprofloxacin adsorption is described by several mechanisms of interaction (Figure II.2). Since ciprofloxacin is an amphoteric molecule, ionic exchanges between the charges of the molecule and of the soil can contribute to the ciprofloxacin adsorption, depending on the soil pH (Vasudevan et al., 2009). The cation exchange capacity (CEC) influences the cation exchange and the cation bridging while the surface complexation depends on the metal oxide content in soil. Sorption of ciprofloxacin to soil could be mostly due to the binding of carboxylic acid negatively charged to clay minerals. Indeed, Nowara et al. (1997) showed that the decarboxylation of enrofloxacin, another fluoroquinolone, led to a lower sorption of the molecule. Other types of interactions such as hydrophobic and van der Waals interactions could be involved in the sorption of ciprofloxacin. The aromatic structure of ciprofloxacin (Figure II.2) and aromatic components of the organic matter as humic substances could facilitate these interactions (Aristilde and Sposito, 2013).

Nevertheless, our soft extraction results provided some information on the ciprofloxacin behavior in soil amended with EOM. The fate of ciprofloxacin in both EOM-amended soils showed similar trends, but slight differences were observed between the two EOM (Figure II.1). Given ^{14}C -ciprofloxacin was added on the EOM and a 1 h-contact was let before the incorporation of EOM into soil, we assumed that the antibiotic was in the first place adsorbed on the EOM. As a consequence, the ciprofloxacin behavior in amended soil was influenced by the properties of the applied EOM. Firstly, the organic matter quality involved different ciprofloxacin behaviors over the incubation (section II.3.3.3). Secondly, regarding the ciprofloxacin sorption depending on the pH (Vasudevan et al., 2009), the pH of the EOM influenced the ionization state of the antibiotic and its extractability (section II.3.3.4).

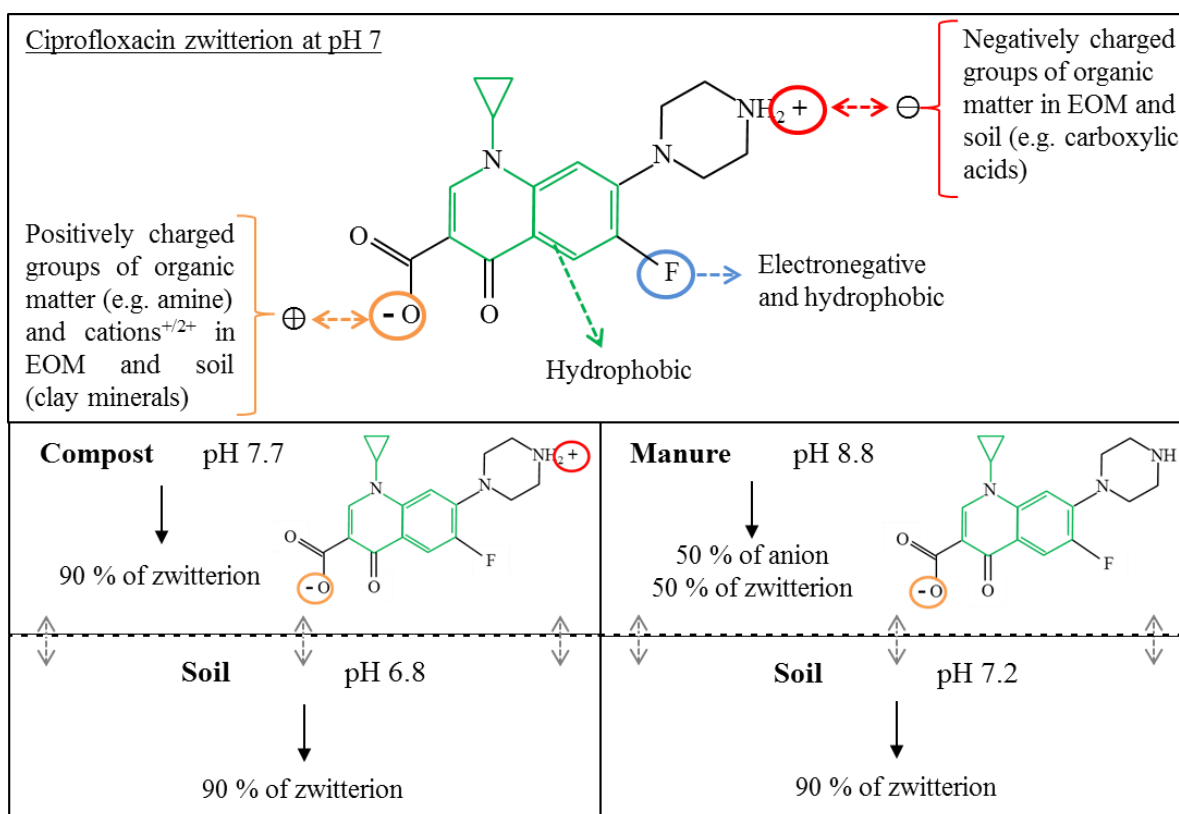


Figure II.2. Key interaction sites of ciprofloxacin adsorption on EOM and soil in function of the pH and the chemical ionic form (Vasudevan et al., 2009). Are not represented the hydrophilic interactions possible with non-bonding electrons.

II.3.3.3. Organic matter effect on ciprofloxacin behavior

The two EOMs were different in terms of organic matter quality. Regarding their biochemical composition, the concentration of water-soluble organic carbon was equal to 72.4 g kg⁻¹ dry mass in compost and 111.6 g kg⁻¹ dry mass in manure (Table II.S1). The water-soluble organic matter is easily extractable. Consequently, considering the capacity of ciprofloxacin to be sorbed on dissolved organic carbon (Carmosini and Lee, 2009), we could suppose that ciprofloxacin was more adsorbed ciprofloxacin on soluble organic matter in manure than in compost. It was confirmed by the soft extractions with greater available fractions in soil/manure mixtures than in soil/compost mixtures (except for Na₂EDTA at day 0; section II.3.3.1). Furthermore, soluble organic matter is the first source of available carbon for microbial degradation. The indicators of residual organic carbon (I_{ROC}; Lashermes et al., 2009) equal to 70% for compost and 56% for manure (Table II.S1) were in accordance with the greater stability of organic matter in compost than in manure. Thus, the organic carbon biodegradation was higher in soil/manure mixtures than in soil/compost mixtures. The mineralized fractions indeed reached 10% in soil/compost mixtures and 27% in soil/manure mixtures after 28 d (Figure II.S1). The biodegradation of the organic matter could be responsible for

the release of initially-adsorbed ciprofloxacin, in particular in soil/manure mixtures in which the available fractions increased between 0 and 7 d of incubation (Figure II.1, section II.3.3.1). Surprisingly, the mineralized fraction of ^{14}C -ciprofloxacin did not increase in soil/manure mixtures. A possible explanation in this respect is that the molecule was not used as a carbon source by soil microorganisms. The presence of ^{14}C -ciprofloxacin at the considered concentration had no effect on the mineralization of the organic carbon in both soil/EOM mixtures (Figure II.S1).

During incubations, the cations present in soil/EOM mixtures were simultaneously redistributed with the organic matter evolution. The Na_2EDTA solution chelated the cations and co-extracted the organic matter as suggested by the color of the extracts (Figure II.S2) and this, whatever the ageing of the soil/EOM mixture. The ^{14}C -ciprofloxacin molecules adsorbed on organic matter by cationic exchange were consequently extracted. Since the organic matter was stable in compost, the available fraction of ciprofloxacin quantified with the Na_2EDTA -based extraction was stable in soil/compost mixtures over the incubation. Comparatively, the mechanisms of extraction of Borax and 2-HP- β -CD solutions were different and the available fractions extracted with both solutions decreased over the incubation in soil/compost mixtures. The non-extractable fraction increased in parallel between 0 and 7 d, and was stable between 7 and 28 d (section II.3.3.1). Even if the mineralized fraction of ^{14}C -ciprofloxacin was very low, the formation of biogenic non-extractable residues cannot be excluded.

In soil/manure mixtures, the available fraction was relatively stable with Na_2EDTA and 2-HP- β -CD-based extractions whereas it slightly decreased with Borax-based extraction (section II.3.3.1) between 7 and 28 d. Considering the HPLC analyses, the extracts were mostly composed of ciprofloxacin after 7 d; consequently, the availability increase in soil/manure mixtures was not due to ciprofloxacin biodegradation. The antibiotic was probably redistributed after its release by the biodegradation of the organic matter between 0 and 7 d. Nevertheless, the organic matter of the manure was not yet stabilized after 28 d (Figure II.S1).

We can conclude that the organic matter quality influenced the initial partitioning of ciprofloxacin in soil/EOM mixtures but also the time evolution of available fractions due to decomposition processes occurring in soil. EOM biodegradability was therefore an important driver of the fate of ciprofloxacin in amended soils.

II.3.3.4. Effects of pH: EOM and extracting solutions

The pH may have governed the ionization state of ciprofloxacin being mostly zwitterionic in the compost (pH 7.7) and both anionic and zwitterionic in the manure (pH 8.8). The greater sorption on compost (Figure II.2) could be explained via increased interactions by ionic exchanges with zwitterionic ciprofloxacin (Vasudevan et al., 2009) and a greater amount of exchangeable cations in compost than in manure (CEC 47.3 and 32.3 $\text{cmol}_+ \text{kg}^{-1}$ dry mass, respectively, Table II.S1). A greater sorption in soil/compost than in soil/manure mixtures was confirmed over the incubation, except at day 0 with the Na_2EDTA solution (Figure II.1).

The pH was measured in soft extracts and the values were consistent with the initial pH of the solution, i.e., 9 for Borax and 7 for Na_2EDTA and 2-HP- β -CD solutions. The pH was stable in all extracts for 28 days. The EOM pH influenced the extract pH given it was slightly higher in extracts from soil/manure mixtures than in extracts from soil/compost mixtures ($p < 0.05$). Consequently, the higher extractability of ^{14}C -ciprofloxacin in soil/manure mixtures than in soil/compost mixtures with the three soft extractants ($p < 0.05$, except at day 0 with Na_2EDTA) was due to the different ionization states of the antibiotic. In the manure, the extraction of anionic ciprofloxacin was easier at alkaline pH with the Borax solution (pH 9), keeping the anionic form and increasing the antibiotic solubility in the aqueous solution (Yu et al., 1994), compared to the two other soft extracting solutions (pH 7), in particular at day 0 (Figure II.1).

Considering the solvent-based extraction, a higher recovery level of ciprofloxacin was expected at pH 2 than at pH 9 due to the ionization state of the molecule modifying its interactions with the solid matrix, as well as the presence of EDTA in the acetonitrile/buffer (Ferhi et al., 2016). The distribution of ^{14}C -activity extracted at pH 9 and pH 2 depended on the EOM type and also on the previously-performed soft extraction (Figure II.3). In soil/compost mixtures, 75-88% of the maximum extractable ^{14}C -activity was recovered at pH 2 over the incubation. In soil/manure mixtures, the extracted fraction at pH 2 decreased in 28 d from 53-82% to 44-62% of maximum extractable residues ($p < 0.05$), with a concomitant increase of the extracted fraction at pH 9. The highest recovery level at pH 9 (56%) was obtained after the Borax-based soft extraction. Indeed, by using extracting solutions at pH 9, the mineral surface of soil/manure mixture progressively became negative that increased the electrostatic repulsions with anionic ciprofloxacin (Wu et al., 2013). Notably, the longer the ^{14}C -ciprofloxacin was in contact with manure in soil, the greater it was extracted at pH 9. As discussed before for the Borax solution, the higher initial pH of manure was

responsible for the presence of both anion and zwitterion of ciprofloxacin and the solvent-based extraction at pH 9 was easier to keep in the anionic form.

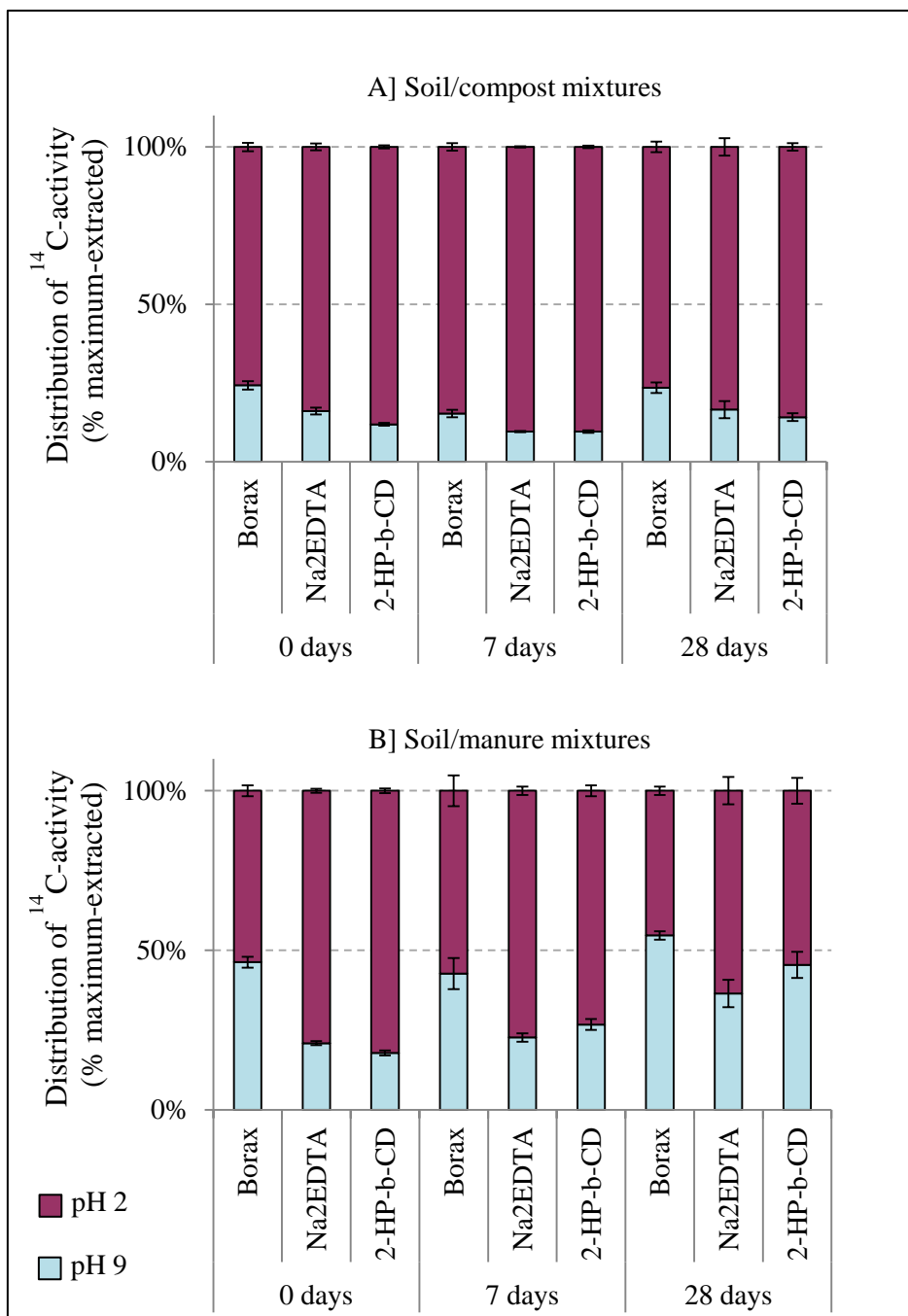


Figure II.3. Partitioning of the ¹⁴C-activity (% maximum extractable, n = 3) in fractions obtained with the solvent-based extraction, depending on the pH of the acetonitrile/buffer mixture for A] soil/compost and B] soil/manure mixtures. Borax, Na₂EDTA, 2-HP-β-CD corresponded to the previously-used soft extractants.

II.3.4. About soft extraction methodology to target the environmental availability

The three selected solutions extracted ciprofloxacin differently, in terms of the mechanism of extraction involved (solubilising *vs* chelating agents; Table II.2), the pH, and the chemical forms of both extractant and ciprofloxacin. It also depended on whether ciprofloxacin was initially in the compost or in the manure.

Borax (0.02 M, pH 9) presents both boric acids and borate ions in water. Ion pair complexes could be formed between Borax and ciprofloxacin by interactions between the negative charges of borate ions and ciprofloxacin zwitterion and also between the positive charges of boric acids and ciprofloxacin zwitterion or anion. In consequence, the Borax solution allowed a better extraction of ¹⁴C-ciprofloxacin from soil/manure mixtures and its efficiency as a surfactant was related to the presence of ciprofloxacin anions in the aqueous solution.

Contrary to Borax, the cage molecule 2-HP-β-CD presents a hydrophilic layer outside and a hydrophobic core inside the molecule, and at neutral pH, the 2-HP-β-CD was not ionic. Ciprofloxacin antibiotic was too strongly adsorbed in soil/EOM mixtures to be extracted by the 2-HP-β-CD solution (0.05 M, pH 7) proceeding as encapsulating agent. The cyclodextrin-based extraction usually works for non-polar compounds and in consequence a polar compound like ciprofloxacin is not extracted well from soil/EOM mixtures. Moreover, the bigger structure of 2-HP-β-CD than Borax probably does not allow the molecule to accede to clay minerals where ciprofloxacin could be sorbed.

Tests of the Na₂EDTA solution (0.1 M, pH 7) as a chelating agent suggest that, at pH 7, the molecule presents negative charges with deprotonated carboxylic acids. The mechanism of extraction is the chelation of metallic cations, and an important co-extraction and solubilisation of organic matter (Figure II.S2), which allows ¹⁴C-ciprofloxacin bound to cations or adsorbed on the organic matter to be released. This explains the greater extraction initially from the soil/compost mixture (6.3%) than from the soil/manure mixture (3.9%) in relation with the greater amount of exchangeable cations in compost. Despite sample ageing, the available fraction of ¹⁴C-ciprofloxacin extracted with the Na₂EDTA solution remained stable in time possibly due to the more stable organic matter in the compost but also to the implication of mineral sorbing phases. In the case of manure containing more biodegradable organic matter, the availability of ciprofloxacin increased after 7 d and remained relatively stable afterwards.

Therefore, the Na₂EDTA solution (0.1 M, pH 7) could be the most adapted to assess the environmental availability of ciprofloxacin in compost-amended soils. In manure-amended soils, the soft extraction was improved with an alkaline solution such as the Borax solution (0.02 M, pH 9). As the results were not concluding for the cyclodextrin-based extraction and considering the high cost of this chemical product, 2-HP-β-CD could be not retained for the assessment of ciprofloxacin availability.

II.4. Conclusion

The soft extraction methods used in this study allowed unravelling the behavior of ciprofloxacin in agricultural soils after EOM application in terms of environmental availability, with respect to different sorption mechanisms. The environmental availability and its time evolution depended on the initial properties of EOM, and mostly on the pH, the quality and biodegradability of organic matter. Na₂EDTA and Borax solutions were selected to assess the ciprofloxacin availability in soil/compost and soil/manure mixtures, respectively.

Considering the strong sorption of ciprofloxacin, precautions concerning the laboratory material must be taken into account, from the soil sampling to the analysis in the laboratory to minimize ciprofloxacin losses. In order to assess the environmental availability of other antibiotics in agricultural soils, the soft extraction method must be tested in controlled conditions before being applied to real samples. We suggest that the choice of the extractant should also consider the type of EOM applied since the behavior of the antibiotic may differ significantly according to EOM properties. One important result is that ciprofloxacin soft extraction is improved when the pH of the extracting solution is close to the pH of the EOM. In a next step, the available fractions extracted with the soft extractants selected in this study have to be confronted with ciprofloxacin effects on microbial activities such as nitrification. This will help us to conclude if the environmentally-available fraction is bioavailable for soil microorganisms.

Acknowledgements

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II. Supplementary material

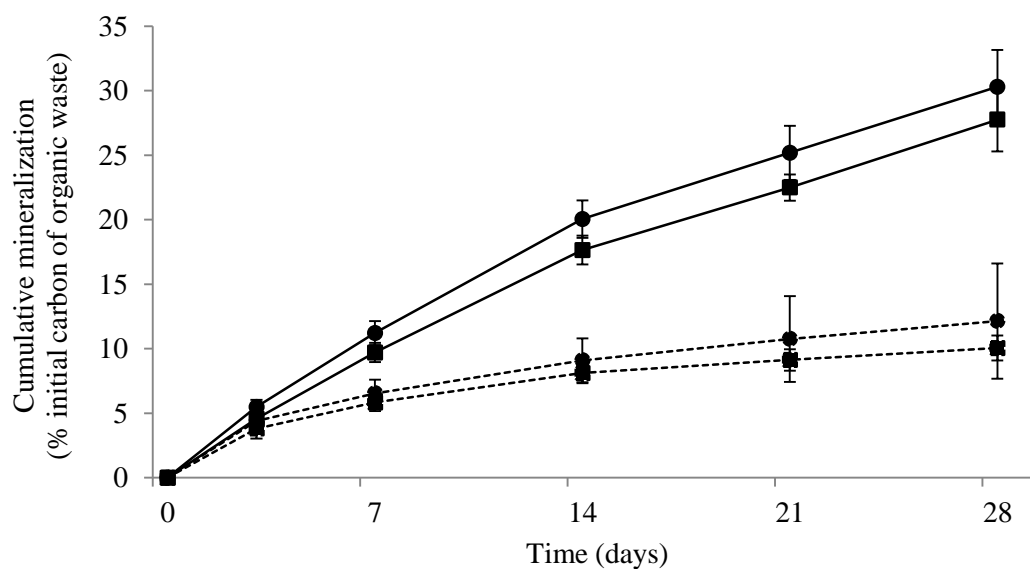


Figure II.S1. Cumulative mineralization of the organic matter (% of the carbon initially added by the EOM, $n = 3$) in soil amended with (—) manure; (---) compost; (■) without ^{14}C -ciprofloxacin; (●) with ^{14}C -ciprofloxacin.

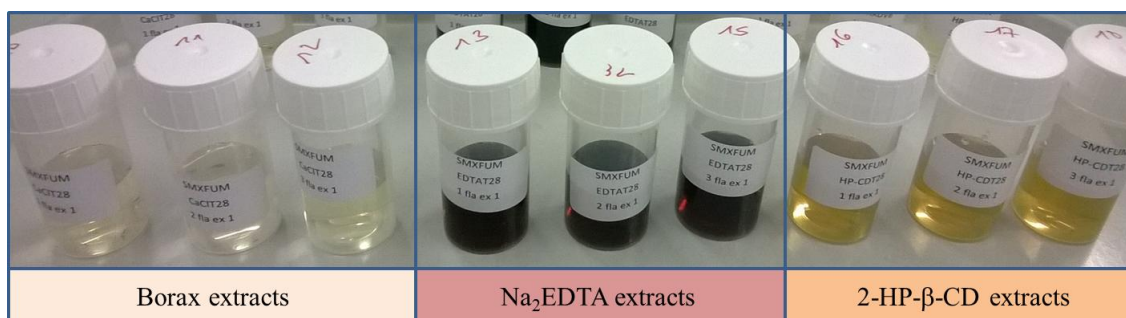


Figure II.S2. Color of Borax, Na_2EDTA and 2-HP- β -CD extracts. The color depended on the amount of co-extracted organic matter: Borax < 2-HP- β -CD <<< Na_2EDTA .

Table II.S1Characteristics and compositions of the compost of sewage sludge/green waste and the bovine manure (n = 3, average \pm standard deviation).

Characteristics and compositions									
	pH (water)	Dry mass %	Organic carbon g kg ⁻¹	Total N g kg ⁻¹	Mineral N g kg ⁻¹	C/N	CEC cmol ₊ kg ⁻¹		
Compost	7.7 \pm 0.1	67.2 \pm 1.3	257 \pm 3	25.9 \pm 0.5	6.4 \pm 0.1	9.9 \pm 0.3	47.3 \pm 2.4		
Manure	8.8 \pm 0.3	33.9 \pm 0.7	355 \pm 14	20.1 \pm 0.7	0.5 \pm 0	17.6 \pm 0.1	32.3 \pm 3.1		
Organic matter (OM)									
	Soluble OM in water			Soluble OM in detergent		Cellulose	Lignin	I _{ROC}	%
	g kg ⁻¹			% OM					%
Compost	464 \pm 8	15.6 \pm 2.7	46.8 \pm 1.5	6.3 \pm 2.0	23.7 \pm 2.5	23.2 \pm 1.7	70.3 \pm 1.4		
Manure	649 \pm 2	17.2 \pm 1.8	32.1 \pm 2.2	14.1 \pm 1.5	35.7 \pm 0.7	18.1 \pm 0.3	55.8 \pm 1.3		
Major and trace metals									
	Cr	Cd	Cu	Ni	Pb	Zn	As	Se	Hg
	mg kg ⁻¹								
Compost	43 \pm 1	0.9 \pm 0.1	209 \pm 12	22 \pm 0	66 \pm 4	471 \pm 11	14.9 \pm 0.1	1.4 \pm 0	0.47 \pm 0.01
Manure	14 \pm 1	0.2 \pm 0.4	44 \pm 0	5 \pm 0	14 \pm 2	215 \pm 3	2.1 \pm 0.2	0.6 \pm 0	0.11 \pm 0.01
	CaCO ₃	P ₂ O ₅	K	Ca	Mg	Na	Al	Fe	Mn
	g kg ⁻¹								
Compost	37 \pm 12	27.7 \pm 0.8	17.1 \pm 0.5	47.9 \pm 1	5.5 \pm 0.1	4.4 \pm 0.3	22.3 \pm 0.2	29.7 \pm 0.3	0.73 \pm 0.01
Manure	46 \pm 3	15.7 \pm 0.3	32.3 \pm 0.6	28.4 \pm 1.5	4.9 \pm 0.1	5.4 \pm 0.2	6.1 \pm 1	4.1 \pm 0.6	0.27 \pm 0.02

Table II.S2Aqueous solutions tested for the extraction of the available fraction of ^{14}C -ciprofloxacin.

Solutions used for ^{14}C -ciprofloxacin		
Name	Concentration (mol L ⁻¹)	pH
KCl	0.01	6.3 ^(a)
Na ₂ EDTA	0.10	4.5 ^(a)
Na ₂ EDTA	0.10	7 ^(b)
Na ₂ EDTA	0.20	7 ^(b)
β -CD ^(c)	0.02	7 ^(a)
2-HP- β -CD ^(c)	0.05	7 ^(a)
2-HP- γ -CD ^(c)	0.05	6.9 ^(a)
2-HE- β -CD ^(c)	0.05	ND
TA- β -CD ^(c)	0.0075	7 ^(a)
SDS	0.005	7 ^(b)
Borax	0.02	9 ^(a)

^(a) measured pH^(b) adjusted pH^(c) cyclodextrins: β -cyclodextrin (β -CD), 2-hydroxypropyl- β -cyclodextrin (2-HP- β -CD), 2-hydroxypropyl- γ -cyclodextrin (2-HP- γ -CD), 2-hydroxyethyl- β -cyclodextrin (2-HE- β -CD), triacetyl- β -cyclodextrin (TA- β -CD)

En comparaison avec les fluoroquinolones, les sulfonamides présentent une adsorption plus faible sur les matrices solides et celle-ci semble se faire en premier lieu sur la composante organique. La disponibilité environnementale des sulfonamides dans les sols peut donc être plus importante, ce qui peut représenter un risque pour l'environnement (par exemple, transfert vers les eaux souterraines) et pour les micro-organismes du sol s'ils sont exposés. Par ailleurs, les métabolites acétylés des sulfonamides excrétés et retrouvés dans les MAFOR peuvent être retransformés lors du stockage des MAFOR et/ou dans les sols amendés. Il est donc essentiel de prendre en considération les produits de transformation et de connaître aussi leur comportement et leur disponibilité dans les sols dans le cadre d'une évaluation des risques.

Certains modèles récemment développés permettent de simuler le devenir de polluants organiques (par exemple : pesticides, HAP) dans les sols après leur apport avec les MAFOR, en couplant la décomposition de la matière organique dans le sol avec l'évolution des fractions extractible, non-extractible et minéralisée du polluant. C'est le cas du modèle COP-Soil (Geng et al., 2015) également adapté au devenir des pesticides dans les mulchs de résidus de culture (Aslam et al., 2014). Cependant, dans ces modèles, le module associé au polluant organique ne décrivait pas la fraction disponible ; le modèle a donc été modifié par Khaled Brimo (doctorant au sein de l'UMR ECOSYS). Les sulfonamides interagissant avec la matière organique et étant introduits dans les sols par les MAFOR, nous avons fait l'hypothèse que le devenir des sulfonamides dans les sols pourrait être prédit au cours du temps grâce à l'utilisation d'un modèle de ce type.

Avec l'aide de Nicolas Sertillanges (stagiaire en Master 2), des extractions douces ont été réalisées pour estimer la disponibilité du sulfaméthoxazole et de son métabolite acétylé radiomarqués au carbone 14 au cours d'incubations de mélanges sol/MAFOR, afin de répondre aux questions suivantes dans le Chapitre III :

- 1) Quelle méthode chimique d'extraction peut être utilisée pour estimer leur fraction disponible ?
- 2) Quelle est leur disponibilité environnementale dans les sols amendés par les MAFOR ?
- 3) Le type de MAFOR influence-t-il leur disponibilité dans les sols ?
- 4) Les comportements du sulfaméthoxazole et du N-acétylsulfaméthoxazole sont-ils différents ?
- 5) Un modèle couplant le devenir d'un polluant avec l'évolution de la matière organique peut-il être utilisé dans le cas de ces deux composés ? Quelles réponses peuvent être apportées par ce type d'outil par rapport aux questions sur le déterminisme de la disponibilité des antibiotiques apportés aux sols via les MAFOR ?

Partie 1. Estimation de la disponibilité environnementale

Approche analytique : radiomarquage (^{14}C -antibiotique)

Chapitre III

Sols amendés par les MAFOR
**Antibiotiques + produits
de transformation**
Adsorption dans des matrices
complexes (organique + minéral)



Extractions aqueuses



Disponibilité du sulfaméthoxazole et du N-acétylsulfaméthoxazole

Adsorption et dégradation des sulfonamides influencées par la
composition des MAFOR



- 1) Quelle méthode chimique d'extraction?
- 2) Quelle disponibilité environnementale dans les sols amendés par les MAFOR?
- 3) Disponibilité influencée par le type de MAFOR?
- 4) Comportements du sulfaméthoxazole et du métabolite différents?
- 5) Utilisation d'un modèle pour décrire leur devenir dans les sols en fonction de l'évolution de la matière organique?

Chapter III: Assessing the environmental availability of sulfamethoxazole and its acetylated metabolite in agricultural soils amended with compost and manure: an experimental and modelling study

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Abstract

The recycling of sludge compost and farmyard manure in agriculture can lead to the introduction of sulfonamide antibiotics and their acetylated metabolites into soils. The quality and the biodegradability of the exogenous organic matter (EOM) containing sulfonamide residues is determinant for their environmental availability and fate in soils. This study combining experimental and modelling approaches aimed at: 1) assessing the fraction of sulfamethoxazole (SMX) and *N*-acetyl-sulfamethoxazole (AcSMX) available in EOM-amended soils by using soft extractions (CaCl₂, EDTA or cyclodextrin solutions) during a 28-day incubation; and 2) better understanding the dynamics of sulfonamide residues in amended soils in connection with their availability and the mineralization of EOM thanks to a model coupling the organic matter evolution to the organic pollutant behavior (COP-Soil model).

The available fraction in both soil/EOM mixtures decreased from 56-96% and 31-63% of the initial ¹⁴C-activity for AcSMX and SMX, respectively, to reach 7-33% after 28 days. The high decrease in the first seven days was mainly due to the formation of non-extractable residues that were more abundant in soil/manure mixtures than in the soil/compost ones. The three aqueous solutions extracted differently the available ¹⁴C-residues according to the incubation time, the EOM and the molecule. The mineralized fractions for both ¹⁴C-molecules were only 2-3% with a little more mineralization in the soil/manure mixtures than in the soil/compost. By using the COP-Soil model, the dynamics of OC and OP were well described using parameter values specific to the organic matter (OC mineralization), and this for the three soft extractants used. Others parameter values were common to both EOM and both sulfonamide compounds (when coupling the dynamics of OP to OC). The set of parameter values describing the pollutant fate strongly differed according

to the soft extractant, confirming different mechanisms of extraction. Globally, the best OP simulations were obtained for the CaCl₂-based extraction.

Keywords

Sulfonamides; environmental availability; recycling; organic matter; modelling

III.1. Introduction

Antibiotics including sulfonamides are largely used as antibacterial treatments for humans and animals (Du and Liu, 2012). They are found in organic waste (sewage sludge, livestock effluents) as parent compound and/or metabolites. The main metabolite of a sulfonamide is the acetylated form (Baran et al., 2011). Sulfonamide concentrations can vary from 0.1 to 35.5 mg kg⁻¹ dry manure (Karcı and Balcıoğlu, 2009; Pan et al., 2011), and from 7 to 178 µg kg⁻¹ in sewage sludge (Lillenberg et al., 2009; Nieto et al., 2010). Concentrations of acetylated metabolites are mainly reported in wastewaters and sludge (Göbel et al., 2005; García-Galán et al., 2013). Post-treatments of organic waste like composting can lead to the formation of transformation products, the dilution of the initial concentrations and/or the formation of non-extractable residues (Lillenberg et al., 2010; Geng et al., 2016).

Sulfonamide residues, i.e., parent compound and transformation products including metabolites, are transferred into agricultural soils when exogenous organic matter (EOM) is spread as organic fertilizers or organic amendments, e.g., farmyard manure or sludge compost. In agricultural soils amended with farmyard manure, the total sulfonamide concentrations may vary from ≤ 2 up to 400 µg kg⁻¹ dry soil (Hamscher et al., 2005; Karcı and Balcıoğlu, 2009; Toth et al., 2011). As sulfonamides can adsorb on organic matter for example by ionic exchange, hydrogen bonding (Thiele-Bruhn et al., 2004), their behavior in soils could depend on the decomposition and humification occurring in soils after the incorporation of EOM.

The behavior of contaminants in soils can be studied using radiolabeled contaminants to follow the distribution of the radioactivity between different fractions, e.g., available, solvent-extractable, non-extractable and mineralized, throughout an incubation period. Thereby, the behavior of ¹⁴C-SMX was studied in soils receiving biosolids or compost (Li et al., 2015), and the behavior of ¹⁴C-SMX and its acetylated metabolite ¹⁴C-AcSMX was followed in soils receiving previously contaminated manure or sludge (Höltge and Kreuzig, 2007). However in these studies, the extractable residues were only quantified by solvent-based extraction. The environmental availability of SMX and AcSMX was not assessed while it is a crucial information to understand

the contaminant behavior in terms of transport in soil, bioavailability for soil microorganisms to be biodegraded, and further potential impacts such as the development and transfer of antibiotic resistances. The environmental availability of contaminants can be assessed by soft extractions with aqueous solutions (Lanno et al., 2004) while the use of organic solvents must be avoided (Alexander, 2000). Moreover, the environmental availability assessment by using different soft extractants can highlight different binding strengths of compounds with soil or EOM components. Indeed, the availability of ciprofloxacin, belonging to another antibiotic family, depended on the organic matter quality and its biodegradability, when the environmental availability was assessed by soft extractions involving different mechanisms of extraction (Chapter II).

Models can also help to understand the contribution of physical, chemical and microbiological processes in the contaminant behavior and in the evolution of its environmental availability. A model was recently developed, coupling the organic pollutant (OP) fate to the organic carbon (OC) evolution in time after the addition of EOM to soils (Geng et al., 2015). This COP-Soil model was used to simulate the release of OP such as polycyclic aromatic hydrocarbons, surfactants and herbicide during the compost decomposition in soils. The model was recently modified by Brimo et al. (2016a; 2016b) to add a module for the available fraction of the OP. However, the OP simulated in Geng et al. (2015) and Brimo et al. (2016), like PAHs, were more hydrophobic than compounds such as sulfonamide antibiotics. Zarfl et al. (2009) used another model to simulate the fate of sulfadiazine and its metabolites in manure-amended soils and the easily-extractable fraction measured by CaCl_2 -based extraction was taken into account in the model. Nevertheless, the decomposition of the manure occurring during the incubation period was not considered whereas the release of sulfonamides from manure decomposition is possible (Höltge and Kreuzig, 2007).

The objective of this study was to investigate in depth the environmental availability of SMX and AcSMX in soils amended with sludge compost or manure, in relation with the transformation of the EOM after its incorporation in soil. Two complementary approaches were used. The experimental one aimed at assessing with soft chemical extractions the environmental availability of SMX and AcSMX in soil/EOM mixtures. Referring to the mechanisms potentially involved in the sorption of sulfonamides in soils (Boxall et al., 2002; Thiele-Bruhn et al., 2004), we selected three aqueous solutions representative of different extraction mechanisms: ionic exchange (CaCl_2), chelation of cations (EDTA), and solubilization (2-hydroxypropyl- β -cyclodextrin, HPCD). Each EOM was initially spiked with ^{14}C -radiolabelled SMX or AcSMX at environmentally relevant concentrations before its incorporation into the soil. The distribution of the ^{14}C -activity among the available fraction, solvent-extractable fraction, non-extractable fraction and mineralized fraction was followed over a 28-day incubation. In the second approach, the use of the COP-Soil model to

simulate the results from the experimental part aimed at: 1) better understanding the dynamics of SMX and AcSMX residues in relation to the dynamics of the exogenous organic matter, taking into account its biochemical composition; 2) identifying if possible, the soft extractant for which the fate of SMX and AcSMX will be the best described in EOM-amended soils.

III.2. Materials and methods

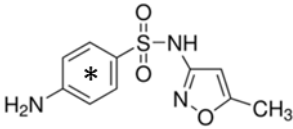
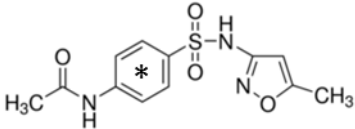
III.2.1. Chemicals and reagents

Radiolabeled [phenyl-¹⁴C] sulfamethoxazole (SMX) and N-acetylsulfamethoxazole (AcSMX) were purchased from the American Radiolabeled Chemicals Incorporation (Saint Louis, MO, USA). Both stock solutions in ethanol had radiochemical purity at 99% and specific activity at 2.85 GBq mmol⁻¹. The concentrations of the diluted solutions in Milli-Q water (Millipore, Saint-Quentin-en-Yvelines, France) were 1.1 and 2.2 mg L⁻¹ for ¹⁴C-AcSMX and ¹⁴C-SMX, respectively. The physicochemical properties of SMX and AcSMX are described in Table III.1.

Calcium chloride (CaCl₂, > 98%; Carlo Erba Reagents, Val-de-Reuil, France), sodium ethylenediaminetetraacetic acid (EDTA, > 99%; Sigma-Aldrich, Saint-Quentin-Fallavier, France) and 2-hydroxypropyl-beta-cyclodextrin (HPCD, > 99%; Sigma-Aldrich) were dissolved in Milli-Q water to prepare the soft extracting solutions. Acetonitrile (HPLC quality, Carlo Erba) was used for the solvent-based extraction.

Table III.1

Physicochemical properties of sulfamethoxazole (SMX) and N-acetylsulfamethoxazole (AcSMX), with *: radiolabeling on the molecule structures.

Molecule	SMX	AcSMX
Structure		
Molecular weight (g mol ⁻¹)	253.3	295.3
pK _a	5.89 ^a	5.07 ^a
Log K _{ow}	0.89 ^b	1.21 ^b

^a (Bonvin et al., 2013)

^b (Majewski et al., 2014)

III.2.2. Exogenous organic matter and soils

A farmyard manure and a compost of sewage sludge and green waste (20:80 % v/v, respectively) were sampled in September 2013 before their spreading on respective plots of the experimental device located in Feucherolles (Yvelines, France). The two EOMs differed according to pH: 7.7 and 8.8, organic carbon content: 257 and 355 g kg⁻¹ and dry mass: 67 and 34%, respectively for compost and manure. The biochemical composition of both EOMs is given in Supplementary material (Table III.S1). The compost was sieved to 5 mm and the manure was ground. Both EOM were freeze-stored at -20°C. The topsoil (0-28 cm) receiving each EOM was sampled in 2013 and 2015, two months after the EOM application. Since 1998 these cultivated soils have received organic amendments equivalent to 4 t carbon/ha/application every two years (Cambier et al., 2014). Soil samples were sieved to 4 mm, dried to 20% (water/dry soil) and stored at 4 °C. The main characteristics were similar for both soil samples: 15% clay, 79% silt, 6% sand; 15.2 g organic carbon kg⁻¹ dry soil; pH 7 and CEC 9.8 cmol₊ kg⁻¹. The initial microbial biomasses were equal to 278 and 287 mg C kg⁻¹ for the soil receiving the compost and the soil receiving the manure, respectively (Chalhoub et al., 2013).

III.2.3. Incubation experiment

III.2.3.1. Preparation and incubation of soil/EOM mixtures

According to the procedure described in Chapter II, the soil/EOM mixtures were prepared in Falcon[®] tubes (50 mL, Corning, NY, USA). First, soil was weighed (equivalent to 5.0 g dry soil) and the moisture content was adjusted to 24% (0.24 g water per gram dry soil). After a 3-day period of pre-incubation of soil at 28 °C, the EOM was weighed (equivalent to 0.15 g dry EOM) above the soil and 100 µL of ¹⁴C-molecule in Milli-Q water (12 and 24 MBq L⁻¹ for ¹⁴C-AcSMX and ¹⁴C-SMX, respectively) were added on the EOM. The ¹⁴C-spiked EOM was mixed with soil after ~1 h of contact. Thereby, the molecule was transferred to soil by the application of contaminated EOM (Boxall et al., 2012). The concentrations of SMX and AcSMX in soils finally reached 44 and 22 µg kg⁻¹ dry soil, respectively. The AcSMX concentration was two times lower than that of SMX to be close to the concentration ratio reported in soils (García-Galán et al., 2013).

In addition to the soil/EOM mixtures spiked with ¹⁴C-SMX or ¹⁴C-AcSMX, three other treatments were incubated: 1) soil alone, 2) soil mixed with the corresponding EOM (compost or manure), and 3) blanks without any soil, EOM or antibiotic. Each treatment was prepared in

triplicate. All centrifuge tubes were individually placed in hermetically-closed jars (1 L, Le Parfait, France) with two vials, one filled with water to keep constant the moisture conditions, and the other one filled with NaOH solution (1 N, 6 mL) to trap the released $^{12}\text{C-CO}_2$ and $^{14}\text{C-CO}_2$ (sections III.2.3.2 and III.2.3.3) during the incubation period. All jars were placed in the dark at 28°C for a period of either 7 or 28 days (section III.2.3.2).

III.2.3.2. Behavior of SMX and AcSMX in EOM-amended soil

The behavior of $^{14}\text{C-SMX}$ ($1.48 \mu\text{g SMX g}^{-1}$ EOM dry mass) and $^{14}\text{C-AcSMX}$ ($0.74 \mu\text{g AcSMX g}^{-1}$ EOM dry mass) in EOM-amended soil was assessed through the quantification of ^{14}C -activity partitioning among: 1) the available fraction; 2) the adsorbed fraction; 3) the non-extractable fraction and 4) the mineralized fraction. The different fractions were quantified at day 0, i.e., immediately after mixing the ^{14}C -spiked EOM with the soil (section III.2.3.1), and after 7 and 28 days of incubation as described below.

First, the available fraction was measured by the soft extraction of both molecules in soil/EOM mixtures with aqueous solutions. Three aqueous solutions, CaCl_2 (0.01 M, pH 4.5), EDTA (0.1 M, pH 7) or HPCD (0.05 M, pH 7), were selected from a preliminary study on the extractability of AcSMX in similar soil/EOM mixtures (Supplementary material III.S1). The CaCl_2 , EDTA and HPCD solutions differed according to the mechanisms of extraction and were used to obtain different fractions of potentially available ^{14}C -residues at this first step. The soft extraction was performed by shaking overnight (17 h) the soil/EOM mixture with 15 mL of aqueous solution with an overhead shaker (15 rpm, Heidolph[®] Reax 20, Schwabach, Germany). After centrifugation of the suspension at $11\ 000 \times g$ for 10 min (Sorvall[®] Evolution-RC, ThermoFisher Scientific), the supernatant was removed and the ^{14}C -activity was measured as described in section III.2.3.3.

Secondly, the solvent-based extraction was performed on the centrifugation pellet to measure a fraction of molecules which are more adsorbed on solid matrix. This method consisted in extracting the ^{14}C -molecules in ultrasonic bath for 15 min with a first 30/70 (v/v) mixture of McIlvaine buffer pH 9 / acetonitrile; and a second 30/70 (v/v) mixture of McIlvaine buffer pH 2 containing 0.41 M Na_2EDTA / acetonitrile (Ferhi et al., 2016). After centrifugation, the ^{14}C -activity was measured in each extract (pH 9 and pH 2) as described in section III.2.3.3.

The mineralized fraction of ^{14}C -molecules was followed over the incubation with the $^{14}\text{C-CO}_2$ trapping in the NaOH solution (section III.2.3.1).

Finally, the non-extractable fraction was quantified after drying at 40°C for 24 h the centrifugation pellets after the soft and solvent-based extractions. The soil/EOM mixtures were

ground and combusted in the oxidizer (Biological Oxidizer, OX 700, Zinsser Analytic, Frankfurt, Germany). The produced $^{14}\text{C-CO}_2$ during the combustion was trapped in Oxysolve 400 (Zinsser Analytic).

III.2.3.3. Analytical measurements

The ^{14}C -activity was measured by liquid scintillation counting (Tri-Carb 2100 TR, Packard Instruments, Meriden, CT, USA) in Oxysolve and in extracts (1 mL) and NaOH solution (1 mL) diluted in Ultima Gold XR scintillation cocktail (Perkin Elmer, Waltham, MA, USA). All counting results were expressed as % initial ^{14}C -activity. The pH was measured in the aqueous extracts over the incubation using a pH-meter (InoLab pH7110, WTW, Weilheim, Germany).

The mineralization of compost and manure organic carbon in soil was measured over the incubation by analyzing the CO_2 trapped in the NaOH solution with a colorimetric method (Colorimetric Skalar Analyzer, Breda, Netherlands). The cumulative mineralization was expressed as % initial total organic carbon (TOC) of compost or manure.

To identify the ^{14}C -molecules present in the extracts, i.e., $^{14}\text{C-SMX}$, $^{14}\text{C-AcSMX}$ or transformation products, the samples containing a sufficient ^{14}C -activity were concentrated and analyzed by high performance liquid chromatography (HPLC). The concentration of aqueous extracts from the incubation with AcSMX was realized by solid-phase extraction (Strata-X cartridges, Phenomenex, Le Pecq, France) or freeze-drying in the case of SMX. The organic extracts were evaporated to a droplet. Then the concentrated extracts were diluted in 1.5 mL of mobile phase. The radioactivity in the injected volume needed to be in the 125-250 Bq range; the injection volume was consequently adapted according to the radioactivity measured in the sample. The HPLC was performed on a Flexar system (Perkin Elmer, Villebon-sur-Yvette, France) equipped with a photodiode array detector and a flow scintillation detector (Radiomatic 150TR). Separation was achieved on a 250 x 4.6 mm 5 μm XBridge-C18 column (Waters, Milford, MA, USA), using a 1 mL min^{-1} mobile phase constituted of A) Milli-Q water + 0.01% TFA (trifluoroacetic acid, Sigma-Aldrich) and B) acetonitrile + 0.01% TFA, with a gradient from 5 to 35% of B in 31 min and to 100 % in 1 min for 5 min. Absorbance was measured continuously in the range of 190-400 nm. The data were processed with the Chromera[®] software from Perkin Elmer. The UV spectra of detected peaks were compared to that of SMX and AcSMX standards, which presented a maximum of absorption at 272 nm and 266 nm, respectively. The delay in retention times was equal to 2 min between the AcSMX and SMX peaks in UV and radioactivity detections.

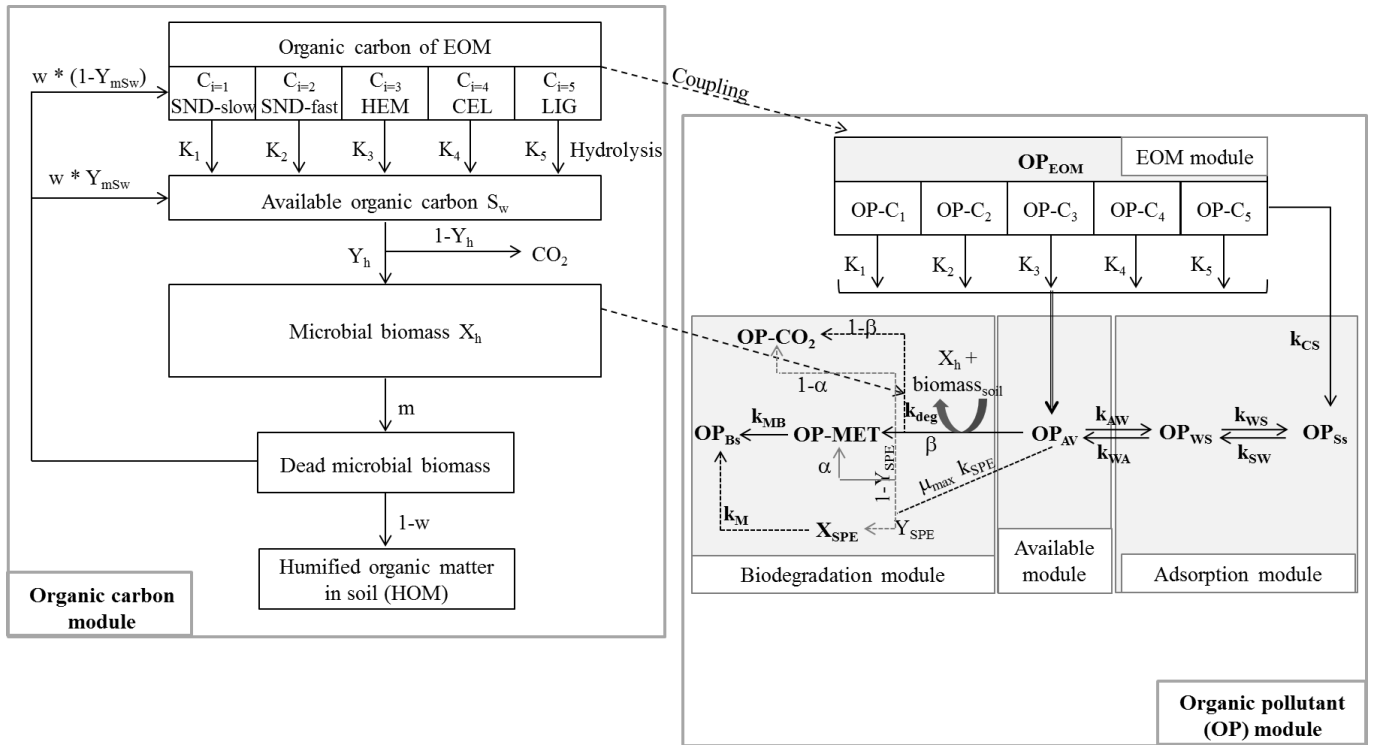


Figure III.1. General scheme of the COP-Soil model coupling the organic carbon module with the organic pollutant module.

III.2.3.4. Statistical analyses

The fractions extracted with aqueous solutions and organic solvent were assumed to correspond to the available and weakly-sorbed fraction, respectively, and the non-extractable fraction corresponded to the strongly-sorbed fraction. Statistical analyses were realized with RStudio software (version 0.98.501, RStudio Inc.) to compare the concentrations ($\mu\text{g } ^{14}\text{C kg}^{-1}$ total dry mass) measured in the available, solvent-extractable, non-extractable and mineralized fractions during the incubation. Differences according to the firstly-used soft extractant and the EOM type added to the soil were highlighted using linear mixed-effects models (ANOVA) and pairwise differences were determined. In the same way, differences were determined between the fractions (% initial ^{14}C -activity) of ^{14}C -AcSMX and ^{14}C -SMX.

III.2.4. Modelling of the behavior of SMX and AcSMX in EOM-amended soil

III.2.4.1. Presentation of the COP-Soil model

The model was adapted from Geng et al. (2015) by Brimo et al. (2016a and b) to simulate the behavior of organic pollutants introduced into soils with EOM. The pollutant fate was supposed to depend on the organic carbon dynamics over the incubation period. Therefore, the model included two main modules: the organic carbon (OC) module coupled to the organic pollutant (OP) module (Figure III.1).

Four submodules compose the organic pollutant module: the EOM module (OP_{EOM}), for the OP release from compost or manure decomposition; the available module (OP_{AV}), the adsorption module (OP_{WS} , OP_{SS}) and the biodegradation module (OP_{MET} , OP_{CO_2} , OP_{BS}). According to the NER classification proposed by Kästner et al. (2014), the non-extractable residues OP_{BS} (biological origin) and OP_{SS} (physical and chemical origin) were summed in the model to obtain the total fraction of non-extractable residues (OP_{NER}). All variables and parameters are presented in Table III.2 and the equations concerning the OP module are detailed by Brimo et al. (2016a and b).

Table III.2
Variables (A) and parameters (B) presented in the COP-Soil model.

A	Symbol	Description	Unit
OC module	$C_{i=1}$ (<i>SND-slow</i>)	Proportion of organic C in the soluble fraction of pH-neutral detergent with slow degradation	% initial TOC
	$C_{i=2}$ (<i>SND-fast</i>)	Proportion of organic C in the soluble fraction of pH-neutral detergent with fast degradation	% initial TOC
	$C_{i=3}$ (<i>HEM</i>)	Hemicelluloses-like fraction	% initial TOC
	$C_{i=4}$ (<i>CEL</i>)	Cellulose-like fraction	% initial TOC
	$C_{i=5}$ (<i>LIG</i>)	Lignin-like fraction	% initial TOC
	S_w	Organic C soluble in hot water	% initial TOC
	<i>HOM</i>	Humified organic matter in soil	% initial TOC
	X_h	Microbial biomass	% initial TOC
	CO_2	Carbon dioxide gas	% initial TOC
	Organic pollutant module		
EOM module	OP_{EOM}	Weakly sorbed OP on EOM	$\mu\text{g }^{14}\text{C kg}^{-1}$
Available module	OP_{AV}	Available OP in soft extracting solution	$\mu\text{g }^{14}\text{C kg}^{-1}$
Adsorption module	OP_{WS}	Weakly sorbed OP extracted with solvent	$\mu\text{g }^{14}\text{C kg}^{-1}$
	OP_{SS}	Strongly sorbed OP (physical and chemical NER)	$\mu\text{g }^{14}\text{C kg}^{-1}$
	$OP-CO_2$	^{14}C -labeled carbon dioxide gas from OP mineralization	$\mu\text{g }^{14}\text{C kg}^{-1}$
Biodegradation module	$OP-MET$	Metabolites from OP biodegradation	$\mu\text{g }^{14}\text{C kg}^{-1}$
	OP_{BS}	Biogenic NER from OP biodegradation	$\mu\text{g }^{14}\text{C kg}^{-1}$
	X_{SPE}	Specific biomass for degradation of OP	$\mu\text{g }^{14}\text{C kg}^{-1}$

Table III.2 (continued)

B	Symbol	Description	Unit
	K_1	Hydrolysis constant of $C_{i=1}$ (<i>SND-slow</i>)	d^{-1}
	K_2	Hydrolysis constant of $C_{i=2}$ (<i>SND-fast</i>)	d^{-1}
	K_3	Hydrolysis constant of $C_{i=3}$ (<i>HEM</i>)	d^{-1}
	K_4	Hydrolysis constant of $C_{i=4}$ (<i>CEL</i>)	d^{-1}
	K_5	Hydrolysis constant of $C_{i=5}$ (<i>LIG</i>)	d^{-1}
OC module	m	Death constant for microbial biomass	d^{-1}
	Y_{msw}	Availability yield for dead microbial biomass	-
	Y_h	Assimilation yield of organic C available for microbial biomass	-
	μ_{maxh}	Maximal growth rate for microbial biomass	d^{-1}
	K_{Sh}	Saturation constant for Monod kinetic	% initial TOC
	w	Proportion of dead biomasses into biodegrading pool	-
	% <i>slow</i>	Proportion of <i>SND-slow</i> in total soluble fraction	-
OP module			
First order kinetic with the first subscript denoting the source and the second the sink of the according process	k_{AW}	First order kinetic: available OP_{AV} and weakly sorbed OP_{WS}	d^{-1}
	k_{WA}	First order kinetic: weakly sorbed OP_{WS} and available OP_{AV}	d^{-1}
	k_{WS}	First order kinetic: weakly sorbed OP_{WS} and strongly sorbed OP_{SS}	d^{-1}
	k_{SW}	First order kinetic: strongly sorbed OP_{SS} and weakly sorbed OP_{WS}	d^{-1}
	k_{MB}	First order kinetic: metabolites OP_{MET} and biogenic sorbed OP_{BS}	d^{-1}
	k_{CS}	First order kinetic: weakly sorbed on EOM OP_{EOM} and strongly sorbed OP_{SS}	d^{-1}
Co-metabolism biomass	k_{deg}	First order kinetic of biodegradation for co-metabolism	d^{-1}
	β	Magnitude of metabolite production by biodegradation for co-metabolism	-
Specific biomass	$biomass_{soil}$	Microbial biomass concentration in soil (before EOM application)	mg C kg^{-1} soil
	Y_{SPE}	Yield of specific biomass	-
	μ_{max}	Maximum specific growth rate	d^{-1}
	k_{SPE}	Half saturation of growth substrate for the specific biomass	$\mu g\ kg^{-1}$ soil
	k_M	Mortality rate constant	d^{-1}
	α	Magnitude of metabolite production by biodegradation for specific biomass	-

III.2.4.2. Adaptation of the COP-Soil model

Since the COP-Soil model was developed for modelling the fate of polycyclic aromatic hydrocarbons (PAHs) in Brimo et al. (2016a), several hypotheses were established to adapt and use the COP-Soil model for more polar compounds such as some antibiotics and other pharmaceuticals. The model itself was not changed but the parameter values were modified in function of the pollutant.

First, the decomposition and humification of compost or manure organic matter in soils during the incubation period could influence the behavior of ^{14}C -SMX and ^{14}C -AcSMX. The ^{14}C -compound brought on the EOM was supposed to be distributed among the different OC biochemical fractions according to the OC fraction proportion (organic carbon of EOM in the OC module, Table III.2). So, the initial concentrations OP-C₁ to OP-C₅ in the OP_{EOM} submodule were calculated from the total OP concentration (OP module, Table III.2).

Then, given the greater solubility of SMX and AcSMX compared to that of PAHs, the availability of both compounds was expected to be higher than the availability of organic pollutants such as PAHs. Therefore, the k_{AW} value for SMX and AcSMX (transfer from available OP_{AV} into weakly sorbed OP_{WS}) was expected to be lower. In the same way, the formation of non-extractable residues OP_{SS} was supposed to occur rather via the available and adsorption modules. As a consequence, the k_{CS} value (transfer of weakly sorbed OP_{EOM} to strongly sorbed OP_{SS}) was expected to be low.

No specific biomass was considered since SMX had been neither detected in the two EOM nor in manure-amended soil (data not shown) and only detected in compost-amended soil below the quantification limit (Ferhi et al., 2016). Therefore we considered that the microorganisms had not been previously exposed and thus were not adapted to biodegrade SMX or AcSMX.

III.2.4.3. Use of the COP-Soil model

The organic matter module (OC module) was first used alone to calibrate the model with experimental data (% initial TOC) related to the mineralization of compost and manure organic carbon in soils during the incubation experiments. The parameter values tested in our OC module were taken or adapted from Geng et al. (2015) who simulated the behavior of several organic contaminants during sludge compost decomposition in soil.

We supposed that after calibrating the OC module for compost and for manure, we could use the same parameters in the OP module to simulate SMX and AcSMX behavior in the soil mixed

with compost or mixed with manure. The experimental data related to antibiotics ($\mu\text{g }^{14}\text{C kg}^{-1}$ dry soil) were used to simulate the evolution of the different fractions of SMX and AcSMX in amended soils, using the COP-Soil model. Moreover, given the possible transformation of AcSMX into SMX in soil (Höltge and Kreuzig, 2007), the parameter values used to model SMX behavior were supposed to be used for AcSMX.

For that, we first optimized the parameters for compost-amended soil and then the model was tested for manure-amended soil. The parameter values in OP module were optimized step by step for each soft extractant determining the modality and each molecule. The first step consisted in optimizing the parameter values with respect to the different mechanisms of extraction of CaCl_2 , EDTA and HPCD solutions and the possible co-extraction of organic matter (Chapter II). Indeed, the available fraction (OP_{AV}) may contain both free dissolved molecules and those adsorbed on colloids (Brimo et al., 2016a). The β value (magnitude of metabolite production by biodegradation for co-metabolism) was kept constant for all treatments assuming that the biodegradation of the ^{14}C -molecule coupled to the OC mineralization was low and independent from the soft extractant used. In this way, the concentrations of OP_{Bs} (biogenic sorbed OP from OP biodegradation) produced after 28 days could be similar whatever the soft extractant used.

The selected parameter values were finally tested to simulate the behavior of SMX and AcSMX in manure-amended soil. The statistical efficiencies of the simulations were compared to highlight the best simulation (section III.2.4.4).

III.2.4.4. Statistical analyses to measure the model efficiency

Experimental data were obtained for the available OP (OP_{AV}), the adsorbed OP ($\text{OP}_{\text{WS+EOM}}$, i.e., weakly adsorbed and adsorbed on EOM), the non-extractable OP (OP_{NER} , i.e., strongly sorbed OP_{Ss} and biogenic sorbed OP_{Bs}) and the mineralized OP (OP_{CO_2}). Thus, the relative root mean squared error (RRMSE, comprised between 0 and 1) was calculated between the experimental and simulated values as follows (Eq. 1):

$$\text{RRMSE} = \frac{100}{\bar{O}} \sqrt{\sum_{i=1}^n \frac{(Si-Oi)^2}{n}} \quad (1)$$

where n is the number of experimental observations, Si and Oi are the simulated and observed values, respectively, and \bar{O} is the average of n experimental values. The objective function $\text{RRMSE}_{\text{tot}}$ was the sum of RRMSE and the smallest $\text{RRMSE}_{\text{tot}}$ represented the best simulation. The effectiveness of the simulations was measured with the efficiency coefficient (Ef , Eq. 2) and the mean difference (\bar{D} , Eq. 3) calculated as follows:

$$Ef = \frac{\sum_{i=1}^n (oi - \bar{o})^2 - \sum_{i=1}^n (si - \bar{o})^2}{\sum_{i=1}^n (oi - \bar{o})^2} \quad (2)$$

$$\bar{D} = \frac{\sum_{i=1}^n (si - oi)}{n} \quad (3)$$

A value of Ef close to 1 and a value of \bar{D} close to 0 indicated a good fit of the model to the experimental data. Throughout our study, the modalities were determined by the soft extractant used to assess the environmental availability, i.e., CaCl_2 , EDTA and HPCD modality.

III.3. Results

III.3.1. Behavior of ^{14}C -SMX and ^{14}C -AcSMX in EOM-amended soils

The results of the ^{14}C -activity partitioning between the available, solvent-extractable, mineralized and non-extractable fractions are presented in Figure III.2.

III.3.1.1. Environmentally-available fractions

Initially in both soil/EOM mixtures, the available fractions ranged between 56 and 96% of initial ^{14}C -activity for ^{14}C -AcSMX, between 31 and 63% for ^{14}C -SMX, depending on the soft extractant used, with the highest ^{14}C -concentrations ($\mu\text{g } ^{14}\text{C kg}^{-1}$ dry soil) measured in the HPCD extracts ($p < 0.05$). The available fractions strongly decreased in the first seven days ($p < 0.05$) and still decreased after ($p < 0.05$) to reach 7-33% at day 28 for both ^{14}C -molecules, depending on the soft extractant used. The highest ^{14}C -concentrations were measured in the EDTA extracts at both days 7 and 28 ($p < 0.05$).

The three aqueous solutions extracted different amounts of both ^{14}C -molecules. According to the $\text{p}K_a$ of SMX and AcSMX (Table III.1), both molecules were mainly as anionic form in both soil/EOM mixtures whose pH was around 7 according to the pH values measured in all the aqueous extracts over the incubation time. Consequently, we assumed that different extraction mechanisms explained the different extractable fractions. For both compounds, the available fractions were higher or lower in soil/manure than in soil/compost mixtures, or similar, depending on the soft extractant used and the incubation time. For example, the CaCl_2 solution extracted higher available fractions in soil/manure mixtures than in soil/compost mixtures over the incubation ($p < 0.05$;

except for AcSMX at day 28, $p > 0.05$), as did also the HPCD solution at days 7 and 28 ($p < 0.05$; except for AcSMX at day 28). Inversely, the EDTA solution extracted higher available fractions from soil/compost mixtures at day 0 and day 28 ($p < 0.05$; except for SMX at day 28).

With respect to the HPLC analyses of available ^{14}C -AcSMX fractions, ^{14}C -SMX was also identified at days 0 and 7 in all aqueous extracts, confirming the reversible transformation of the metabolite into the parent compound immediately after the EOM application to soils (Höltge and Kreuzig, 2007). For ^{14}C -SMX, the HPLC analyses of the CaCl_2 and HPCD extracts obtained at day 0 showed a main radioactive peak corresponding to ^{14}C -SMX and different transformation products, including ^{14}C -AcSMX in both soil/EOM mixtures. However, after 7 and 28 days, there was no ^{14}C -AcSMX in the chromatograms. In the EDTA extracts, there was no ^{14}C -SMX detected and the main radioactive peak appeared at the start of the chromatograms suggesting that ^{14}C -SMX and/or ^{14}C -transformation products could be associated to the co-extracted organic matter. Any difference between soil/compost and soil/manure mixtures, was observed considering the HPLC analyses.

III.3.1.2. Weakly-sorbed fractions

The solvent-based extraction enabled to recover a more adsorbed fraction after the soft extraction. The sum of the available and solvent-extractable fractions or cumulated fraction decreased for both ^{14}C -molecules in both soil/EOM mixtures between 0 and 7 days ($p < 0.05$). The cumulated fractions also decreased between 7 and 28 days in soil/manure mixtures ($p < 0.05$) while in soil/compost mixtures, either it decreased ($p < 0.05$, ^{14}C -SMX in EDTA modality; ^{14}C -AcSMX in EDTA and HPCD modalities), or it remained stable ($p > 0.05$, other modalities). For both ^{14}C -molecules, the cumulated fractions were higher in soil/compost than in soil/manure mixtures ($p < 0.05$).

During the solvent-based extraction, ^{14}C -AcSMX was initially more extracted at pH 9 than at pH 2 in both soil/EOM mixtures except in soil/manure mixtures with HPCD as first extractant (Figure III.S2). Indeed, 80-87% of the solvent-extractable ^{14}C -activity was counted in the hydro-organic phase at pH 9 after performing soft extractions with CaCl_2 or EDTA solutions. The ^{14}C -activity fraction in the phase at pH 9 was lower (47-67%) after the HPCD-based extraction since the highest radioactivity level was quantified in the available fraction. At day 0, ^{14}C -SMX was more extracted at pH 9 than at pH 2 in soil/compost mixtures according to Ferhi et al. (2016), but here only when the CaCl_2 extractant was first used; otherwise the fractions corresponding to hydro-organic phase at pH 9 were below 50%. Except in the case of HPCD, ^{14}C -SMX was as well extracted at pH 9 as at pH 2 in soil/manure mixtures; these variations were attributed to the low

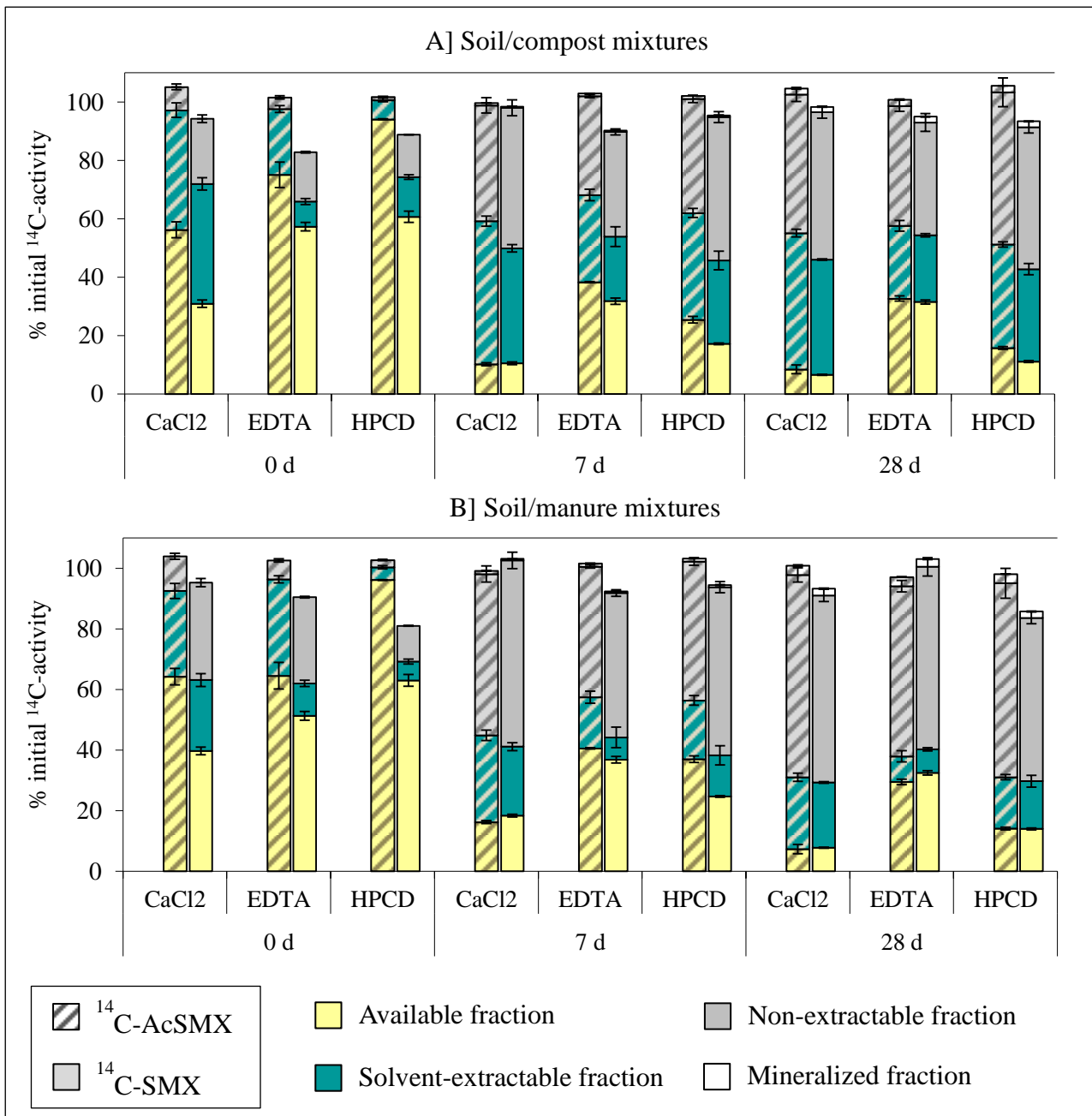


Figure III.2. Repartition of the ^{14}C -activity (% initial ^{14}C -activity, $n = 3$) in the different fractions for ^{14}C -AcSMX and ^{14}C -SMX in function of the incubation time (d) in A] soil/compost mixtures and in B] soil/manure mixtures.

solvent-extractable fractions in these modalities, according to the radioactivity level measured in the available fraction and the extraction mechanism of the aqueous solution (Figure III.2). Indeed, the solvent-extractable fractions were low at day 0 in soil/compost mixtures after the HPCD-based extraction, at day 7 in soil/manure mixtures after the EDTA-based extraction; therefore the partitioning of the ^{14}C -activity between the phases at pH 9 and pH 2 varied. The partitioning of the ^{14}C -activity also varied with the incubation time; in particular, after 28 days, depending on the first extractant used, more ^{14}C -residues could be extracted at pH 2 in proportion and quantified both in organic and aqueous phases. Consequently, we assumed that the formation of transformation products over the incubation experiment was responsible for the variation in the ^{14}C -activity partitioning. This hypothesis was supported by the HPLC analysis of the hydro-organic phases (pH 9) and the organic phases (pH 2) where unidentified transformation products were detected.

III.3.1.3. ^{14}C - and ^{12}C -mineralization

The mineralized fraction of ^{14}C -molecules reached 1.9-2.2% in soil/compost mixtures and 2.3-3.0% in soil/manure mixtures after 28 days. The ^{14}C -molecules were more mineralized in soil/manure mixtures than in soil/compost mixtures at the end of the incubation ($p < 0.05$ after 21 and 28 days for SMX; $p < 0.05$ after 14, 21 and 28 days for AcSMX).

The mineralization of compost OC reached 10% while the mineralization of manure OC reached 28% or 48%, depending on the incubation experiment (Figure III.S3). The OC mineralization was in accordance with the values of the index of residual organic carbon (I_{ROC}), i.e., 70% for compost and 56% for manure (Table III.S1), reflecting that compost OM was more stable, while manure OM was more biodegradable. As soils probably received manures of different quality in 2013 and 2015 and given the OM instability in manure, we assumed that slight differences between the two soil samples may have led to different mineralization rates of OC during the two different incubation experiments.

Moreover, the highest OC mineralization in soil/manure mixtures could reflect a greater microbial activity in soil/manure mixtures than in soil/compost mixtures although the microbial biomass was similar in both soils (section III.2.2). The OC mineralization was the same in the soil/EOM mixtures contaminated with either SMX or AcSMX, and not contaminated ($p > 0.05$, data not shown). Consequently, we assumed that SMX and AcSMX had no impact on the microbial biomass degrading the organic matter.

III.3.1.4. Non-extractable residues

Globally, the mass balance recoveries were comprised between 90 and 105% for all modalities in both incubation experiments. Unfortunately, some mass balance recoveries were lower than 90% in the incubation experiment with ^{14}C -SMX, i.e., 83 and 89% at day 0 in soil/compost mixtures using EDTA and HPCD solutions as soft extractants, respectively; 86% at day 0 in soil/manure mixtures using HPCD solution; and 86% at day 28 in soil/manure mixtures using HPCD solution.

The fractions of non-extractable residues (NER) strongly increased between 0 and 7 days ($p < 0.05$) for both ^{14}C -molecules and in both soil/EOM mixtures. Then, the NER fraction increased less between 7 and 28 days for ^{14}C -AcSMX in both soil/EOM mixtures ($p < 0.05$); it was stable for ^{14}C -SMX ($p > 0.05$), except for EDTA modality, for which the NER fraction increased ($p < 0.05$).

For both molecules, the non-extractable fractions were higher in soil/manure mixtures than in soil/compost mixtures ($p < 0.05$).

III.3.2. Modelling of ^{14}C -SMX and ^{14}C -AcSMX in EOM-amended soils

III.3.2.1. Calibration of the organic carbon module

The parameter values were adapted or taken from Geng et al. (2015) (Table III.S3). Considering the proportion of SND-fast equal to 0 at the end of composting, (Zhang et al., 2012; Geng et al., 2015), the proportion of SND-slow was equal to 1 at the beginning of the soil/compost mixture incubation. Inversely, the proportion of SND-fast in manure was equal to 1 since the mineralization of manure OC was high (Figure III.S3). According to the different mineralized fractions of manure OC, i.e., 28% and 43% (section III.3.1.3), the assimilation yield Y_h was equal to 0.7 in the experiment with ^{14}C -AcSMX and 0.5 in the experiment with ^{14}C -SMX (Table III.S3).

Some parameter values were different between compost and manure. The hydrolysis constants for SOL-S and cellulose were higher for manure ($K_1 = 0.060 \text{ d}^{-1}$, $K_4 = 0.020 \text{ d}^{-1}$) than for compost ($K_1 = 0.0001 \text{ d}^{-1}$, $K_4 = 0.009 \text{ d}^{-1}$). The hydrolysis constants were adapted from Aslam et al. (2014) who studied a pesticide dynamic during the decomposition of maize mulch whose hemicellulose and cellulose contents were high. For both soil/EOM mixtures, the organic carbon mineralization was well simulated, with Ef values comprised between 0.96 and 1.00 (Table III.S4).

III.3.2.2. Simulation of ^{14}C -AcSMX and ^{14}C -SMX behavior in compost-amended soil

Different parameter values were tested to calibrate and optimize the simulation of AcSMX and SMX behavior using the COP-Soil model. A set of parameters was retained for each soft extractant (Table III.3) based on the values of statistical efficiencies (Table III.S5). The simulations of ^{14}C -SMX concentrations in the different fractions are presented in Figure III.3 (^{14}C -AcSMX in Figure III.S4). For the three soft extractants, the decrease of the available fraction (OP_{AV}) was well-described with the parameter values given in Table III.3. The extractability difference clearly appeared between the aqueous solutions; the OP_{AV} fraction was nearly always higher with the EDTA solution than with the CaCl_2 and HPCD solutions. The decrease of the OP_{AV} fraction in soil was mainly explained by the strong increase of the non-extractable fraction ($\text{OP}_{\text{Ss}}+\text{OP}_{\text{Bs}}$), particularly on the 0-7 day period.

Table III.3

Parameter values (in d^{-1}) optimized in the simulations using the COP-Soil model for AcSMX and SMX in soil/compost mixtures.

First order kinetic	Parameter	CaCl_2	EDTA	HPCD
Available OP_{AV} to weakly sorbed OP_{WS}	k_{AW}	0.600	0.250	0.300
Weakly sorbed OP_{WS} to available OP_{AV}	k_{WA}	1.000	1.000	0.300
Weakly sorbed OP_{WS} to strongly sorbed OP_{Ss}	k_{WS}	0.500	0.500	0.300
Strongly sorbed OP_{Ss} to weakly sorbed OP_{WS}	k_{SW}	0.050	0.100	0.100
Weakly sorbed OP_{EOM} on EOM to strongly sorbed OP_{Ss}	k_{CS}	0.001	0.010	0.001
Metabolites OP-MET to biogenic sorbed OP_{Bs}	k_{MB}	1.0	1.0	1.0
Biodegradation for co-metabolism	k_{deg} AcSMX	3.1×10^{-5}	1.4×10^{-5}	1.6×10^{-5}
	SMX	4.1×10^{-5}	1.3×10^{-5}	2.1×10^{-5}
Magnitude of metabolite production by biodegradation for co-metabolism	β	0.85	0.85	0.85

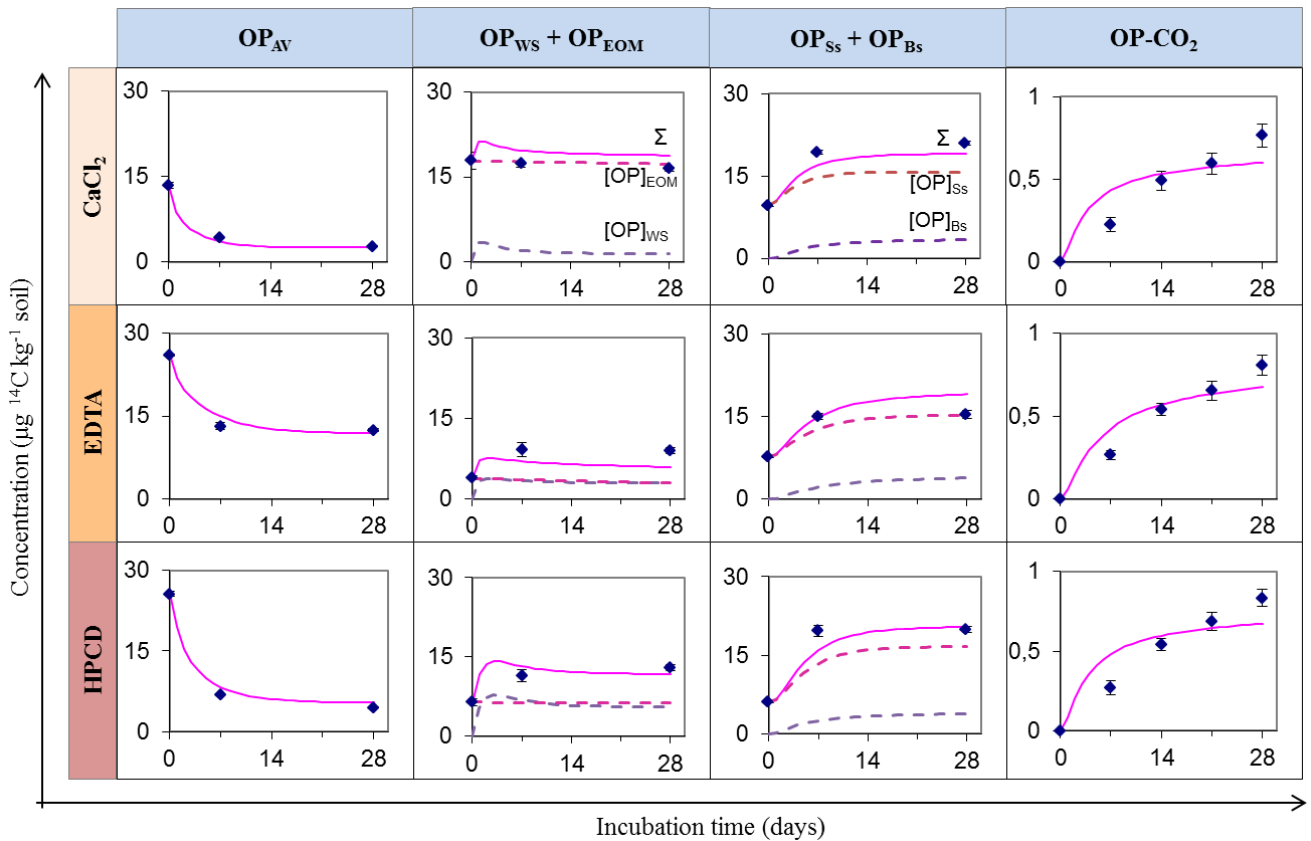


Figure III.3. Soil/compost mixtures: Simulations of ¹⁴C-SMX concentrations in available (OP_{AV}), weakly adsorbed (OP_{WS} + OP_{EOM}), non-extractable (OP_{SS} + OP_{BS}) and mineralized (OP-CO₂) fractions. CaCl₂, EDTA and HPCD were the soft extractants used to assess the available fraction and determined the modalities.

The adsorbed fraction (OP_{WS} + OP_{EOM}) contained weakly-sorbed compounds distributed in the soil and in the compost. There were some differences in the initial concentrations of OP_{WS} + OP_{EOM} according to the soft extractant, the amplitude of variations, and the division between the OP_{WS} and OP_{EOM} fractions. In all cases, the OP_{WS} + OP_{EOM} fraction increased in the 2-3 first days and then slowly decreased to become nearly constant. In the absence of experimental point before the day 7, this increase could not be verified. If most of ¹⁴C-SMX in this fraction was in the compost in the case of CaCl₂, it was nearly equally distributed in the case of EDTA and HPCD between the OP_{WS} and the OP_{EOM}, but the concentrations for HPCD were twice lower than for EDTA.

In accordance with the modelling hypotheses (Kästner et al., 2014; Brimo et al., 2016a), the majority of NER formed were of physical/physicochemical origins (OP_{SS}). The concentrations of biological NER (OP_{BS}), initially at zero, increased at a rate depending on the biological activity, here the mineralization of compost OC (Figure III.4 in section III.4.1). The low value of k_{cs} was well adapted to the situation where ¹⁴C-SMX was brought on the compost with only a short contact

period between the antibiotic and the compost matrix before mixing with soil. This meant that the progressive formation of more-tightly bound residues (OP_{Ss}) resulted first of all from the ^{14}C -SMX released from compost and available in soil.

The mineralization curves were rather identical in the three simulations with a slight overestimation of the fraction in the first 14 days and underestimation on the last 7 days.

For the $CaCl_2$ modality, the best simulation resulted in $RRMSE_{tot}$ equal to 0.45 and 0.56 for AcSMX and SMX, respectively. For AcSMX, the efficiency coefficients (Ef) were ≥ 0.77 with good simulations of the available OP (OP_{AV}), the adsorbed OP (OP_{WS+EOM}), the non-extractable OP (OP_{NER}) and the mineralized OP (OP_{CO_2}). For SMX, the efficiency coefficients were ≥ 0.80 . Only the adsorbed fraction (OP_{WS+EOM}) had a negative Ef due to the stability of the OP_{WS+EOM} concentrations over the incubation period.

For the EDTA modality, $RRMSE_{tot}$ were equal to 0.36 for AcSMX and 0.73 for SMX. OP_{AV} , OP_{WS+EOM} , OP_{NER} and OP_{CO_2} concentrations were well simulated ($Ef \geq 0.62$) for both molecules, except for OP_{WS+EOM} fraction for SMX with Ef equal to 0.21.

For the HPCD modality, $RRMSE_{tot}$ were equal to 0.73 for AcSMX and 0.61 for SMX. For both molecules, OP_{AV} , OP_{WS+EOM} , OP_{NER} and OP_{CO_2} concentrations were well simulated with $Ef \geq 0.63$.

Finally, based on the value of $RRMSE_{tot}$ for soil/compost mixtures, the best simulations were obtained: i) with $CaCl_2$ and EDTA solutions for AcSMX; and ii) with $CaCl_2$ and the HPCD solutions for SMX.

For all modalities, the k_{sw} value was low (0.05 or 0.10 d^{-1}) to limit the remobilization of non-extractable residues (Zarfl et al., 2009). The k_{CS} value was low according to the weak instantaneous formation of strongly sorbed OP (OP_{Ss}). The k_{MB} value was set at 1.0 d^{-1} by default to confound the transformation products (OP_{MET}) with the biogenic non-extractable residues (OP_{Bs}) at this modelling step. The corresponding concentrations of OP_{Bs} were equal to 1.98-2.10 μg ^{14}C kg^{-1} dry soil for ^{14}C -AcSMX and 3.39-3.81 μg ^{14}C kg^{-1} dry soil for ^{14}C -SMX.

The β value (magnitude of metabolite production by biodegradation through co-metabolism) was optimized at 0.85 and the k_{deg} value was optimized for each modality and molecule. As a result, k_{deg} values were comprised between 1.4×10^{-5} and $3.1 \times 10^{-5} d^{-1}$ for ^{14}C -AcSMX; between 1.3×10^{-5} and $4.1 \times 10^{-5} d^{-1}$ for ^{14}C -SMX, depending on the soft extractant used. These parameter values were very low in accordance with the low concentrations of mineralized ^{14}C -molecules.

III.3.2.3. Application of the selected parameter values to simulate the behavior of ^{14}C -AcSMX and ^{14}C -SMX in manure-amended soil

The parameter values optimized for soil/compost mixtures (OP module) were tested to simulate the behavior of AcSMX and SMX in soil/manure mixtures. The other parameter values in OC module were those given in Table III.S3 for manure. The statistical values are given in Table III.S6 and simulated curves are presented in Figure III.S5 for SMX and Figure III.S6 for AcSMX. The model simulated a slightly higher mineralization of antibiotics in soil/manure mixtures compared to soil/compost mixtures. In both cases, the evolution of the available fraction OP_{AV} , the non-extractable fraction ($\text{OP}_{\text{Ss}} + \text{OP}_{\text{Bs}}$) and the mineralized fractions OP-CO_2 were globally well-simulated when using for each soft extractant a unique set of parameters common to SMX and AcSMX and to compost and manure. The simulation results were not as good for the weakly-sorbed fraction. Indeed the statistical efficiencies indicated good simulations of the OP_{AV} , OP-NER and OP-CO_2 concentrations, for both molecules. For the $\text{OP}_{\text{WS+EOM}}$ concentrations, only one Ef value was ≥ 0.6 (AcSMX, HPCD modality) while the other Ef values were negative. As explained before (section III.3.2.2), the Ef was negative because the $\text{OP}_{\text{WS+EOM}}$ concentrations were relatively stable over the time (e.g., CaCl_2 modality).

The optimized k_{deg} values were comprised between 8.6×10^{-6} and $1.7 \times 10^{-5} \text{ d}^{-1}$ for ^{14}C -AcSMX; between 1.0×10^{-5} and $2.0 \times 10^{-5} \text{ d}^{-1}$ for ^{14}C -SMX. The simulated concentrations in OP_{Bs} produced after 28 days were equal to $2.99\text{-}3.17 \mu\text{g } ^{14}\text{C kg}^{-1}$ dry soil for ^{14}C -AcSMX, and $4.42\text{-}5.45 \mu\text{g } ^{14}\text{C kg}^{-1}$ dry soil for ^{14}C -SMX.

Finally, if we consider $\text{RRMSE}_{\text{tot}}$ in Table III.S5 and Table III.S6, we noticed that simulations for the CaCl_2 extractant gave the lowest $\text{RRMSE}_{\text{tot}}$ compared to the simulations with the two others extractants.

III.4. Discussion

III.4.1. Effect of the exogenous organic matter

The two antibiotics were directly applied to compost or manure, and a short contact period was observed before mixing with soil. At initial time, the interactions of SMX and AcSMX with the EOM may have first influenced the availability of compounds in soil/EOM mixtures. According to the Table III.S1, the manure contained a higher concentration of soluble OC, i.e., 60 g kg^{-1} dry mass

compared to 41 g kg^{-1} in compost. As sulfonamides can adsorb on dissolved organic matter (Wegst-Uhrich et al., 2014), the environmental availability of AcSMX and SMX might have been enhanced in soil/manure mixtures compared to in soil/compost mixtures thanks to a facilitated transport of compounds by the dissolved organic matter of manure (Zhou et al., 2016). This may explain the higher fraction recovered with CaCl_2 solution at day 0 in manure-amended soil for both molecules in particular SMX.

In the modelling, we tested the hypothesis that the availability and the fate of SMX and AcSMX residues were linked to the dynamics of the EOM organic matter. The two contaminants were initially distributed among the EOM fractions whose parameter values could differ between compost and manure (Table S3). The hydrolysis of EOM fractions was supposed to release SMX and AcSMX (same constants) in soil thus enriching the available fraction OP_{AV} . There were some differences in the OC module regarding the hydrolysis constants of the SND-slow and CEL fractions, whose values were for manure respectively 600 times and about 2 times the values for compost. Besides the manure contained more than 3 and 2 times the concentrations of hemicellulose-like and cellulose-like OC, respectively. However, for a given soft extractant and a given contaminant, the parameter values in the OP module except the k_{deg} were identical between compost and manure.

The Figure III.4 shows for SMX with CaCl_2 that the dynamics of the antibiotic and the organic carbon were well simulated for the two EOM. The quality of the organic matter influenced first the mineralization of organic carbon that was higher in the case of manure as seen in the simulation of OC- CO_2 . The available fraction OP_{AV} though decreasing in time was always higher in the soil/manure mixture than in the soil/compost mixture. The weakly-sorbed concentration stayed rather stable after a small increase in the first days for both EOM. The main difference came from the concentration of SMX weakly-sorbed to compost and manure (OP_{EOM}), its evolution depending on the SDN-fast fraction.

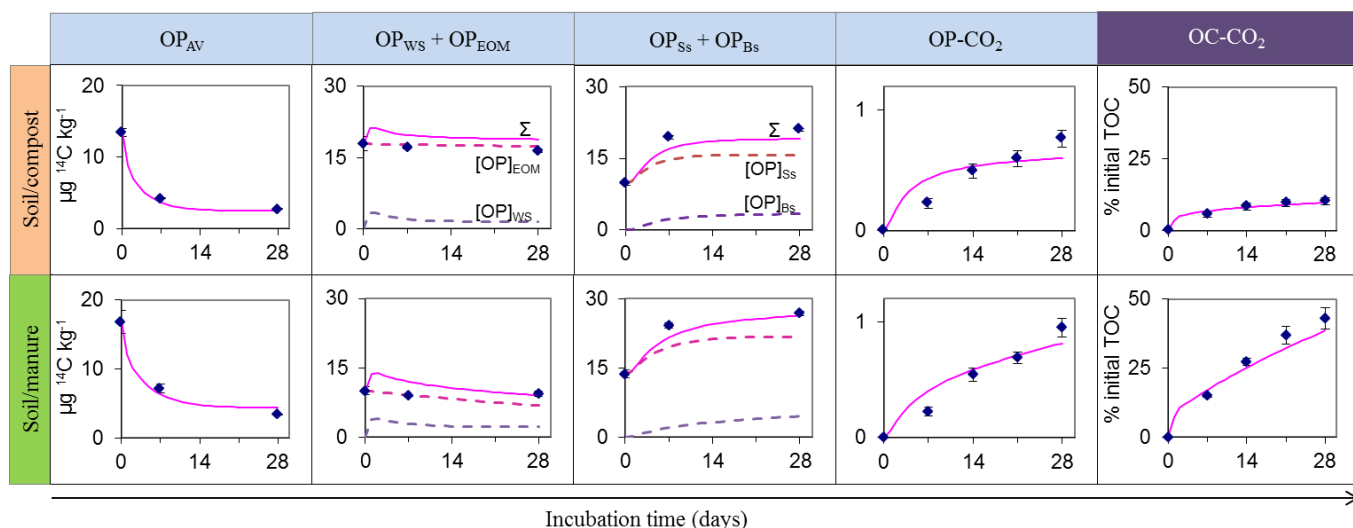


Figure III.4. Comparison of simulations between soil compost/mixtures and soil/manure mixtures for OC mineralization and ^{14}C -SMX concentrations in available (OP_{AV}), weakly adsorbed ($OP_{WS} + OP_{EOM}$), non-extractable ($OP_{SS} + OP_{BS}$) and mineralized ($OP-CO_2$) fractions ($CaCl_2$ modality).

The NER fraction was greater in soil/manure mixtures than in soil/compost mixtures after 28 days (section III.3.1.4). As the OC mineralization was also higher in soil/manure mixtures, a greater microbial activity could be responsible for an increase in the formation of OP_{BS} , i.e., the NER of biological origin (Kästner et al., 2014). This hypothesis was supported by the simulation results as the concentrations of biogenic NER (OP_{BS}) produced after 28 days were higher in soil/manure mixtures than in soil/compost mixtures. The increased formation of OP_{SS} in the manure-amended soil could be due to a higher addition of organic matter and to the composition/characteristics of manure (Table III.S1). There was a little more mineralization of ^{14}C -labelled residues in the case of manure. For both compost and manure, the ^{14}C -concentration mineralized was overestimated before the day 14 and a little underestimated after the day 21 (Figure III.4). Since the ^{14}C -concentrations mineralized were similar and the microbial biomass was higher in the manure-amended soil than in the compost-amended soil, this could explain why the kinetic parameter k_{deg} was lower with manure (section III.3.2.2.).

As a conclusion, the fate of AcSMX and SMX was closely dependent on the quality of the organic matter brought. Through the use of a model coupling the OP and OC dynamics, it was possible to describe the continuous evolution of the available fractions of SMX and AcSMX in connection with the other fractions in soil and the residues still in the EOM. However, this result strongly relied on the interactions that sulfonamides may have with organic matter in the EOM and

then in soil/EOM mixtures. This might be different for other classes of antibiotics with some more adaptations needed for modelling.

The adsorption of sulfonamides depends on the matrix pH (Strauss et al., 2011; Schwarz et al., 2012). Considering the pK_a of compounds (Table III.1), both AcSMX and SMX were anionic in soil/compost and soil/manure mixtures. Consequently, the pH did not play a major role in the differences between soil/EOM mixtures. However, the pH should be taken into account in the model if the initial pH of the EOM is below the pK_a of compounds.

III.4.2. Comparison between SMX and AcSMX

The behaviors of ^{14}C -SMX and ^{14}C -AcSMX over the 28-day period were globally similar in both soil/EOM mixtures. Statistical tests were applied to compare the fractions between ^{14}C -AcSMX and ^{14}C -SMX only for modalities with mass balance recoveries $\geq 90\%$ (section III.3.1.4). AcSMX was more available than SMX in both soil/EOM mixtures at day 0 ($p < 0.05$ for all soft extracting solutions) and day 7 ($p < 0.05$ for EDTA and HPCD solutions), with respect to the adsorption coefficients (K_d) in manure-amended soils of arable land (pH 6.9, Luvisol) reported by Hölting and Kreuzig (2007): 2.9 and 0.7 L kg⁻¹ for SMX and AcSMX, respectively.

The mineralized fraction of ^{14}C -SMX after 28 days was comparable to the mineralized fraction measured by Li et al. (2015), i.e., around 2% in compost-amended soils. In the same way, the mineralized fraction of ^{14}C -AcSMX after 28 days was similar to the fraction measured by Hölting and Kreuzig (2007), i.e., around 3% in manure-amended soils. The higher environmental availability of ^{14}C -AcSMX compared to ^{14}C -SMX could be related to a higher bioavailability for microbial degradation, given the higher mineralized fractions for ^{14}C -AcSMX after 14, 21 and 28 days in both soil/EOM mixtures ($p < 0.05$).

Considering the COP-Soil model, the same parameter values were used to simulate the distribution of ^{14}C -AcSMX and ^{14}C -SMX residues over the incubation time, and this, in both soil/EOM mixtures. Only the k_{deg} parameter value was different, for which the optimization was necessary to adjust the OP-CO₂ simulation. As previously described, the β value was kept constant to 0.85 to obtain the same amount of OP_{Bs} after 28 days in each soil/EOM mixture, whatever the soft extractant used. Thereby, the concentrations of OP_{Bs} produced after 28 days were equal to 1.98-3.17 $\mu\text{g } ^{14}\text{C kg}^{-1}$ dry soil for AcSMX; 3.39-5.45 $\mu\text{g } ^{14}\text{C kg}^{-1}$ dry soil for SMX, depending on the soil/EOM mixture (sections III.3.2.2 and III.3.2.3). These OP_{Bs} concentrations were consistent with

the fact that the initial ^{14}C -activity was doubled in the experiments with ^{14}C -SMX compared to the experiments with ^{14}C -AcSMX. However, when the OP-NER fraction was expressed as the percentage of the total radioactivity, the fractions after 28 days were similar between ^{14}C -AcSMX and ^{14}C -SMX (section III.3.1.4).

In parallel, the simulation of the OP- CO_2 fraction depended on the $(1-\beta)$ and k_{deg} values. The concentrations of mineralized ^{14}C -SMX were higher after 28 days than the concentrations of mineralized ^{14}C -AcSMX in both soil/EOM mixtures. Therefore the k_{deg} values were higher for ^{14}C -SMX (1.0×10^{-5} to $4.1 \times 10^{-5} \text{ d}^{-1}$) than for ^{14}C -AcSMX (8.6×10^{-6} to $3.1 \times 10^{-5} \text{ d}^{-1}$). Nevertheless, as described in section III.3.1.3, the mineralized fractions (in % initial ^{14}C -activity) of ^{14}C -AcSMX were higher than the mineralized fractions of ^{14}C -SMX in both soil/EOM mixtures.

Finally, considering the HPLC analyses (section III.3.1.1), AcSMX was transformed in SMX in amended soils (Höltge and Kreuzig, 2007; Zarfl et al., 2009). As the parameter values used in the COP-Soil model were the same to model ^{14}C -AcSMX and ^{14}C -SMX behaviors in soils, the fate of ^{14}C -AcSMX was supposed to be closely linked to the ^{14}C -SMX one.

III.4.3. Comparison between the soft extracting solutions

The set of parameter values varied in function of the experimental data obtained for each soft extractant. Indeed, the CaCl_2 , EDTA and HPCD solutions differently extracted the available fraction and could have an impact on the adsorbed and the non-extractable fractions (Figure III.3). Therefore, we assumed that differences in the parameter values were mainly due to the mechanism of extraction targeted by each soft extractant. The best simulations were obtained with CaCl_2 extractant.

The CaCl_2 solution extracted available AcSMX and SMX compounds in the soil/EOM mixture by miming the soil solution regarding pH, concentration and composition (Förster et al., 2009). The CaCl_2 -available fraction strongly decreased between 0 and 7 days. Considering this mechanism of extraction, the ^{14}C -compounds were not easily desorbed from soil/EOM mixtures and the adsorbed fraction $\text{OP}_{\text{WS}+\text{EOM}}$ remained relatively stable over the incubation. Consequently, the OP-NER fraction increased between 0 and 7 days. In the COP-Soil model, the kinetic of available OP_{AV} to adsorbed OP_{WS} concentrations was better simulated with k_{AW} value equal to 0.6, while the k_{WA} value was equal to 1.0 d^{-1} . The k_{AW} value was the highest compared to values used in EDTA and HPCD modalities.

The EDTA solution chelated the cations of soil and EOM, and co-extracted organic matter as suggested by the dark-brown color of extracts and the saturated signal on chromatograms in UV detection. The possible adsorption of AcSMX and SMX on organic matter could promote their extraction by EDTA solution. Moreover, the higher abundance of cations in compost compared to manure (Table III.S1) could have influenced the retention of anionic SMX and AcSMX at a pH above 7. The chelation of cations by the EDTA solution might have led to the co-extraction of either free compounds or associated compounds to organic matter. Consequently, the EDTA solution extracted initially more SMX and AcSMX in soil/compost mixtures than in soil/manure mixtures.

Then, the organic matter biodegradation occurring during the incubation processes could lead to a redistribution of cations in soil/EOM mixtures that could release SMX and AcSMX (Chapter II). Moreover, the possible association of compounds to co-extracted organic matter (Wegst-Uhrich et al., 2014) in EDTA extracts led to the highest available fraction after 7 and 28 days compared to CaCl₂- and HPCD-based extractions. Therefore, the EDTA-available fraction decreased less rapidly. In the COP-Soil model, the k_{AW} value for the kinetic of available OP_{AV} to OP_{WS} concentrations was thus equal to 0.25 d⁻¹ and was lower than in CaCl₂ modality. The k_{WA} value for the kinetic of OP_{WS} to OP_{AV} was similar (1.0 d⁻¹) since the adsorbed OP_{WS+EOM} fraction was stable or slightly decreased over the incubation like in CaCl₂ modality.

The kinetic of adsorbed OP on EOM (OP_{EOM}) to strongly sorbed OP_{Ss} was well simulated with a k_{CS} value ten times higher for EDTA modality (0.01 d⁻¹) than for CaCl₂ and HPCD modalities (0.001 d⁻¹). This order was probably due to the co-extraction of ¹⁴C-molecules adsorbed on the EOM colloids by EDTA. Consequently, the concentration of OP_{EOM} decreased over the incubation time (Figure III.3 for ¹⁴C-SMX and Figure III.S4 for ¹⁴C-AcSMX). This was mostly observed in soil/manure mixtures for both ¹⁴C-molecules, for which the simulations were enhanced by increasing the k_{CS} value: RRMSE_{tot} equal to 0.24 with k_{CS} optimized to 0.15 d⁻¹ for ¹⁴C-AcSMX, and RRMSE_{tot} was equal to 0.15 with k_{CS} optimized to 0.91 d⁻¹ for ¹⁴C-SMX (data not shown).

The HPCD solution encapsulated SMX and AcSMX in soil/EOM mixtures. The extraction resulted in the highest available fraction at day 0 compared to the CaCl₂- and EDTA-based extractions, since a part of the weakly sorbed fraction was extracted in the same time. The HPCD-available fraction rapidly decreased between 0 and 7 days, implying an increase of the adsorbed fraction. Therefore, in the COP-Soil model, the simulation of the OP_{AV} and the OP_{WS+EOM} fraction was better with k_{AW} and k_{WA} values equal to 0.3 d⁻¹, resulting in the equilibrium between available OP_{AV} and OP_{WS} concentrations. The k_{WA} value was consequently lower for HPCD modality than

that in CaCl_2 and EDTA modalities given the adsorbed fraction was generally stable or slightly decreased for the latter.

The other parameter values are less discussed since they mainly depended on the k_{AW} value. Indeed the k_{AW} value was the first parameter optimized using the COP-Soil model. The main difference between the modalities determined by the soft extractants used was based on the available fraction. Differences were observed only at day 0 between the cumulative fractions (available and adsorbed fractions) and between the NER fractions. Then, these fractions were similar between soft extractants after 7 and 28 days of incubation. Considering the OP-NER fraction, the lowest was measured at day 0 in HPCD modality, according to the highest OP_{AV} fraction at day 0. The kinetic of weakly sorbed OP_{WS} to strongly sorbed OP_{SS} was well simulated with the k_{WS} value equal to 0.3 d^{-1} , which was lower than the k_{WS} value used for CaCl_2 and EDTA modalities (0.5 d^{-1}).

The kinetic of strongly sorbed OP_{SS} to weakly sorbed OP_{WS} was low, with k_{SW} values equal to 0.05 or 0.1 d^{-1} . The lowest k_{SW} value obtained in CaCl_2 modality could be related to the extraction mechanism of the solution. Indeed, the EDTA and the HPCD solutions could extract a higher part of more adsorbed ^{14}C -molecules compared to the CaCl_2 solution.

III.5. Conclusion

As sulfonamide residues can adsorb on organic matter, the behavior of these compounds in soils depends on the exogenous organic matter (EOM) applied. The assessment of the environmental availability is crucial to understand the behavior of antibiotics in soils. Soil/manure and soil/compost mixtures were incubated during one month; the EOM was previously contaminated with ^{14}C -sulfamethoxazole (SMX) or its acetylated ^{14}C -metabolite (AcSMX). The experiments enabled to acquire original data on the behavior of both compounds. In particular, their environmental availability was assessed by soft extractions with aqueous solutions (CaCl_2 , EDTA or cyclodextrin) exhibiting different extraction mechanisms. The availability of both compounds was higher in soil/manure mixtures than in soil/compost mixtures. By using a model coupling the organic matter biodegradation to the organic pollutant behavior, we simulated on 28 days the distribution of both sulfamethoxazole and its acetylated metabolite in the available, adsorbed, non-extractable and mineralized fractions. The biodegradation of the organic matter led to the low mineralization of ^{14}C -SMX and ^{14}C -AcSMX by co-metabolism and also to the formation of biogenic non-extractable residues.

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III. Supplementary material

Table III.S1

Characteristics and compositions of the compost of sewage sludge/green waste and the bovine manure (n = 3, average ± standard deviation).

Dry mass	pH	OC	Total N	Mineral N	C/N	SOL	NDF	HEM	CEL	LIC	I _{ROC}	CEC	%		
													%OC	%	
		g kg ⁻¹											cmol ₊ kg ⁻¹		
Compost	67 ± 1	7.7 ± 0.1	257 ± 3	26 ± 0.5	6.4 ± 0.1	10 ± 0.3	16 ± 3	47 ± 1.5	6 ± 2	24 ± 2	23 ± 2	70 ± 1	47 ± 2		
Manure	34 ± 1	8.8 ± 0.3	355 ± 14	20 ± 0.7	0.5 ± 0	18 ± 0.1	17 ± 2	32 ± 2	14 ± 2	36 ± 1	18 ± 0.3	56 ± 1	32 ± 3		

		mg kg ⁻¹										
Cr	Cd	Cu	Ni	Pb	Zn	As	Se	Hg				
Compost	43 ± 1	0.9 ± 0.1	209 ± 12	22 ± 0	66 ± 4	471 ± 11	14.9 ± 0.1	1.4 ± 0	0.47 ± 0.01			
Manure	14 ± 1	0.2 ± 0.4	44 ± 0	5 ± 0	14 ± 2	215 ± 3	2.1 ± 0.2	0.6 ± 0	0.11 ± 0.01			

		g kg ⁻¹									
CaCO ₃	P ₂ O ₅	K	Ca	Mg	Na	Al	Fe	Mn			
Compost	37 ± 12	28 ± 0.8	17 ± 0.5	48 ± 1	6 ± 0.1	4 ± 0.3	22 ± 0.2	30 ± 0.3	0.7 ± 0.01		
Manure	46 ± 3	16 ± 0.3	32 ± 0.6	28 ± 1.5	5 ± 0.1	5 ± 0.2	6 ± 1	4 ± 0.6	0.3 ± 0.02		

III.S1. Soft extraction tests of ^{14}C - acetylsulfamethoxazole (0.74 mg kg^{-1} dry mass of EOM) from soil/compost and soil/manure mixtures.

[phenyl- ^{14}C]-N-acetylsulfamethoxazole (AcSMX, radiochemical purity 99%, specific activity $2.85 \text{ GBq mmol}^{-1}$) was purchased from American Radiolabeled Chemicals Incorporation (Saint Louis, MO, USA). The molecule was selected as a reference to test the soft extractant efficiencies and to compare the obtained recoveries. The soil/compost and soil/manure mixtures were prepared with the same procedure described before. The absence of ^{14}C -acetylsulfamethoxazole loss by adsorption on the container was checked with the shaking of the molecule ($6.8 \mu\text{g L}^{-1}$, $n = 2$) in each extracting solution (Table III.S2). No loss was found regarding 90 to 99% initial ^{14}C -activity was quantified in the solution after the overnight shaking.

Table III.S2

Counted ^{14}C -activity (% initial ^{14}C -activity, average \pm standard deviation) obtained after the shaking of ^{14}C -acetylsulfamethoxazole in the extracting solution ($6.8 \mu\text{g L}^{-1}$, $n = 2$).

Extracting solution	Concentration M	pH	Recovery % ^{14}C -activity
CaCl_2	0.01	4 ^(a)	99 ± 1.2
EDTA	0.10	4.5 ^(a)	95 ± 0.5
EDTA	0.10	7 ^(b)	97 ± 2.0
EDTA	0.20	7 ^(b)	93 ± 2.7
β -CD	0.02	7 ^(a)	93 ± 3.7
2-HP- β -CD	0.05	7 ^(a)	90 ± 1.5
2-HP- γ -CD	0.05	6.9 ^(a)	91 ± 5.3
2-HE- β -CD	0.05	ND	92 ± 3.2
TA- β -CD	0.0075	7 ^(a)	97 ± 1.5
SDS	0.005	7 ^(b)	94 ± 4.4
SDS	0.0005	7 ^(b)	94 ± 4.9

^(a) measured pH; ^(b) adjusted pH

The aqueous solutions were used to extract ^{14}C -AcSMX from soil/compost and soil/manure mixtures after 24 h of contact between the EOM spiked with the ^{14}C -compound and the soil. The available fractions of ^{14}C -AcSMX were different regarding the nature of the extracting solutions. The mean extracted fractions varied from 35 to 43% with the CaCl_2 solution; from 15 to 42% with EDTA solutions; and from 49 to 76% with cyclodextrin or surfactant solutions (Figure III.S1). The mass balances were measured only for samples extracted with CaCl_2 , EDTA (0.1 M pH 7) and 2-HP- β -CD solutions. The total recovered fraction reached 76 to 91%, with the lowest fraction obtained for the EDTA extracts (Figure III.S1). It was previously checked that ^{14}C -AcSMX in the

extracting solution was not lost by adsorption on the container (Table III.S2). In the EDTA case, the ^{14}C -counting was underestimated owing to a Quenching effect due to the dark-colored extract.

With respect to the main properties of AcSMX, i.e. $\text{Log } K_{ow}$ 0.86 (Majewski et al., 2014), K_d 2.1 in arable soil and $\text{p}K_a$ 5.1 (Höltge and Kreuzig, 2007), all the extracted ^{14}C -fractions were consistent with the low expected adsorption in soil or in EOM. Moreover, given the $\text{p}K_a$ of AcSMX, the amide attached to the sulfur was deprotonated at $\text{pH} > 5.1$. Therefore the molecule was mostly anionic at pH around or above 7, i.e. in both EOM and soil/EOM mixtures. All extracting solutions were at pH 7 except for the CaCl_2 and EDTA solutions (Table III.S2) for which the pH was equal to 4.0-4.5. As the extracted ^{14}C -fractions were the lowest with these last extracting solutions, we could suppose that the extraction of AcSMX was easier at $\text{pH} > \text{p}K_a$. As a result, the fractions extracted with the different aqueous solutions varying from 15 to 76% involved different mechanisms of extraction. Therefore we selected three of the aqueous solutions to test them to assess the environmental availability of AcSMX and its parent compound SMX. The CaCl_2 solution was selected since its regular use in adsorption/desorption batch experiments. Then, the EDTA solution (0.1 M, pH 7) was selected since it is a chelating agent. Finally, the 2-HP- β -CD solution was selected with respect to the highest extracted fraction.

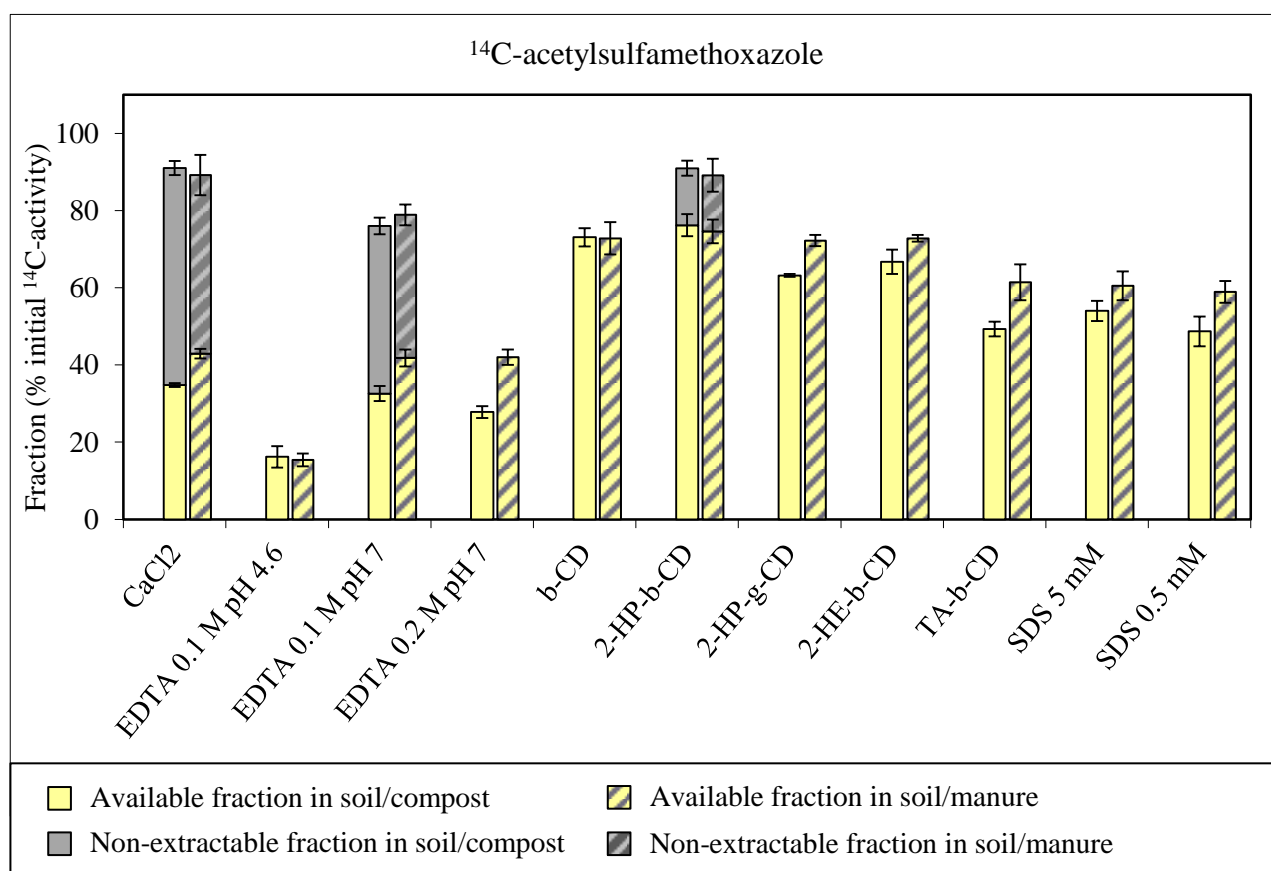


Figure III.S1. Available and non-extractable fractions (% initial ^{14}C -activity) of ^{14}C -AcSMX ($n = 3$, missing data for non-extractable fractions, not measured) in soil/EOM mixtures (24 h-contact, $\sim 20^\circ\text{C}$).

Table III.S3

Parameter values of the organic matter module for compost and manure.

	Parameters of the model	Parameter values		Unit
		Compost	Manure	
K_1	Hydrolysis constant of $C_{i=1}$ (<i>SND-slow</i>)	0.0001 ^a	0.0600 ^b	d^{-1}
K_2	Hydrolysis constant of $C_{i=2}$ (<i>SND-fast</i>)	0.0612 ^a		d^{-1}
K_3	Hydrolysis constant of $C_{i=3}$ (<i>HEM</i>)	0.0200 ^c		d^{-1}
K_4	Hydrolysis constant of $C_{i=4}$ (<i>CEL</i>)	0.0090 ^a	0.0200 ^c	d^{-1}
K_5	Hydrolysis constant of $C_{i=5}$ (<i>LIG</i>)	0.0001 ^a		d^{-1}
m	Death constant for microbial biomass	0.2290 ^a		d^{-1}
Y_{ndsw}	Availability yield for dead microbial biomass	0.4096 ^a		-
Y_h	Assimilation yield of organic C available for microbial biomass	0.70 ^b	0.70 ^{b,d} / 0.5 ^{b,d}	-
μ_{maxh}	Maximal growth rate for microbial biomass	5.9958 ^a		d^{-1}
K_{sh}	Saturation constant for Monod kinetic	101.10 ^a		% initial TOC
w	Proportion of dead biomasses into biodegrading pool	1 ^a		-
% <i>slow</i>	Proportion of SND-slow in total soluble fraction	1 ^b	0 ^b	-
X_h	Quantity of carbon in biomass	3.6 ^e	2.7 ^e	% initial TOC

^a The parameter values used for the OC module were taken from Geng et al. (2015).^b The parameter values were estimated in the present study.^c The parameter values were adapted from Aslam et al. (2014).^d The Y_h values depended on the incubation experiment with ¹⁴C-AcSMX or ¹⁴C-SMX.^e The parameter values were calculated in the present study.

Table III.S4

Statistical evaluation of model efficiency in the simulations of the carbon mineralization (5 observations) using the OC module alone.

EOM	Incubation experiment	RRMSE	Ef	\bar{D}
Compost	1) ^{14}C -AcSMX	0.07	0.98	0.15
	2) ^{14}C -SMX	0.09	0.97	-0.05
Manure	1) ^{14}C -AcSMX	0.04	1.00	0.18
	2) ^{14}C -SMX	0.13	0.96	-1.84

Table III.S5

Statistical evaluation of model efficiency in the scenarios retained using the COP-Soil model for A] ^{14}C -AcSMX and B] ^{14}C -SMX in soil/compost mixtures.

A] ^{14}C -AcSMX						
Modality		OP_{AV}	OP_{WS+EOM}	OP-NER	OP-CO ₂	RRMSE _{tot}
CaCl ₂	RRMSE	0.09	0.03	0.12	0.21	0.45
	Ef	0.99	0.77	0.95	0.87	
	\bar{D}	0.14	-0.17	0.61	0.00	
EDTA	RRMSE	0.05	0.00	0.16	0.15	0.36
	Ef	0.99	1.00	0.93	0.94	
	\bar{D}	-0.15	0.00	0.33	0.00	
HPCD	RRMSE	0.05	0.32	0.16	0.19	0.73
	Ef	1.00	0.63	0.95	0.89	
	\bar{D}	0.32	-1.32	0.71	0.00	
B] ^{14}C -SMX						
Modality		OP_{AV}	OP_{WS+EOM}	OP-NER	OP-CO ₂	RRMSE _{tot}
CaCl ₂	RRMSE	0.05	0.12	0.10	0.29	0.56
	Ef	0.99	-11.82	0.88	0.80	
	\bar{D}	-0.26	1.65	-1.42	0.01	
EDTA	RRMSE	0.06	0.30	0.17	0.21	0.73
	Ef	0.97	0.21	0.62	0.89	
	\bar{D}	0.40	-1.76	1.23	0.01	
HPCD	RRMSE	0.08	0.12	0.14	0.26	0.61
	Ef	0.99	0.80	0.89	0.83	
	\bar{D}	0.80	0.20	-1.07	0.01	

Table III.S6

Statistical evaluation of model efficiency in the simulations obtained using the COP-Soil model for A] ^{14}C -AcSMX and B] ^{14}C -SMX in soil/manure mixtures.

A] ^{14}C -AcSMX						
Modality		OP_{AV}	$\text{OP}_{\text{WS+EOM}}$	OP-NER	OP-CO ₂	$\text{RRMSE}_{\text{tot}}$
CaCl ₂	RRMSE	0.03	0.11	0.05	0.15	0.34
	<i>Ef</i>	1.00	-0.47	0.99	0.95	
	\bar{D}	0.10	0.29	-0.13	0.02	
EDTA	RRMSE	0.09	0.65	0.24	0.05	1.02
	<i>Ef</i>	0.93	-0.83	0.84	0.99	
	\bar{D}	-0.59	1.91	-1.21	0.00	
HPCD	RRMSE	0.08	0.32	0.03	0.13	0.57
	<i>Ef</i>	0.98	0.62	1.00	0.96	
	\bar{D}	-0.62	0.28	0.03	0.01	
B] ^{14}C -SMX						
Modality		OP_{AV}	$\text{OP}_{\text{WS+EOM}}$	OP-NER	OP-CO ₂	$\text{RRMSE}_{\text{tot}}$
CaCl ₂	RRMSE	0.12	0.19	0.05	0.22	0.58
	<i>Ef</i>	0.97	-17.59	0.97	0.90	
	\bar{D}	-0.47	0.94	-0.54	0.02	
EDTA	RRMSE	0.04	0.67	0.12	0.09	0.92
	<i>Ef</i>	0.97	-16.20	0.77	0.98	
	\bar{D}	-0.55	2.13	-1.77	0.02	
HPCD	RRMSE	0.00	0.60	0.19	0.29	1.08
	<i>Ef</i>	1.00	-2.37	0.85	0.84	
	\bar{D}	-0.03	2.20	-2.25	0.03	



Figure III.S2. Partitioning of the ^{14}C -activity (% solvent-extractable; average, $n = 3$) between hydro-organic phase (pH 9), organic phase (pH 2) and aqueous phase (pH 2), for ^{14}C -AcSMX or ^{14}C -SMX in soil/compost and soil/manure mixtures. CaCl₂, EDTA and HPCD referred to the soft extractant used before the solvent-based extraction.

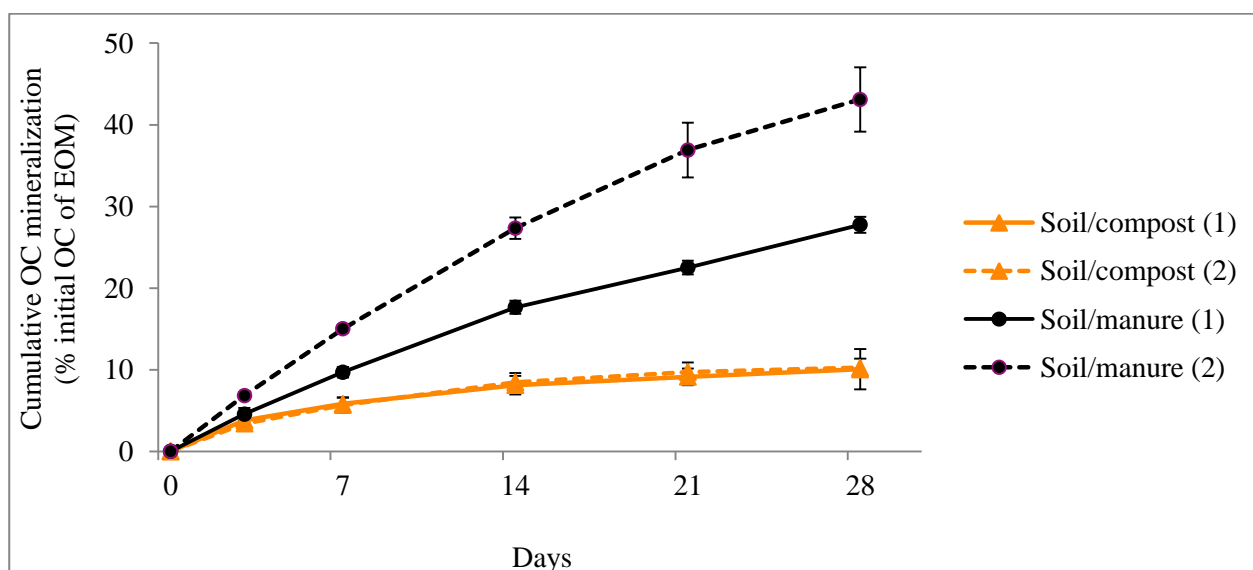


Figure III.S3. Cumulative mineralization of organic carbon (OC) of compost or manure in soil during the incubation experiments (1) with ^{14}C -AcSMX and (2) with ^{14}C -SMX.

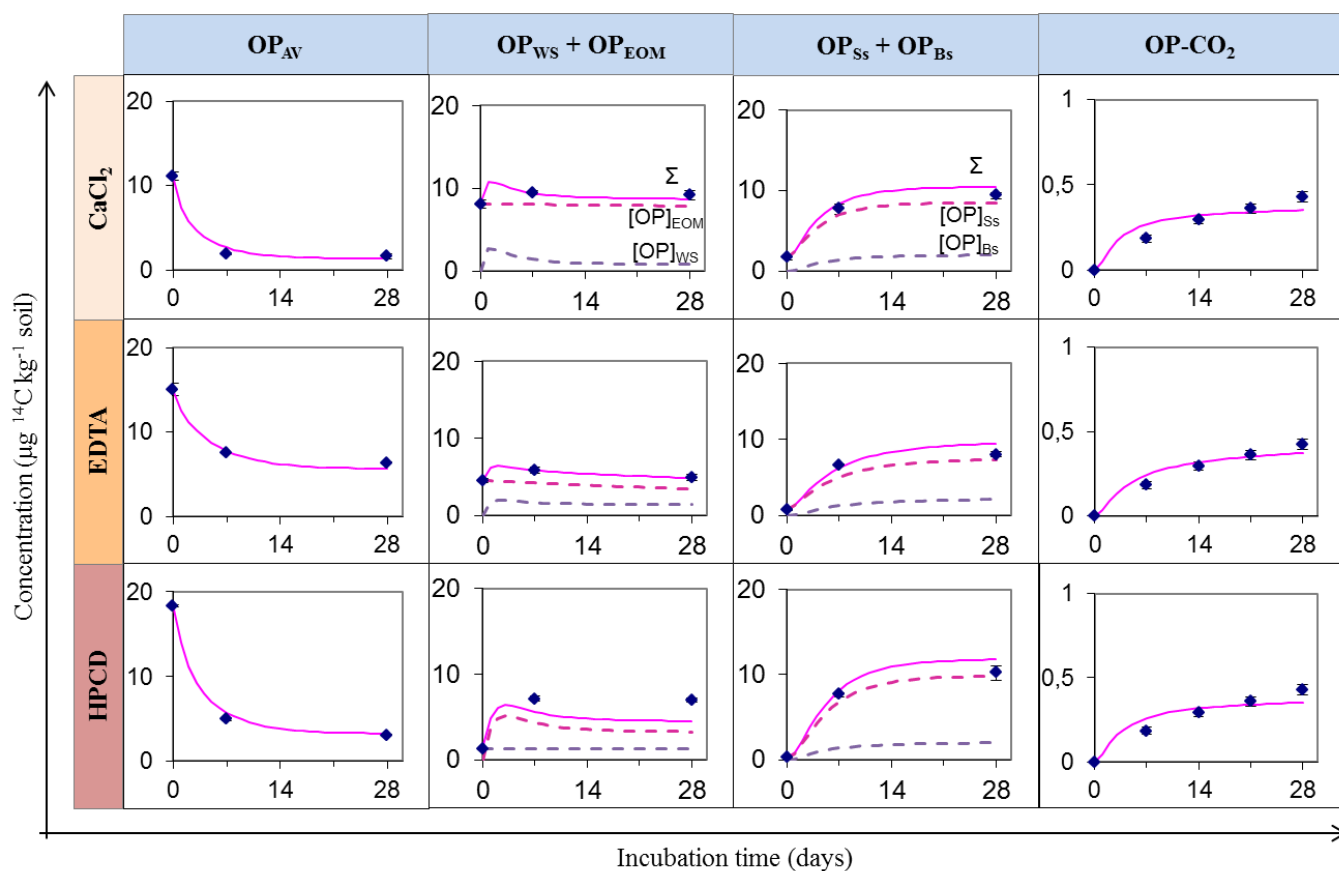


Figure III.S4. Soil/compost mixtures: Simulations of ^{14}C -AcSMX concentrations in available (OP_{AV}), weakly adsorbed (OP_{WS} + OP_{EOM}), non-extractable (OP_{Ss} + OP_{Bs}) and mineralized (OP-CO₂) fractions. CaCl₂, EDTA and HPCD were the soft extractants used to assess the available fraction and determined the modalities.

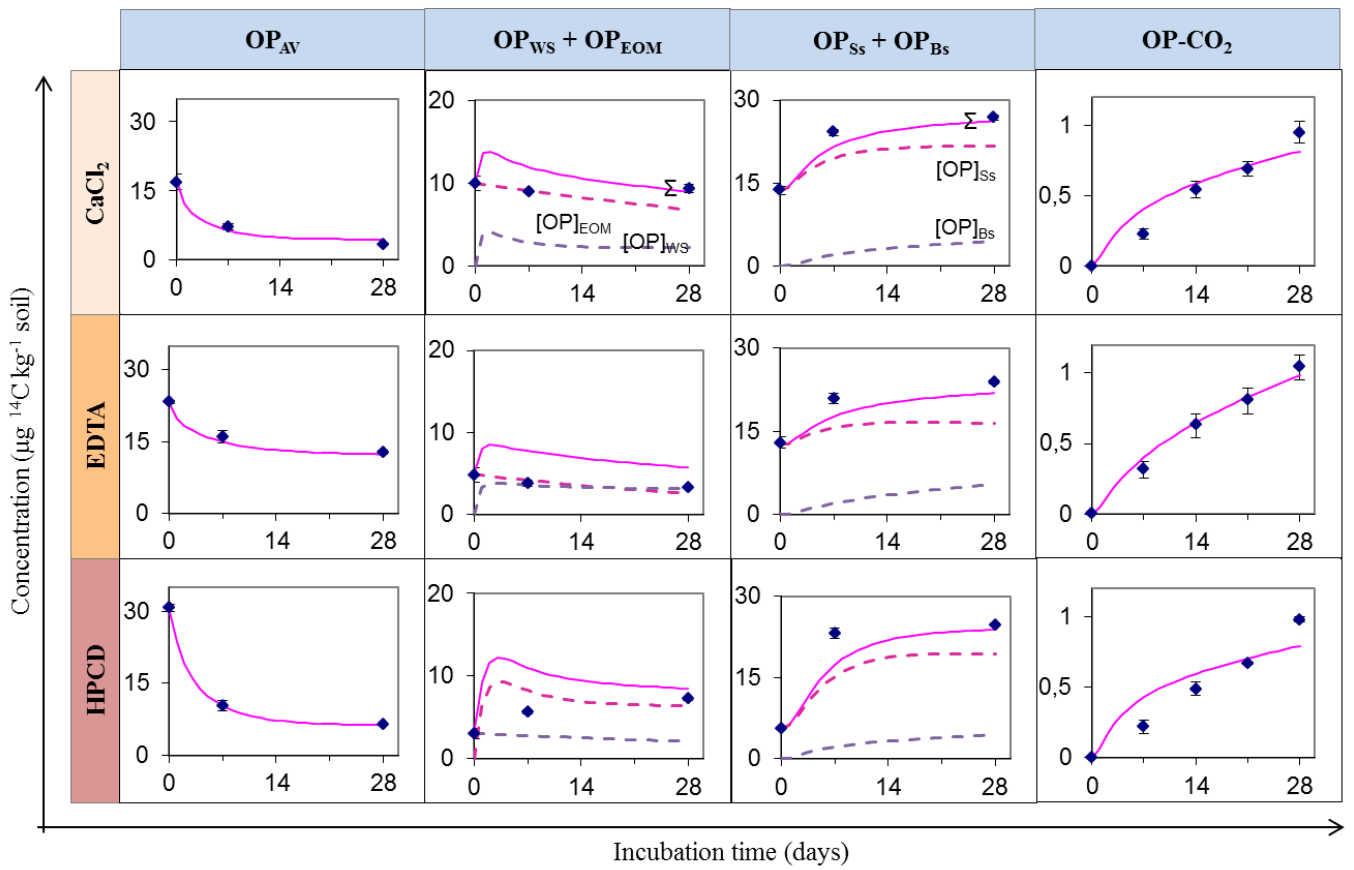


Figure III.S5. Soil/manure mixtures: Simulations of ¹⁴C-SMX concentrations in available (OP_{AV}), weakly adsorbed (OP_{WS} + OP_{EOM}), non-extractable (OP_{Ss} + OP_{Bs}) and mineralized (OP-CO₂) fractions. CaCl₂, EDTA and HPCD were the soft extractants used to assess the available fraction and determined the modalities.

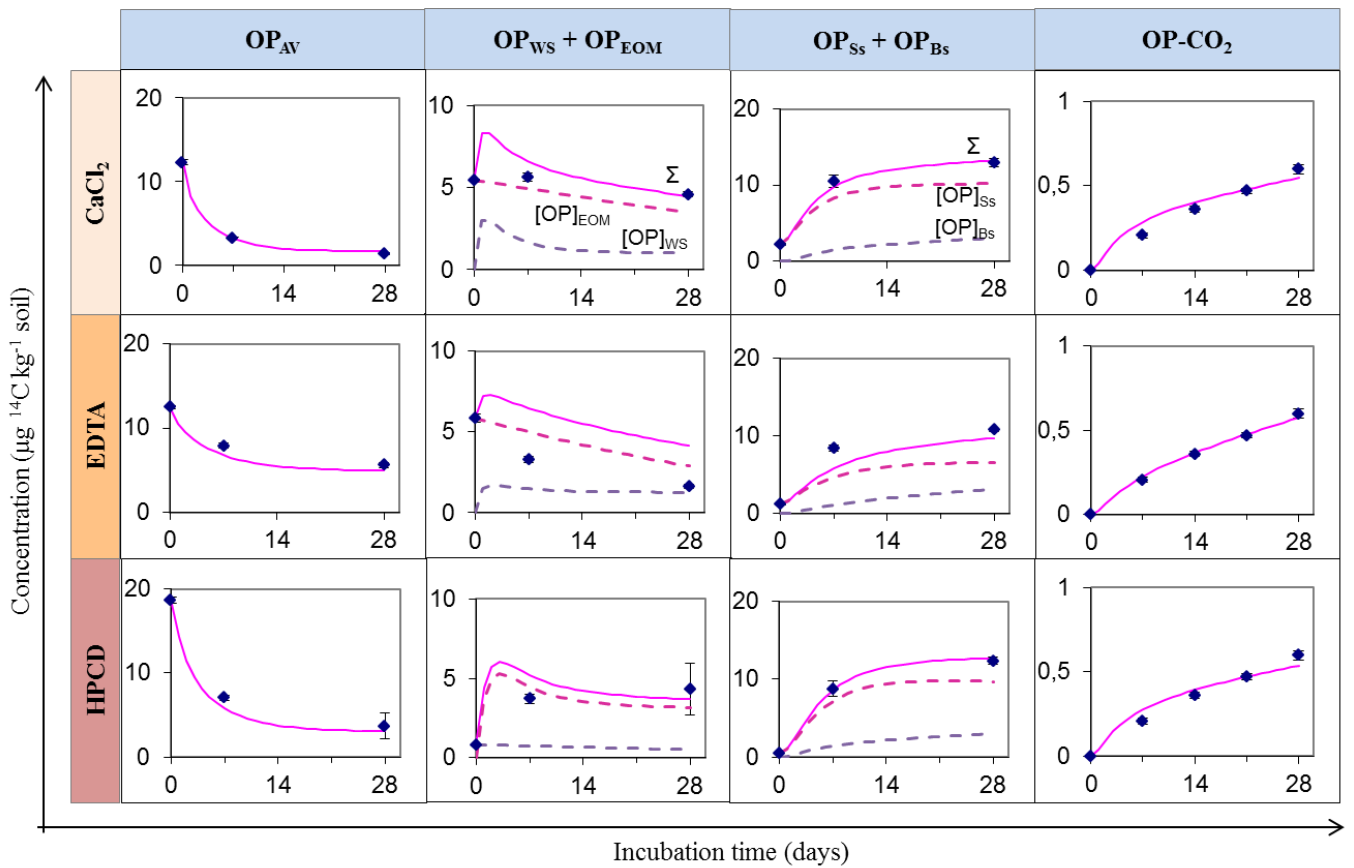


Figure III.S6. Soil/manure mixtures: Simulations of ^{14}C -AcSMX concentrations in available (OP_{AV}), weakly adsorbed ($\text{OP}_{\text{WS}} + \text{OP}_{\text{EOM}}$), non-extractable ($\text{OP}_{\text{SS}} + \text{OP}_{\text{BS}}$) and mineralized (OP-CO_2) fractions. CaCl_2 , EDTA and HPCD were the soft extractants used to assess the available fraction and determined the modalities.

Trois solutions d'extraction ont été sélectionnées pour estimer la disponibilité environnementale du sulfaméthoxazole et de son métabolite acétylé (Chapitre III). Les extractions douces avec les solutions de CaCl₂, d'EDTA ou de cyclodextrine ont permis de mettre en évidence une forte diminution de la disponibilité des deux molécules dans les sols amendés par les MAFOR au cours de la première semaine d'incubation. Selon le mécanisme d'extraction de la solution considérée, la co-extraction plus ou moins forte de matière organique pourrait expliquer les différences observées entre les fractions extraites. Par ailleurs, l'analyse de certains extraits a permis de mettre en évidence la biotransformation possible du sulfaméthoxazole et du N-acétylsulfaméthoxazole dans les sols amendés par les MAFOR.

Des produits de transformation ont déjà été identifiés dans la littérature, dans différents contextes. Il serait intéressant de les mettre en évidence et de suivre leur évolution lors de nouvelles incubations de sols amendés par des MAFOR contaminées par du sulfaméthoxazole.

Ces analyses représentent un challenge, car les antibiotiques et leurs produits de transformation doivent être extraits de matrices solides complexes, et leurs concentrations peuvent être relativement faibles. Une méthode d'analyse sensible et spécifique doit donc être développée en vue de leur quantification. La technique d'extraction sur phase solide en ligne de la chromatographie liquide ultra-haute performance couplée à la spectrométrie de masse en tandem (SPE-UHPLC-MS-MS) a été choisie afin de développer puis caractériser une méthode analytique adéquate, en vue de la quantification du sulfaméthoxazole et trois produits de transformation dans les extraits CaCl₂, EDTA et cyclodextrine.

De plus, afin de mieux comprendre les interactions entre le sulfaméthoxazole et le mélange sol/MAFOR, les matières organiques co-extraites ont été caractérisées grâce à la technique de spectrométrie de fluorescence 3D utilisée au LBE de Narbonne.

Dans le Chapitre IV, les questions traitées étaient les suivantes :

- 1) Quels sont les effets matrices ? La matière organique co-extraite peut-elle expliquer les interactions limitant la disponibilité du sulfaméthoxazole ?
- 2) La méthode analytique permet-elle de suivre l'évolution de la disponibilité du sulfaméthoxazole dans les sols amendés par les MAFOR ?
- 3) Les produits de transformation sélectionnés sont-ils formés et quantifiés dans les sols amendés par les MAFOR contenant du sulfaméthoxazole ?
- 4) Le type de MAFOR influence-t-il la disponibilité et la biodégradation du sulfaméthoxazole dans les sols ?

Chapitre IV

Sols amendés par les MAFOR
**Antibiotiques + produits
de transformation**
Adsorption dans des matrices
complexes (organique + minéral)



Extractions aqueuses



**Disponibilité du sulfaméthoxazole et des produits de
transformation**

Adsorption et dégradation des sulfonamides influencées par la
composition des MAFOR



- 1) Quantification des molécules dans les extraits : quels effets matrices? La matière organique co-extraite peut-elle expliquer les interactions limitant la disponibilité?
- 2) Méthode analytique adaptée pour le suivi de la disponibilité du sulfaméthoxazole dans les sols amendés?
- 3) Biotransformation du sulfaméthoxazole?
- 4) Influence du type de MAFOR sur la disponibilité et la biotransformation du sulfaméthoxazole?

Chapter IV: Investigating the effects of dissolved organic matter on the environmental availability assessment of sulfamethoxazole and transformation products in soils amended with sludge compost or manure by a new soft extraction method

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Abstract

Agricultural recycling of organic waste containing antibiotics such as sulfamethoxazole (SMX) may lead to their transfer into soils. The environmental availability of SMX and three transformation products (*N*-acetylsulfamethoxazole, 3-amino-5-methylisoxazole and aniline) was assessed in soils amended with sludge compost or manure by three different aqueous solutions: CaCl₂, EDTA or cyclodextrin. In these different extracts, the concentration of the four compounds was measured by a new method based on online solid phase extraction - ultra high performance liquid chromatography coupled to tandem mass spectrometry (online SPE-UHPLC-MS-MS). Freeze-storing soil samples was first shown to change the concentrations of available SMX. Then, the environmental availability of SMX and its transformation products was followed over a three-month incubation in soils amended with either sludge compost or manure, initially spiked with SMX at concentrations ranging from 22 µg kg⁻¹ to 2.2 mg kg⁻¹ dry soil. In parallel, the dissolved organic matter (DOM) in the extracts was characterized thanks to 3D-fluorescence spectrometry.

The SMX availability depended on the initial concentration, the extracting solution and the amendment at day 0. From 1.9 up to 63% of the SMX total content was initially available. The lowest available fractions were quantified in EDTA extracts in which the DOM was the most complex and responsible for high matrix effects in mass spectrometry compared to CaCl₂ extracts. QuEChERS purification of cyclodextrin extracts highly reduced the matrix effects. The available fraction of SMX strongly decreased in the first seven days of incubation to finally reach 0.4-0.8% after 84 days, whatever the initial conditions. This high decrease could be related to the DOM

complexification during the incubation. Two transformation products were quantified in the extracts for the highest initial SMX concentration levels: around 1% of acetyl-SMX at day 0, and < 0.1% of 3-amino-5-methylisoxazole at day 84.

In conclusion, the low environmental availability of SMX could be mainly due to the adsorption of the antibiotic on organic matter and probably resulted in the low SMX biotransformation in amended soils.

Keywords

Sulfonamide; availability; aqueous extraction; fluorescence spectrometry; ageing effect

IV.1. Introduction

Sewage sludge and manure are recycled in agriculture as exogenous organic matter (EOM) for their amending and fertilizing properties. However, via the EOM application, small amounts of antibiotics such as sulfonamides can be introduced also into cultivated soils. Sulfonamide concentrations can vary from 0.1 to 35.5 mg kg⁻¹ dry manure (Karcı and Balcıoğlu, 2009; Pan et al., 2011), and from 7 to 178 µg kg⁻¹ dry matter of sewage sludge (Lillenberg et al., 2009; Sabourin et al., 2009; Nieto et al., 2010). In agricultural soils amended with manure, the sulfonamide concentrations can be from less than 2 up to 400 µg kg⁻¹ dry soil (Hamscher et al., 2005; Karcı and Balcıoğlu, 2009; Toth et al., 2011). The behavior of sulfonamides in soils depends on the environmental conditions and is governed by the physicochemical properties of the molecule, the EOM and the soil. Generally, the sorption of sulfonamides to soils is low; as a consequence, the molecules are mobile in soils and can be leached to groundwater (Thiele-Bruhn et al., 2004; Kurwadkar et al., 2011; Bailey et al., 2016).

The possible biodegradation of sulfonamides in EOM-amended soils can lead to the formation of transformation products (TPs). However TPs may have been formed before EOM spreading, during wastewater treatments (Jelic et al., 2012), EOM post-treatments like composting (Lillenberg et al., 2010; Martín et al., 2015) or liming (Geng et al., 2016) and during EOM storage (Wang et al., 2006). The manure storage can also lead to an increase of the sulfonamide concentration as a result of the deacetylation of *N*₄-acetylated metabolite (Lamshöft et al., 2010). As a result, not only sulfonamides are present in EOM, but TPs and/or human or animal metabolites (e.g., *N*₄-acetylated metabolites) can also be found (García-Galán et al., 2008). As an example, the microbial transformation of sulfamethoxazole (SMX) can result in two types of transformation products (Majewski et al., 2014): 1) the derivatives from SMX transformation at the *para* position

like *N*₄-acetylsulfamethoxazole, *N*₄-hydroxy-acetylsulfamethoxazole, *N*₄-hydroxysulfamethoxazole, 4-nitro-sulfamethoxazole; and 2) the breakdown products from SMX cleavage like 3-amino-5-methylisoxazole, benzenesulfonamide, aniline. Some derivatives like nitroso-SMX are unstable and can be further decomposed or transformed (Majewski et al., 2014).

The presence of sulfonamides in the environment and their impacts on microbial communities were shown in a large number of publications (e.g., Kotzerke et al., 2008; Liu et al., 2009). Even if their concentrations may be low, the impacts of antibiotic residues on soil microorganisms should not be neglected, especially the antibiotic resistance acquisition and its potential dissemination (Wright, 2010; Boxall et al., 2012). Moreover, some transformation products of sulfonamides could still have toxic effects, and particularly the compounds preserving the sulfanilamide toxicophore with respect to the quantitative structure-activity relationship (Nouws et al., 1985; García-Galán et al., 2012; Bonvin et al., 2013; Majewski et al., 2014).

In order to measure the microbial exposure to sulfonamide residues, i.e., parent compound and transformation products, their bioavailability in soil has to be assessed. It could be defined as the fraction of molecules available to interact with living organisms in soil (Semple et al., 2004). Assessing the environmental availability of sulfonamides could be considered as a first step in the bioavailability assessment and indirect chemical methods such as aqueous extractions could be engaged (Lanno et al., 2004; Harmsen, 2007). Nowadays, most of the analytical methods are developed to measure the total concentrations of antibiotic residues in agricultural soils (Kemper, 2008). However, these measures are generally not representative of the microbial exposure (Alexander, 2000).

Our study focuses on the assessment of the environmental availability of SMX in EOM-amended soils. Two EOMs were considered with respect to their different characteristics and organic matter quality, i.e., a compost of sewage sludge and green waste and a farmyard manure. Aqueous extractions were performed with three different solutions, i.e., calcium chloride (CaCl₂), ethylenediaminetetraacetic acid (EDTA) and hydroxypropyl-beta-cyclodextrin (HPCD), representing different mechanisms of extraction (Chapter III). Based on their possible formation, commercial availability and physicochemical properties, three of the SMX transformation products were included in the analytical method development. The *N*₄-acetylsulfamethoxazole (AcSMX) derivative was selected as main metabolite, given its presence in sewage sludge (García-Galán et al., 2008) and its reversible transformation back to SMX in manure-amended soil (Höltge and Kreuzig, 2007). Then, two breakdown products were selected: 3-amino-5-methylisoxazole (3A5MI) considering its stability in the SMX biodegradation reaction (Müller et al., 2013), and aniline (ANI). This study had three objectives: i) to develop a suitable method to measure the potentially-low

amounts of SMX, AcSMX, 3A5MI and ANI in environmentally-available fractions in amended soils; ii) to evaluate the impact of soil freeze-storage on the environmental availability of the compounds; and iii) to study the effects of the composition of co-extracted organic matter in aqueous extracts on the quantification of available fractions.

For the first objective, the analytical method was developed using online solid-phase extraction-ultra-high performance liquid chromatography coupled to tandem mass spectrometry (online SPE-UHPLC-MS-MS), due to the high sensitivity and selectivity of this technique. After assessing the method's characteristics, we tested the method over a three-month incubation of soils amended with contaminated EOM, from environmentally relevant concentrations ($22 \mu\text{g SMX kg}^{-1}$ dry soil; Toth et al., 2011, Baran et al., 2011) to high concentrations ($2.2 \text{ mg SMX kg}^{-1}$ dry soil). The time-evolution of SMX environmental availability and the formation of transformation products were targeted.

Several authors stored the collected soil samples at -20°C until extraction and analysis (Kodešová et al., 2016; Zhao et al., 2010), without assessing the potential effect of soil freezing on the extractability of the compounds. As the concentrations of compounds in aqueous extracts could be low, it was crucial to study these potential impacts, particularly in case of large number of soil samples collected.

For the third objective, we supposed that: 1) the aqueous extracts could contain different types of dissolved organic matter (DOM) depending on the extraction mechanism and on the EOM composition; 2) the available fraction may contain both free-dissolved compounds and compounds associated to soluble or colloidal organic matter (Harmsen, 2007); and 3) co-extraction of DOM and SMX could depend on the extracting solution. Thus, DOM was characterized in the CaCl_2 , EDTA and HPCD extracts with the method of three-dimensional fluorescence spectrometry (Jimenez et al., 2014).

Thereby, the effects of initial SMX concentrations, amendment type, ageing, i.e., incubation time and organic matter evolution, on the SMX extractability and the possible biotransformation, were considered in the environmental availability assessment.

IV.2. Materials and methods

IV.2.1. Chemicals and reagents

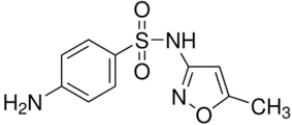
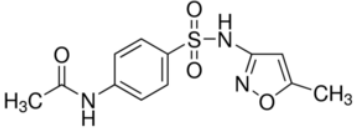
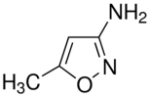
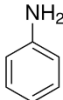
Analytical standards (purity > 99%), sulfamethoxazole (SMX) and *N*₄-acetylsulfamethoxazole (AcSMX) were purchased from Dr Ehrenstorfer GmbH, (Ausburg, Germany); aniline (ANI) and 3-amino-5-methylisoxazole (3A5MI) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Deuterated labelled analytical standard sulfamethoxazole-d₄ (benzene-d₄) was obtained from CDN isotopes (Pointe-Claire, Canada). The physicochemical properties of SMX and selected transformation products are given in Table IV.1.

The stock solutions were prepared in methanol (Carlo Erba, Val-de-Rueil, France), at 500 mg L⁻¹ for AcSMX, ANI and 3A5MI; at 242 mg L⁻¹ for SMX and at 285 mg L⁻¹ for SMX-d₄. The stock solutions were stored in the dark at -20°C.

The extracting solutions were prepared by dissolution in Milli-Q water (Millipore, Saint-Quentin-en-Yvelines, France) of respective chemical products (purity > 99%): calcium chloride (CaCl₂; VWR, Fontenay-sous-Bois, France), disodium ethylenediaminetetraacetic acid (Na₂EDTA; Sigma-Aldrich) and 2-hydroxypropyl-β-cyclodextrin (HPCD; Sigma-Aldrich). Sodium hydroxide (NaOH; Sigma-Aldrich) and hydrochloric acid (HCl; Sigma-Aldrich) were used to adjust the pH. Anhydrous sodium sulfate (Na₂SO₄), anhydrous sodium acetate (NaOAc) and Na₂EDTA composed the salts for quick, easy, cheap, effective, rugged and safe extraction (QuEChERS; Bourdat-Deschamps et al., 2014). HPLC and LC-MS grade acetonitrile and LC-MS grade methanol were purchased from Carlo Erba and HPLC grade trifluoroacetic acid (TFA) from Sigma-Aldrich.

Table IV.1

Chemical structures and physicochemical properties of sulfamethoxazole and the three selected transformation products.

Molecule (abbreviation)	Structure	Molecular weight g mol ⁻¹	pK _a	Log K _{ow}
Sulfamethoxazole (SMX)		253.3	1.6 - 5.7 ^a	0.89 ^b
N ₄ -acetylsulfamethoxazole (AcSMX)		295.3	5.07 ^b	1.21 ^b
3-amino-5-methylisoxazole (3A5MI)		98.1	Unknown	Unknown
Aniline (ANI)		93.1	4.6 ^c	0.9 ^b

^a (Boreen et al., 2004); ^b (Majewski et al., 2014); ^c (Lee et al., 1997).

IV.2.2. Soil and exogenous organic matter (EOM)

Topsoils of non-amended plots in the long-term experimental field device QualiAgro (Yvelines, France) were sampled and sieved to 5 mm. The main soil characteristics were: 79% silt, 6% sand, 15% clay, pH 6.6, 1.1% organic carbon content and 20% water content (g water/100 g dry soil). The farmyard manure and the compost of sewage sludge and green waste (20/80: v/v) were ground and sieved to 2 mm. The compost and the manure were respectively characterized by pH 7.7 and 8.8; 71% and 52% dry matter content; 25.7% and 35.5% organic carbon content. All samples were stored at 4 °C until the experiment for less than 6 months. Following chemical analyses according to Ferhi et al. (2016), no SMX was detected in EOM and soil.

All samples or microcosms of soil/EOM mixtures were prepared according to the field rate application of EOM (4 t C ha⁻¹) in the QualiAgro site (Obriot et al., 2016). The soil was amended with compost or manure to reach a EOM/soil ratio equal to 3% in dry mass.

IV.2.3. Aqueous extraction method

The three extracting solutions were aqueous solutions of 0.01 M CaCl₂, 0.1 M EDTA and 0.05 M HPCD. The pH of CaCl₂ and HPCD solutions were around 4.5 and 7, respectively, and the

pH of EDTA solution was adjusted to 7 with NaOH (Chapter III). The aqueous extraction method consisted in adding 15 mL of the aqueous solution to soil sample (equivalent to 5 g dry soil) in Falcon[®] tubes (50 mL, Corning, NY, USA), shaking overnight with overhead shaker (~17 h, 15 rpm; Heidolph[®] Reax 20, Schwabach, Germany) and centrifuging to collect the supernatant (11 000 x g for 10 min; Sorvall RC6+, ThermoFisher Scientific, Waltham, MA, USA). Another centrifugation was performed on 1.5 mL extract (MiniSpin, 10 min at 12 100 x g, Eppendorf, Hamburg, Germany) to ensure the absence of particles before the online SPE-UHPLC injection.

Different conditions of extract treatment (modification of pH, QuEChERS purification) prior to UHPLC analysis were tested in order to limit the matrix effects generally observed with electrospray ionization in MS detection. As the initial pH of the extracts was neutral (soil pH), the acidification to pH 2-3 with diluted HCl (0.1%) and the alkalization to pH 9 with NaOH were tested. To perform the QuEChERS purification, a volume of 700 µL of spiked water, spiked extract or real extract were introduced in an Eppendorf tube with 700 µL of acetonitrile, 70 mg of NaOAc, 100 mg of Na₂EDTA, 280 mg of Na₂SO₄ (Bourdat-Deschamps et al., 2014). The mixture was then vortex shaken (Vortex Genie, Scientific Industries Inc., Bohemia, NY, USA) for 10 s. After centrifugation at 12,100 x g (MiniSpin) during 10 min, 500 µL of the upper phase were transferred into a HPLC vial and evaporated under nitrogen flow. A volume of 500 µL Milli-Q water was added to the residual drop in the HPLC vial before its swirling on vortex. The sample was then injected in online SPE-UHPLC-MS-MS.

IV.2.4. Analytical conditions for online SPE-UHPLC-MS-MS

The UHPLC device was composed of an Acquity UPLC system (Waters, Milford, MA, USA) with a high pressure binary solvent manager, an autosampler (10 °C) and a column oven (30 °C). A two-position valve was used to switch between the loading and elution modes of the SPE cartridge (Oasis HLB, 2.1 x 20 mm, 25 µm particle size, Waters). The mobile phase was composed of water + 0.01% TFA (A) and acetonitrile + 0.01% TFA (B). The maximal injection volume was determined to avoid the loss of compounds through the SPE cartridge during the loading step. As the smallest breakthrough volume was obtained for the 3A5MI compound (between 200 and 400 µL), the injection volume was set at 200 µL. The sample was loaded on the SPE cartridge with 100% A at 1 mL min⁻¹ during 1.9 min. The equilibrium of the UHPLC column was kept with an isocratic pump (model 515, Waters) during the loading step of the SPE cartridge. Then, after switching the valve to elution mode, the molecules retained on the SPE cartridge were eluted to the

UHPLC column in the back-flush mode and the separation of the compounds was carried out on a BEH C₁₈ column (2.1 x 100 mm, 1.7 μm particle size, Waters) with a flow rate at 0.4 mL min⁻¹, with an isocratic elution at 100% A for 2 min followed by a gradient profile from 100% A to 100% B in 15 min. An UV detector (TUV, Waters) was placed before the mass spectrometer and set at 232 nm.

The UHPLC effluent was introduced in the triple-quadrupole mass spectrometer (TQD, Waters). The temperature of the electrospray source was equal to 150°C with a cone gas flow rate at 20 L h⁻¹. The desolvation temperature and gas flow rate (nitrogen) were fixed at 400°C and 800 L h⁻¹, respectively. The capillary voltage value was 3.0 kV for all the compounds, the extractor voltage, 3 V and the pressure of the dissociation gas (argon) in the collision cell was set at 3.4 x 10⁻³ mbar. The electrospray ionization (ESI) was performed in the positive mode, involving the protonation of the precursor ion: [M + H]⁺. For each standard compound, the precursor ion and the optimum cone voltage were determined in MS full-scan mode, and two product ions and the associated optimum collision energies were determined in daughter ion scan mode. The most abundant product ion (I) was selected for the quantification transition (precursor ion > product ion I) and the second most abundant product ion (II) was selected for the confirmation transition (precursor ion > product ion II; Table IV.2). Acquisition and treatment of data were respectively realized with MassLynx and QuanLynx software (Waters).

Table IV.2

MS-MS parameters of the different molecules (electrospray ionization in positive mode): cone voltage, quantification and confirmation transitions with respective collision energies.

Molecule	Cone (V)	Quantification		Confirmation	
		Transition	Collision energy (eV)	Transition	Collision energy (eV)
SMX	35	254.0 > 156.0	16	254.0 > 108.0	21
AcSMX	40	296.0 > 134.1	25	296.0 > 93.0	30
3A5MI	30	99.0 > 44.0	12	99.0 > 72.0	12
ANI	40	94.0 > 77.0	20	94.0 > 51.0	25

IV.2.5. Characterization of the method

IV.2.5.1. Matrix effects and QuEChERS recoveries

According to the aqueous extraction method (section IV.2.3), the CaCl₂, EDTA and HPCD extracts were prepared and then spiked with SMX (10 µg L⁻¹) and the three selected transformation products (25 µg L⁻¹), in order to determine the matrix effect for each compound in mass spectrometry following equation (1):

$$\text{Matrix effect (\%)} = \left(\frac{A_{\text{extract}} - A_{\text{control}}}{A_{\text{water}}} - 1 \right) \times 100 \quad (1),$$

where A_{extract} is the area of the compound in CaCl₂, EDTA or HPCD extract; A_{control} is the area of the compound in a negative control, which consisted in a non-spiked extract (area supposed to be equal to 0); and A_{water} is the area of the compound in water spiked at the same concentration level and in which the matrix effect is not supposed to occur (equal to 0%).

The QuEChERS purification recoveries were calculated following equation (2):

$$\text{QuEChERS recovery (\%)} = \frac{A_{\text{matrix}} - A_{\text{control}}}{A_{\text{water}}} \times 100 \quad (2),$$

where A_{matrix} is the area of the compound in the spiked water or spiked extract; A_{control} is the area of the compound in the negative control (supposed to be equal to 0); and A_{water} is the area of the compound in water spiked at the same concentration level.

IV.2.5.2. Limits of detection and quantification

The CaCl₂, EDTA and HPCD extracts spiked with SMX and the three transformation products (20 µg L⁻¹) were progressively diluted to determine for each compound the limits of detection and quantification (µg kg⁻¹ dry mass) calculated from the extract concentrations (µg L⁻¹) for which the signal-to-noise ratio was equal to 3 and 10, respectively.

IV.2.5.3. Aqueous extractability

The aqueous extractability was assessed in manure-amended soil for SMX and its main transformation product AcSMX given its possible reversible transformation to SMX (Höltge and Kreuzig, 2007). According to the preparation of soil/EOM mixtures (section IV.2.2), the soil was

weighed (equivalent to 5 g dry soil) in a Falcon[®] tube (50 mL) and the manure was added on the soil (equivalent to 0.15 g dry matter). The manure was spiked with the solution of SMX or AcSMX in water. Two concentration levels were used for each molecule: 22 µg kg⁻¹ ([SMX] ×1 or [AcSMX] ×1) and 1.32 mg kg⁻¹ dry soil ([SMX] ×60 or [AcSMX] ×60). The moisture content of soil/manure mixture was adjusted to 30% (g water/100 g dry soil). A contact time of 30 to 60 min was let between the compound and the manure before its incorporation into soil by thoroughly mixing with a spatula. Thereby, in a similar way as in the field conditions, the compound transfer to the soil was simulated by the EOM application. The short-term availability of SMX and AcSMX was assessed in triplicate by performing the aqueous extraction method (section IV.2.3). The aqueous extractability was calculated with the equation (3):

$$\text{Aqueous extractability (\%)} = \frac{([c]_{\text{extract}} - [c]_{\text{blank}})}{[c]_{\text{initial}}} \times 100 \quad (3),$$

where $[c]_{\text{extract}}$ is the concentration of SMX or AcSMX in CaCl₂, EDTA or HPCD extract (µg L⁻¹); $[c]_{\text{blank}}$ is the concentration in non-spiked soil/manure mixture (supposed to be equal to 0 µg L⁻¹); and $[c]_{\text{initial}}$ is the theoretical initial concentration (µg L⁻¹).

IV.2.6. Effect of freezing storage of soil samples on the aqueous extractability of SMX

The soil/manure mixtures spiked at three SMX concentration levels, i.e., 0.55, 1.32 and 2.22 mg SMX kg⁻¹ dry soil, were sampled in three replicates of microcosms incubated at 15 °C in the dark (section IV.2.7). The aqueous extractions (section IV.2.3) were performed on 6-g wet soil samples, either immediately after incubation or after one-month storage at -20°C. Thereby, the SMX concentrations were compared between fresh soil/manure samples and frozen soil/manure samples.

IV.2.7. Application of the aqueous extraction method to incubated soils

The aqueous extraction method was applied to assess the SMX availability over a three-month incubation in manure-amended soil and also in compost-amended soil. According to the preparation of soil/EOM microcosms (section IV.2.2), the soil was first weighed (equivalent to 170 g dry soil) in a 500-mL jar (Le Parfait, France) two weeks before the beginning of the incubation experiment. The moisture content of the mixtures was adjusted to 50% of the water holding capacity and the microcosms were stored at 15 °C in the dark to adapt the soil microorganisms to the experimental conditions. At day 0, the EOM was weighed (equivalent to 5 g dry matter) and as

described in section IV.2.5.4, it was spiked with the solution of SMX in water to obtain a gradient of concentrations from 22 $\mu\text{g kg}^{-1}$ ([SMX] $\times 1$) up to 2.22 mg kg^{-1} dry soil ([SMX] $\times 100$). Four replicates were prepared for each treatment. After mixing the spiked EOM with the soil, the microcosm moisture was finally adjusted to 80% of water holding capacity, i.e., 27% for soil/compost mixtures and 30% for soil/manure mixtures. All jars were placed at 15°C in the dark for 84 days. During the incubation, the oxygen renewal in the jars was realized each week with their opening and the moisture was adjusted if necessary by weighing the microcosms. After 0, 7, 28 and 84 days of incubation, the aqueous extraction method was applied (section IV.2.3) on 6-g fresh soil samples in Falcon[®] tubes (50 mL). Thereby, the availability of SMX was assessed in function of the SMX concentration level, the incubation time and the EOM. Moreover, the SMX biotransformation was followed with respect to the possible quantification of the three transformation products selected.

IV.2.8. Fluorescence spectroscopy analysis

After the SPE-UHPLC-MS-MS analysis, the CaCl_2 , EDTA and HPCD extracts obtained during the incubation experiment were stored at -20 °C. The absence of any impact on the organic matter quality due to freeze-storing the extracts was previously confirmed (Jimenez et al., personal communication). Two of the four replicates of extracts were filtered ($< 0.45 \mu\text{m}$) and analyzed by fluorescence spectroscopy (Perkin Elmer LS55, Courtaboeuf, France) in liquid phase fluorescence mode. As described by Jimenez et al. (2014), excitation wavelengths varied from 200 to 600 nm with 10 nm steps. Excitation and emission monochromator slit width were set at 10 nm and the emission monochromator scan speed was 1200 nm per second and fluorescence values were recorded every 0.5 nm from 200 to 600 nm. The Perkin Elmer FL Winlab software was used to record and treat the fluorescence spectra obtained. The absence of fluorescent compounds in the CaCl_2 , EDTA and HPCD solutions was confirmed before the analysis of the respective extracts. The fluorescence spectra obtained were decomposed in seven zones (zones I to VII) according to Jimenez et al. (2014), each zone corresponding to a DOM type (Figure IV.1). The proportion of fluorescence (P_i) of each zone (i) was calculated with the following equations (4) and (5):

$$V_i (\text{U. A.} / \text{mg DOC. L}^{-1}) = \frac{V_{\text{image } j \ i}}{\text{DOC sample}} \times 1 / \frac{S_i}{\sum_{i=1}^7 S_i} \quad (4),$$

$$P_i (\%) = \frac{V_i}{\sum_{i=1}^7 V_i} \times 100 \quad (5),$$

where V_i (U.A. / mg DOC L^{-1}) is the fluorescence zone volume of the zone i; $V_{\text{image } j \ i}$ (U.A.) is the raw volume obtained with Image J for the zone i; $\text{DOC}_{\text{sample}}$ (mg L^{-1}) is the concentration of

Dissolved Organic Carbon in the analyzed sample; S_i (nm^2) is the area of the zone i ; and P_i (%) is the fluorescence proportion of the zone i . The concentrations of DOC were measured in respective extracts by using a total organic carbon analyzer in liquid phase (TOC, Shimadzu, Marne-la-Vallée, France). The DOC analyses were also carried out in CaCl_2 , EDTA and HPCD solutions to determine the initial DOC content and to subtract this value to the DOC value in the corresponding extract.

A complexity index was calculated (Eq. 6) by the ratio of the sum of the fluorescence volumes (%) of the most complex zones (IV to VII) over the sum of the fluorescence volumes of the protein-like zones (I to III):

$$\text{Complexity index} = \frac{\sum_{i=4}^7 Z_i}{\sum_{j=1}^3 Z_j} \quad (6),$$

where Z_k is the % of fluorescence in zone k , $k \in [1;7]$.

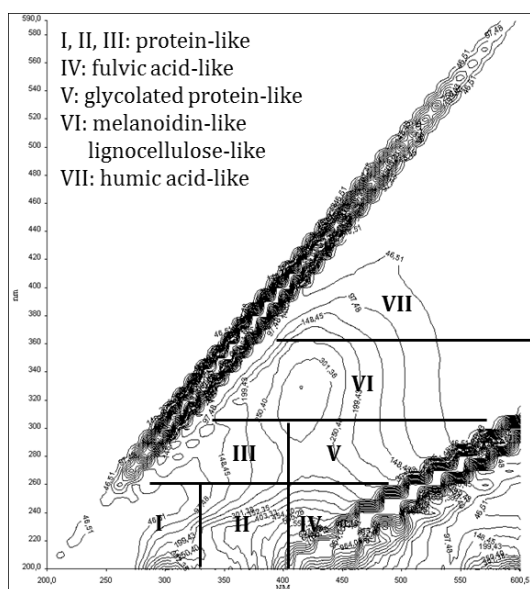


Figure IV.1. 3D-fluorescence spectra of an extract using the liquid phase fluorescence (Jimenez et al., 2014).

IV.2.9. Statistical analyses

Statistical analyses were realized with R-Studio software (version 0.98.501, RStudio Inc.) using linear mixed-effects models (ANOVA) and non-parametric tests (Kruskal-Wallis). Thus, the available fractions were compared in function of the aqueous extractant, the soil/EOM mixture and the incubation time. The complexity indexes of the DOM in aqueous extracts were also compared.

IV.3. Results and discussion

IV.3.1. Characterization of the analytical performances of the aqueous extracts

Negative value of matrix effect ($< 0\%$) corresponded to signal suppression in MS whereas a positive value ($> 0\%$) corresponded to signal enhancement. The matrix effects were generally less important in CaCl_2 extracts (up to -49% ; Figure IV.2A) than in EDTA extracts (up to -60% ; Figure IV.2B) for all the compounds. The higher signal suppression in EDTA extracts could be attributed to the co-extraction of organic matter. This result may be linked to the DOC concentration, significantly higher in EDTA extracts than in CaCl_2 extracts (804 and 50 mg C L^{-1} , respectively). Moreover, the dark brown color of EDTA extracts was in accordance with matrix effects, as well as the UV traces showing a very intense signal on chromatograms of EDTA extracts from 6 to 18 min, with a maximum absorbance around 11 min. The maximum UV absorbance in EDTA extracts was 5 times higher than in CaCl_2 extracts in which the UV absorbance started from 6 min and ended at around 12 min. Comparing the four compounds together, the signal suppression was the highest for ANI. The matrix effects considerably depended on the compound but were not related to the hydrophobicity of compounds since for example, the signal suppression was lower for 3A5MI than for SMX. The attempts to minimize the matrix effects by pH adjustment globally showed that acidification increased the ion suppression, and alkalization was not efficient. Therefore, the decision to keep the initial pH of extract was taken.

In HPCD extracts, the signal suppression was complete for all compounds (matrix effect -100%). This could be due to the huge DOC concentration in HPCD extracts (16.7 g C L^{-1} , even after subtracting the initial organic carbon content of the HPCD solution). A purification step was necessary before the analysis of HPCD extracts; thereby, the QuEChERS purification was tested. Before testing the QuEChERS purification directly on HPCD extracts, the potential loss of compounds was evaluated, performing this purification with compounds in pure water and then in HPCD solution at neutral pH (Figure IV.3). Indeed, the most polar compounds may not be extracted in the organic phase. At neutral pH, QuEChERS recoveries in water were: $82 \pm 5\%$ for SMX, $70 \pm 4\%$ for AcSMX, $73 \pm 2\%$ for 3A5MI and $35 \pm 5\%$ for ANI. The recoveries were lower for ANI since this small and very polar molecule (Table IV.1) tended to stay in the aqueous phase despite the high amount of salts. In the HPCD solution, the QuEChERS recoveries decreased slightly for SMX and AcSMX to $74 \pm 2\%$ and $66 \pm 2\%$, respectively; and more markedly for the two smallest compounds, to $55 \pm 1\%$ for 3A5MI and only $5 \pm 1\%$ for ANI. The QuEChERS recoveries decreased

in presence of cyclodextrins that could modify the solubility of compounds in water or acetonitrile resulting in a lower transfer of compounds in the organic phase. Finally, in HPCD extracts, the QuEChERS recoveries ranged between 68 and 89% for SMX, 67 and 71% for AcSMX, 56 and 66% for 3A5MI, depending on the pH of the HPCD extract (Figure IV.3). The QuEChERS extraction recoveries for SMX were consistent with those in soil water (Bourdat-Deschamps et al., 2014). The mean recovery of the most polar compound ANI was evaluated about 10%, with a high variability due to the difficult quantification of the low concentrations. As the pH modification did not impact much the recoveries, it was decided not to adjust the pH of HPCD extracts. Even though ANI was lost during this step, the QuEChERS purification highly reduced the matrix effects since before the extract purification, the detection signal was totally suppressed.

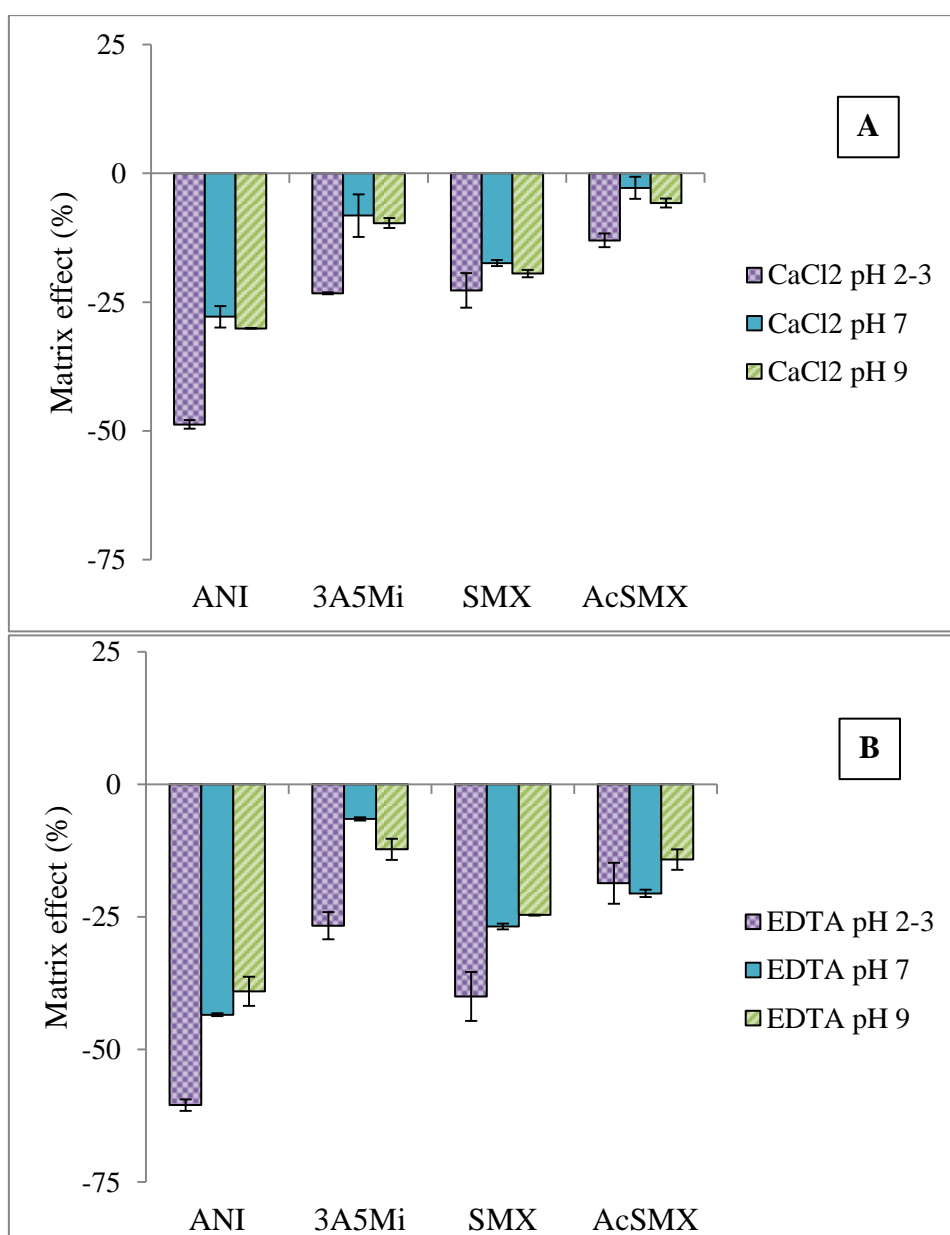


Figure IV.2. Matrix effects (% , n = 2) for the compounds in A] CaCl₂ extracts and B] EDTA extracts without modification (pH 7) or with modification (pH 2-3 and 9) of the extract pH.

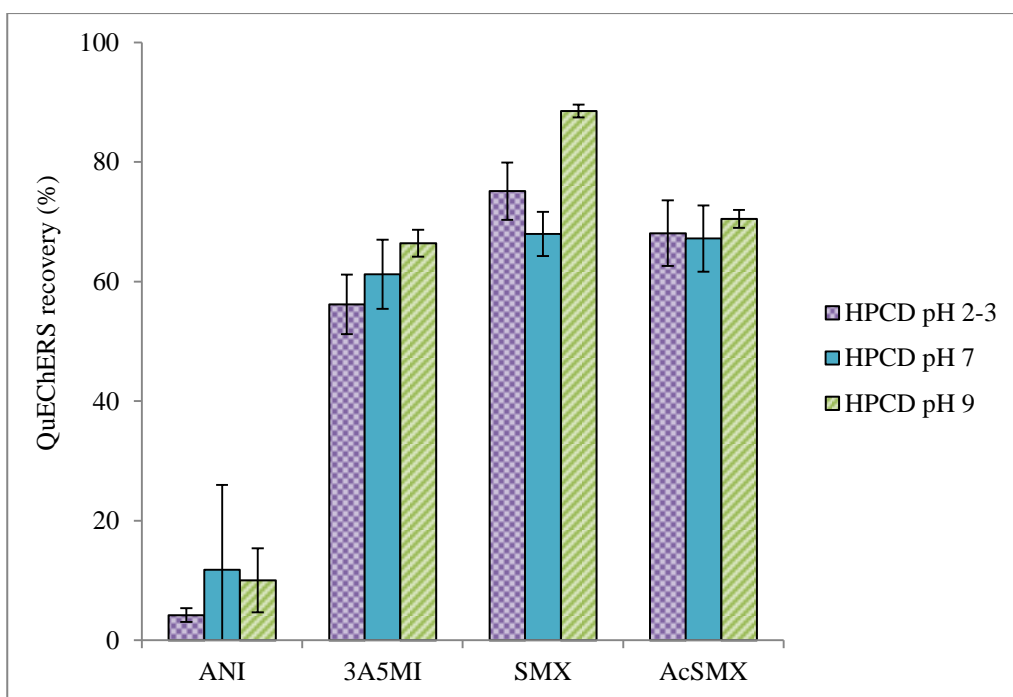


Figure IV.3. QuEChERS recoveries (%) for the compounds in HPCD extracts (n = 3), with and without modification of pH.

Limits of quantification (LOQ) were determined for each compound in aqueous extracts after applying the pretreatment conditions (Table IV.3). In our study, the LOQ integrated the instrumental LOQ of the online SPE-UHPLC-MS-MS method, the matrix effects and also the QuEChERS recoveries for HPCD extracts while the aqueous extractability was not taken into account with respect to “soft” extractability of compounds in the environmental availability assessment. Comparatively to LOQ obtained for SMX and AcSMX with exhaustive methods using solvent-based extractions (Stoob et al., 2006; Martínez-Carballo et al., 2007; Salvia et al., 2013; García-Galán et al., 2013), our LOQ was comprised in the same range of 0.1-10 $\mu\text{g kg}^{-1}$ dry soil. The LOQ obtained in HPCD extracts (0.58-0.73 $\mu\text{g kg}^{-1}$) were two to four times lower than LOQ obtained in CaCl_2 or EDTA extracts (1.17-5.83 $\mu\text{g kg}^{-1}$), depending on the compound. The LOQ of ANI in HPCD extract was not determined given the variability of QuEChERS recoveries. The QuEChERS purification enabled to decrease the LOQ, with respect to cleaner extracts after the purification. Nevertheless, we decided not to perform the QuEChERS purification on CaCl_2 and EDTA extracts to shorten the handling time and to avoid the loss of the ANI compound.

The SMX- d_4 internal standard was added to the CaCl_2 and EDTA extract aliquots directly in the vial (71 $\mu\text{g L}^{-1}$) prior to injection to correct the matrix effects. In case of HPCD extracts, the

internal standard was added to the extract aliquot before performing the purification to correct both matrix effects and QuEChERS recoveries. The internal standard was not added to soil samples before performing the aqueous extraction because we did not want to correct the extraction recoveries. The deuterated compound was used to overcome matrix effects for SMX and AcSMX since their physicochemical properties are close (Table IV.1).

Table IV.3

Limits of detection and quantification (LOD / LOQ, $\mu\text{g kg}^{-1}$ dry soil) of ANI, 3A5MI, SMX and AcSMX extracted by CaCl_2 , EDTA or HPCD solutions (no pretreatment for CaCl_2 and EDTA extracts; QuEChERS purification for HPCD extract).

	ANI	3A5MI	SMX	AcSMX
	LOD / LOQ	LOD / LOQ	LOD / LOQ	LOD / LOQ
CaCl_2	0.70 / 2.33	0.70 / 2.33	0.35 / 1.17	0.44 / 1.46
EDTA	1.75 / 5.83	0.44 / 1.46	0.52 / 1.75	0.52 / 1.75
HPCD	Not determined	0.17 / 0.58	0.17 / 0.58	0.22 / 0.73

IV.3.2. Freeze-storage of soil samples and effect on the aqueous extractability of SMX

To study the potential effect of the soil freezing on the SMX extractability, soil/manure mixtures contaminated with SMX were stored during one month at $-20\text{ }^\circ\text{C}$. The freeze-storage led to a decrease of SMX extractability using the aqueous extraction method, whatever the aqueous extractant used (Figure IV.4). The SMX concentrations were indeed 77 % lower in aqueous extracts from frozen samples than from fresh samples ($R^2 = 0.96$, $p = 2.2 \times 10^{-16}$). The storage of soil samples at $-20\text{ }^\circ\text{C}$ could modify the soil properties and constituents (e.g., organic matter) and the accessibility of the extractant. Significant losses of pharmaceuticals were reported in freeze-stored aqueous extracts or biosolids (EPA, 2010). Another hypothesis could be that during thawing, additional interactions of SMX with soil components could happen, leading to a lower extractability. Consequently, we decided to always perform the aqueous extraction on fresh soil samples to correctly assess the environmental availability.

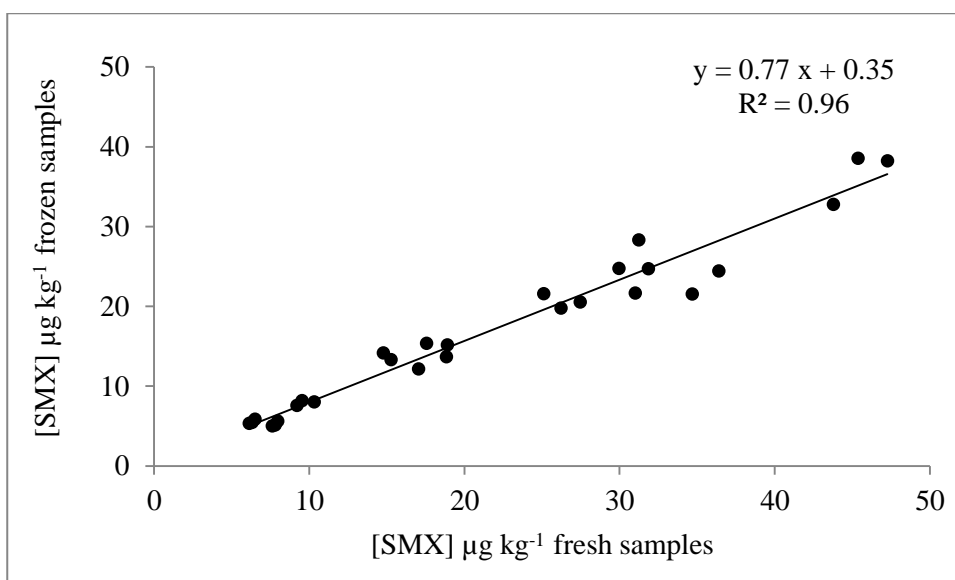


Figure IV.4. Concentrations of available SMX ($\mu\text{g kg}^{-1}$ of dry mass) quantified in CaCl_2 , EDTA and HPCD extracts after the freezing of soil/manure samples in function of the concentrations quantified in fresh soil/manure samples ($n = 27$).

IV.3.3. Aqueous extractability of SMX and AcSMX

The aqueous extraction method was applied to measure the extractability of SMX and AcSMX in soil/manure mixtures at two different concentration levels after a 30-60 min contact with manure before the incorporation into soil and the extraction (Figure IV.5). SMX was less available than AcSMX at the two concentration levels chosen, as the *N*-acetylation of the aniline could lead to a lower adsorption (Förster et al., 2009). The higher aqueous extractability for AcSMX than for SMX was consistent with the sorption coefficients in manure-amended Luvisoil (K_d) obtained by Hölting and Kreuzig (2007): 0.7 L kg^{-1} for AcSMX vs 2.9 L kg^{-1} for SMX.

For SMX, the extractability was different in function of the initial concentration: the fractions for $[\text{SMX}] \times 1$ (2-9%) were lower than the fractions for $[\text{SMX}] \times 60$ (14-56%) as a result of a possible saturation of immediately accessible sorption sites for SMX. Moreover, the three aqueous extractants differently extracted SMX from soil/manure mixtures and for both SMX concentration levels ($p < 0.05$), with the highest available fractions extracted with the HPCD solution and the lowest with the EDTA solution. The different extraction mechanisms of CaCl_2 , EDTA and HPCD solutions are discussed in section IV.3.5.

Inversely, the available fractions of AcSMX were high (70-92%) at both concentration levels and for all aqueous extractants used (Figure IV.5). Less contrast was observed between the available fractions for $[\text{AcSMX}]_{x1}$ and $[\text{AcSMX}]_{x60}$ compared to SMX, suggesting different

mechanisms of adsorption for both compounds. The HPCD solution extracted the lowest available fraction of AcSMX compared to the EDTA solution ($p < 0.05$) for $[\text{AcSMX}]_{x1}$ and compared to CaCl_2 and EDTA solutions ($p < 0.05$) for $[\text{AcSMX}]_{x60}$. The AcSMX extractability was slightly higher for $[\text{AcSMX}]_{x1}$ than for $[\text{AcSMX}]_{x60}$ ($p < 0.05$) with respect to the higher fraction of SMX quantified in the aqueous extracts in the second case. Indeed, from $2.9 \pm 0.4\%$ to $8.8 \pm 0.9\%$ of SMX was quantified in extracts of soil/manure mixtures spiked at the lowest $[\text{AcSMX}]_{x1}$ level and from $3.4 \pm 0.6\%$ to $12.6 \pm 1.2\%$ in extracts of soil/manure mixtures spiked at the highest $[\text{AcSMX}]_{x60}$ level, depending on the aqueous extractant. The quantification of SMX in our extracts confirmed the reversible transformation of AcSMX to SMX (Höltge and Kreuzig, 2007; García-Galán et al., 2008). On the other hand, less than 1% of AcSMX was detected in aqueous extracts from soil/manure mixtures initially spiked with SMX. Some hypotheses could explain the easier transformation of AcSMX to SMX than that of SMX to AcSMX: 1) the deacetylation of AcSMX could result in a non-specific catabolism while the *N*-acetylation of SMX needs both the tolerance of microorganisms (fungal and/or bacterial) to SMX and the production of the *N*-acetyltransferase enzymes by the microorganisms (Martins et al., 2009); and 2) the SMX availability could be too low for microbial transformation comparatively to the availability of AcSMX, as shown in Figure IV.5.

The SMX available fractions quantified in this experiment were 7 to 25 times lower than the same fractions in the experiment using radiolabeled ^{14}C -SMX (Chapter III), with the highest difference for the EDTA-based extraction. Only the free-dissolved molecules in the extracts were supposed to be quantified with the online SPE-UHPLC-MS-MS system that is equipped with $0.2\ \mu\text{m}$ filters, whereas both free-dissolved ^{14}C -molecules and ^{14}C -molecules associated to colloids in the extracts were quantified with the radioactivity counter (the extracts were only centrifuged). Indeed, the organic matter content in the extracts can lead to the SMX adsorption on this co-extracted organic matter (Wegst-Uhrich et al., 2014), in particular in EDTA extracts according to the DOC value and the dark-brown color of the extracts (section IV.3.1). Inversely, few differences were observed between the two experiments with ^{12}C -AcSMX and ^{14}C -AcSMX assuming that the AcSMX adsorption on organic matter was lower than SMX.

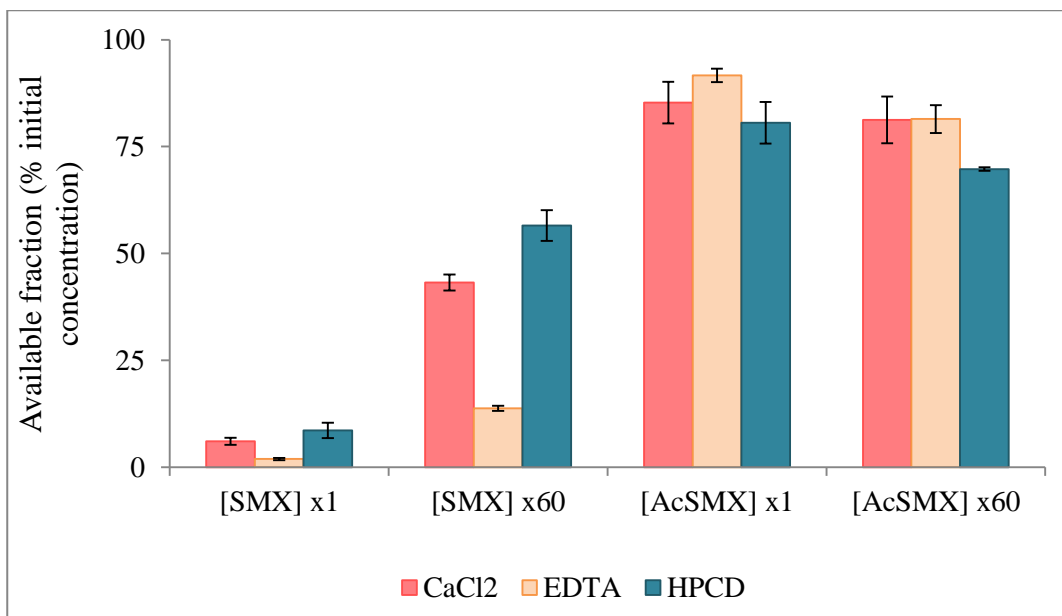


Figure IV.5. Available fractions (%) of SMX (n = 4) and AcSMX (n = 3) in soil/manure mixtures in function of the initial concentration and the aqueous extractant. A 30-60 min contact was let between the molecule and manure before the incorporation of manure into soil and the extraction. The lowest concentration level (x1) corresponded to 22 μg SMX or AcSMX kg^{-1} dry soil and the highest (x60) to 1.32 mg SMX or AcSMX kg^{-1} dry soil.

IV.3.4. Availability of SMX in compost- and manure-amended soil over a three-month incubation

The available fractions of SMX extracted with CaCl₂, EDTA or HPCD solutions from fresh soil/EOM mixtures samples decreased over the three-month incubation (Figure IV.6). At day 0, the results of aqueous extraction in both soil/EOM mixtures were similar to those described in section IV.3.3 for soil/manure mixtures. We can also note that the available fractions of SMX were higher in soil/manure mixtures (1.9-63.3%) than in soil/compost mixtures (0-42.3%) with all extracting solutions ($p < 0.05$).

The fractions strongly decreased during the first 8 days of incubation with a decrease ranging from 2 to 17 fold in soil/compost mixtures and from 2 to 15 fold in soil/manure mixtures. As observed at day 0, SMX extractability was higher in soil/manure mixtures than in soil/compost mixtures at day 8 ($p < 0.05$ with the EDTA and HPCD solutions) and it slightly depended on the initial concentration level in both soil/EOM mixtures ($p < 0.05$). However, the clear differences of efficiency of the three extracting solutions at day 0 became less clear for longer incubation times. At day 8, the aqueous solutions extracted equivalent amounts of SMX in soil/manure mixtures ($p > 0.05$) while in soil/compost mixtures, the CaCl₂ solution extracted greater amounts of SMX than the

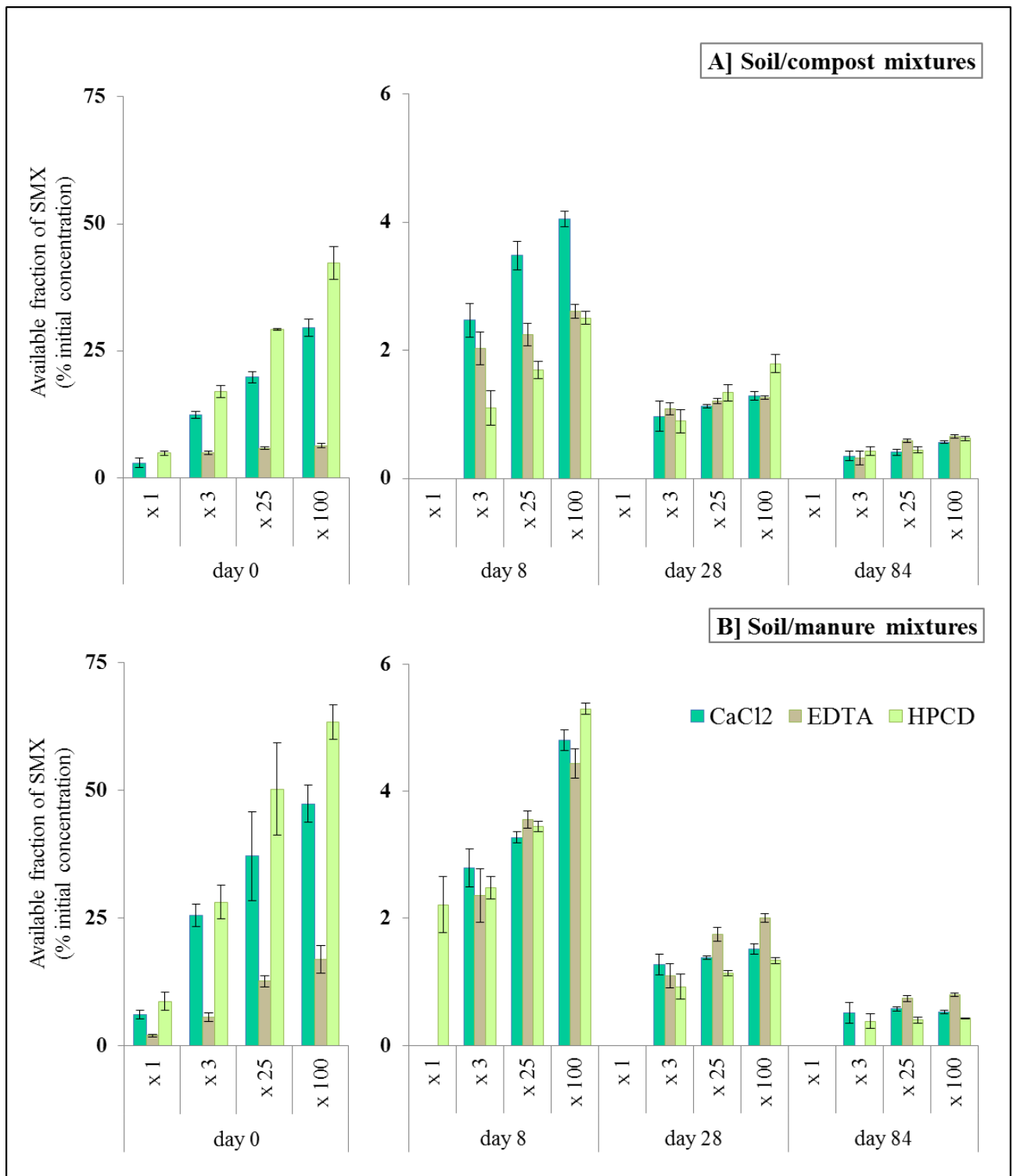


Figure IV.6. Available fractions of SMX (% initial concentration, n = 4) in A] soil/compost mixtures and B] soil/manure samples, extracted with CaCl₂, EDTA and HPCD solutions over the incubation time.

HPCD solution ($p < 0.05$). The different extraction mechanisms of the aqueous solutions are discussed in section IV.3.5.

For the lowest initial concentration $[\text{SMX}] \times 1$, the antibiotic was quantified at day 8 only in soil/manure mixtures with the HPCD-based extraction and was not anymore detected after 28 and 84 days of incubation. For the other SMX concentration levels, the available fractions reached 0.9-2.0% after 28 days and only 0.4-0.8% after 84 days, whatever the soil/EOM mixture and the initial SMX concentration.

The strong decrease of SMX environmental availability in less than 28 days in our soil samples was in accordance with the dissipation rate of another sulfonamide (sulfadiazine) in manure-amended Luvisol (Förster et al., 2009): the time period for which 50% of initial concentration disappears (DT_{50}) reached 19 days for this sulfonamide.

Concerning the transformation products, two compounds were quantified in both soil/EOM mixtures for the two highest SMX concentration levels. At day 0, AcSMX was quantified at around 1% (of SMX molarity) in CaCl_2 extracts; 0.4-0.6% in EDTA extracts and 0.7-0.8% in HPCD extracts. Then, the available AcSMX decreased with the available SMX to less than 0.1% over the incubation. Förster et al. (2009) also evidenced the decrease of the *N*-acetylsulfadiazine availability assessed with CaCl_2 -based extraction. At day 84, 3A5MI was detected in CaCl_2 and EDTA extracts (0.02-0.05%). Finally, ANI was not detected over the incubation time for several hypothetical reasons: 1) SMX was not biodegraded till the ANI formation; 2) the ANI formation could result more from the SMX photodegradation (Majewski et al., 2014); 3) ANI was formed but its adsorption was too high and irreversible (Lee et al., 1997) so that we could neither extract nor detect it.

Other transformation products may be formed during the incubation of soil/EOM mixtures, for example, the hydroxylated compound (OH-SMX). Nevertheless, the quantification of hydroxylated sulfadiazine (another sulfonamide) could be low due to its possible binding to soil organic matter (Förster et al., 2009; Zarfl et al., 2009). An unknown transformation product was detected in UV (232 nm) over the incubation time, with a peak height higher at day 84 than at day 0. According to the retention times, the compound was more retained than SMX and AcSMX in our analytical conditions. This transformation product could be an isomer of 4-nitrosulfamethoxazole, with respect to the similar order of chromatographic retentions described for these compounds by Bonvin et al. (2013). However the amount of the unknown compound was too low to detect the molecular mass in the extracts.

Given the mineralization of SMX was low (2.3 % after 28 days, Chapter III), we assumed that the formation of non-extractable residues was responsible for the rapid dissipation of SMX during the first 8 days of incubation. Hölting and Kreuzig (2007) reported 81% of non-extractable residues at day 0 in manure-amended soil after an ethyl acetate-based extraction. In our previous study, we found 12 to 32% of non-extractable residues at day 0, depending on the aqueous extractant used and the soil/EOM mixture (Chapter III). The strong sorption of sulfonamides to soil particles could be due to exchange reactions between cations and anionic SMX at soil pH, complexation of SMX in the soil matrix and covalent bonding, and biogenic non-extractable residues (Gulkowska et al., 2012; Bailey et al., 2016; Chapter III).

IV.3.5. Characterization of co-extracted organic matter

IV.3.5.1. Quantity and quality of co-extracted organic matter in aqueous extracts

The concentrations of dissolved organic carbon (DOC) extracted from soil/EOM mixtures were different in the aqueous extracts, even after subtracting the part due to EDTA or HPCD. In soil/manure extracts, it reached 50 ± 3 , 807 ± 5 and $16\,680 \pm 41$ mg C L⁻¹ (n = 2) in CaCl₂, EDTA and HPCD, respectively at day 0 (section IV.3.1). Comparatively at the same date, the DOC concentrations were equal to 76 ± 4 , 684 ± 95 and 16800 ± 48 mg C L⁻¹ (n = 2) in CaCl₂, EDTA and HPCD extracts from soil/compost mixtures, respectively. Furthermore, independently of their concentration, the DOM types in extracts were supposed to be different. Therefore, fluorescence analyses on aqueous extracts were carried out and the DOM quality could be determined with the three-dimensional fluorescence spectra.

First, the CaCl₂, EDTA and HPCD solutions co-extracted different DOM types at day 0 (Figure IV.7). For both soil/EOM mixtures, there was a more complex DOM in the EDTA extract than in the CaCl₂ and HPCD extracts. Fluorescence zones II (20-25%) and IV (31-36%) contained the main peaks in CaCl₂ and HPCD extracts, whereas the EDTA extract had a low fluorescence zone II percentage (6%) and the main fluorescence peaks were observed in the zones IV, V and VI (19 to 31%; Figure IV.S1 in IV.Supplementary material). Moreover a higher fluorescence zone VII percentage was observed in the EDTA extract (7-11%) than in CaCl₂ and HPCD extracts (2% for all spectra). Therefore, the EDTA solution mainly extracted organic compounds like fulvic acids (IV), glycolated proteins (V), melanoidin, lignocellulose (VI) and humic acids (VII), according to the molecules previously characterized (Chen et al., 2003; Jimenez et al., 2014). These OM types were

less extracted with the CaCl₂ and HPCD solutions, which extracted more compounds like proteins (fluorescence zones I, II, III). The complexity index was consequently very high for EDTA extracts (5.9-6.9) compared to those for CaCl₂ (1.6-1.9) and HPCD (1.3-1.6) extracts. These results were consistent with the mechanism of extraction of the EDTA solution that chelates the cations and therefore co-extracts more organic matter than the CaCl₂ and HPCD solutions (Chapter III).

The mechanisms of extraction of CaCl₂, EDTA and HPCD solutions were responsible for different DOC contents and DOM types in the extracts, and for different available fractions of SMX quantified. The dark brown color of the EDTA extracts was consistent with the co-extraction of complex DOM that resulted in stronger matrix effects in MS and higher limits of quantification compared to CaCl₂ extracts (section IV.3.1). The more complex the DOM in extracts was, the lower the available fraction of SMX quantified was (Pearson correlation with the complexity index: $r = -0.81$, $p = 0.01$; Figure IV.8). The available fraction could contain both free-dissolved SMX and easily-desorbed SMX (Harmsen, 2007); and some SMX molecules could be associated to soluble organic matter. As suggested in section IV.3.3, only the free-SMX was quantified in the extracts by the analytical method. The content of the EDTA extract in complex organic matter confirmed the possibility for SMX to adsorb on DOM in solution (Wegst-Uhrich et al., 2014). This may explain why the concentration of free SMX quantified in EDTA extracts was lower than that in CaCl₂ and HPCD extracts at day 0 (section IV.3.4). Lastly, the necessity to perform purification for HPCD extracts could be related to the high content of organic carbon and/or cyclodextrin molecules but not to the complex DOM content in these extracts.

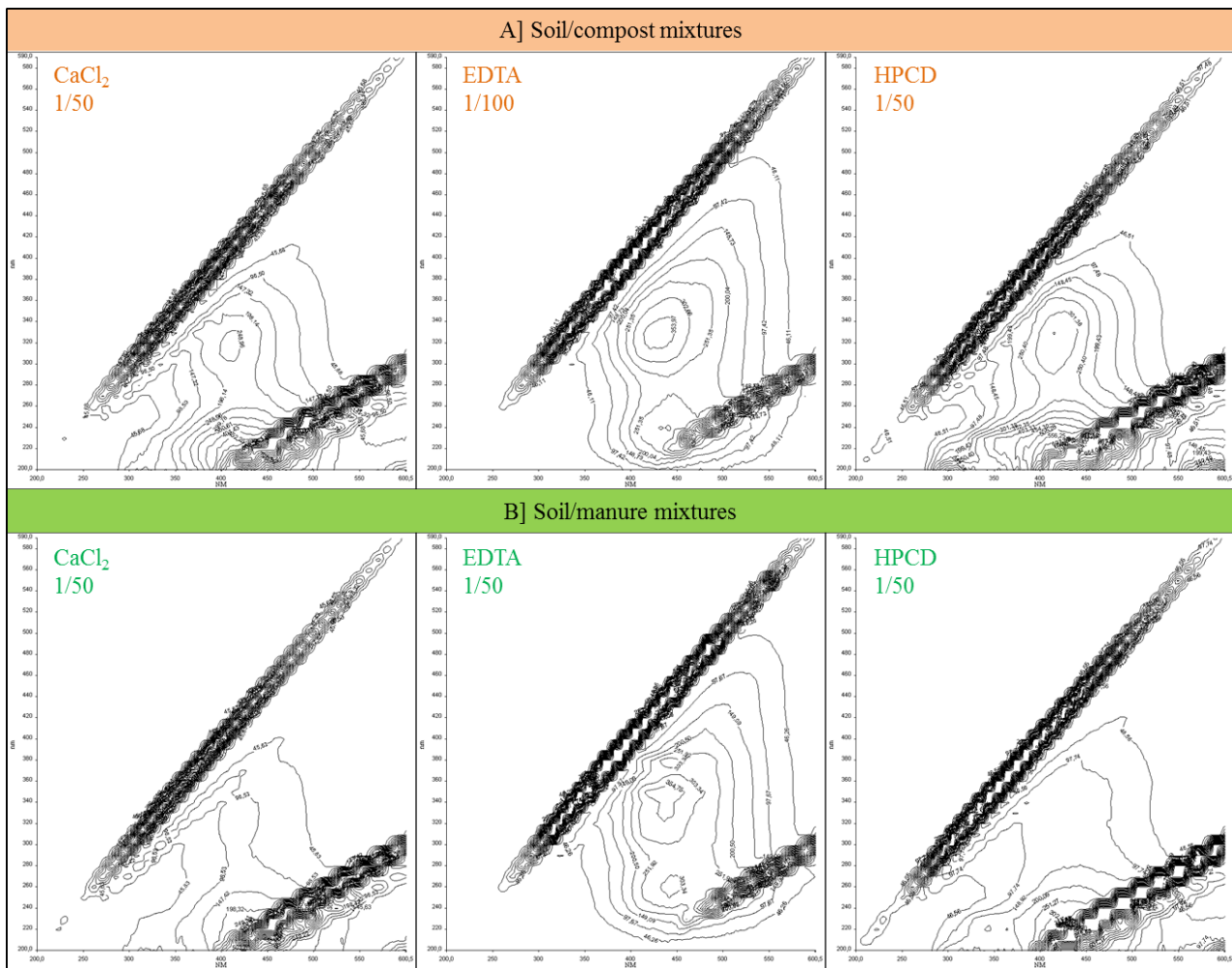


Figure IV.7. 3D-fluorescence spectra obtained for CaCl_2 , EDTA and HPCD extracts diluted to 1/100 or 1/50 from A) soil/compost mixtures and B) soil/manure mixtures, at day 0, for $[\text{SMX}] \times 3$.

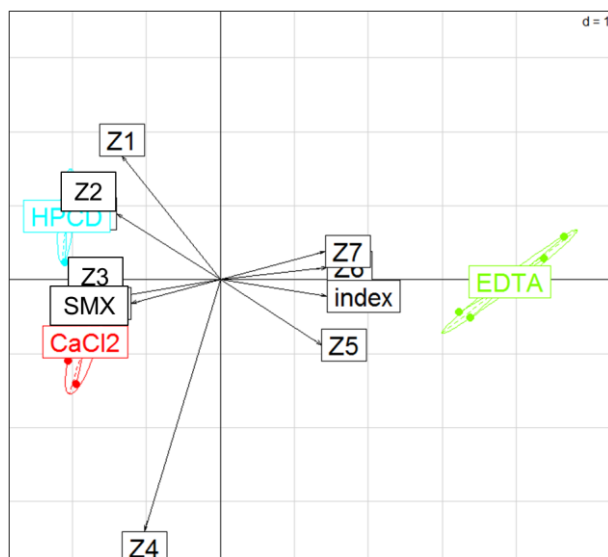


Figure IV.8. Principal component analysis (PCA): SMX concentration measured at day 0 in CaCl_2 , EDTA and HPCD extracts in function of the fluorescence zones (Z1 to Z7) and the complexity index, in both soil/compost and soil/manure mixtures, initially spiked with the concentration $[\text{SMX}] \times 3$. 94% of the dataset variability was explained on the first plan (axe 1: 86% and axe 2: 8%).

IV.3.5.2. Evolution of SMX availability and co-extracted organic matter in aqueous extracts

Considering the CaCl_2 extracts as most representative of soil solution, the DOM types were similar between soil/compost and soil/manure mixtures at day 0 with close fluorescence zones (Figure IV.7 and Figure IV.S1). Nevertheless, the DOM became more complex in soil/compost mixtures than in soil/manure mixtures with time (Figure IV.9), with a decrease of fluorescent compounds from the zones I, II, III and an increase of the compounds from the zones IV, V, VI and VII over the incubation time (Figure IV.S2). This trend corresponded to the humification of DOM during ageing of soil/EOM mixtures. Indeed, the complexity index increased over the incubation time ($p < 0.05$), from 1.6 to 3.5 in soil/compost mixtures and from 1.9 to 3.0 in soil/manure mixtures (Figure IV.10). The increase of the complexity index was previously shown during the anaerobic digestion of sludge (Muller et al., 2014; Aemig et al., 2016). The complexity index was higher in soil/compost mixtures than in soil/manure mixtures after 28 and 84 days ($p < 0.05$) with respect to the highest stability and lowest biodegradability of the organic matter in compost. The indicator of residual organic carbon (I_{ROC}) was indeed equal to 70% for the compost vs 50% for the manure (Chapter III, Table III.S1). The DOC concentrations decreased in CaCl_2 extracts from both soil/EOM mixtures to 24 mg C L^{-1} after 28 days and to 18 mg C L^{-1} after 84 days (data not shown), according to the biodegradation and humification of DOM. Concerning the EDTA and HPCD extracts, the DOC values did not vary between 0 and 28 days of incubation, probably due to the non-targeting of accessible DOM with these extractions.

The antibiotic probably probably had no impact on the OM biodegradability since there was no difference between the complexity index for the two concentration levels $[\text{SMX}] \times 3$ and $[\text{SMX}] \times 100$ and for both soil/EOM mixtures (Figure IV.10, $p > 0.05$, Kruskal-Wallis test).

As humic acid-like molecules can be preferential binding sites for sulfonamides (Gao and Pedersen, 2010) and considering the characterization of DOM in CaCl_2 extracts, the ageing of soil/EOM mixtures resulted in an increase of the complexity index over the incubation time that could have led to a lower SMX availability (section IV.3.5). Actually the complexity index was well correlated with the SMX concentration measured in CaCl_2 extracts (example of principal component analysis in Figure IV.S3): in soil/compost mixtures spiked at the $[\text{SMX}] \times 3$ level, the highest was the complexity index, the lowest was the measured SMX concentration ($r = -0.94$, $p = 0.005$). The same correlation was made for soil/compost mixtures spiked at the $[\text{SMX}] \times 100$ level ($r = -0.91$, $p = 0.011$). In soil/manure mixtures, the same correlation was made only for the lowest SMX concentration level ($[\text{SMX}] \times 3$: $r = -0.80$, $p = 0.05$) and the correlation was weaker than for

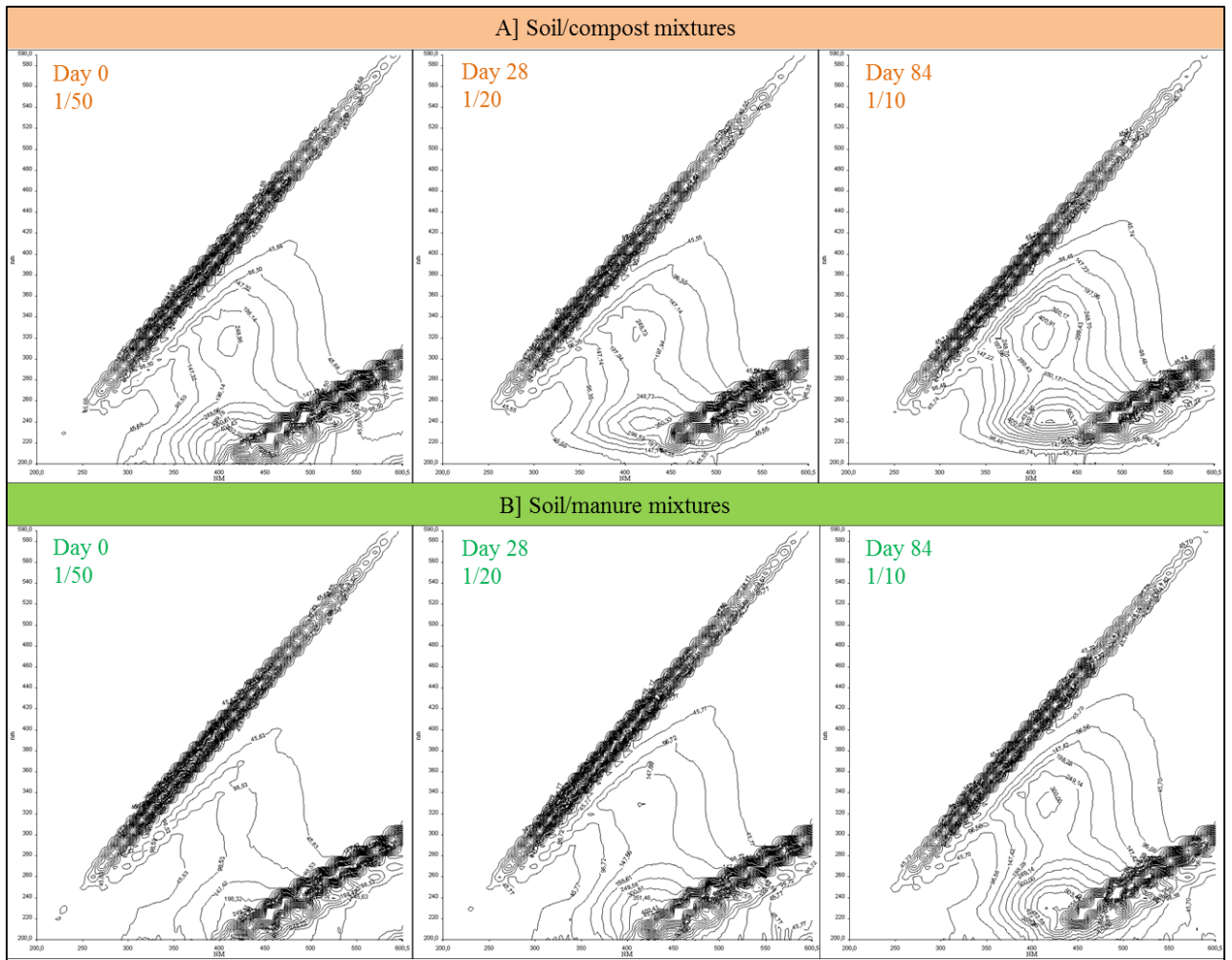


Figure IV.9. 3D-fluorescence spectra obtained for CaCl_2 extracts diluted to 1/50, 1/20 or 1/10 from A) soil/compost mixtures and B) soil/manure mixtures, after 0, 28 and 84 days of incubation for $[\text{SMX}] \times 3$.

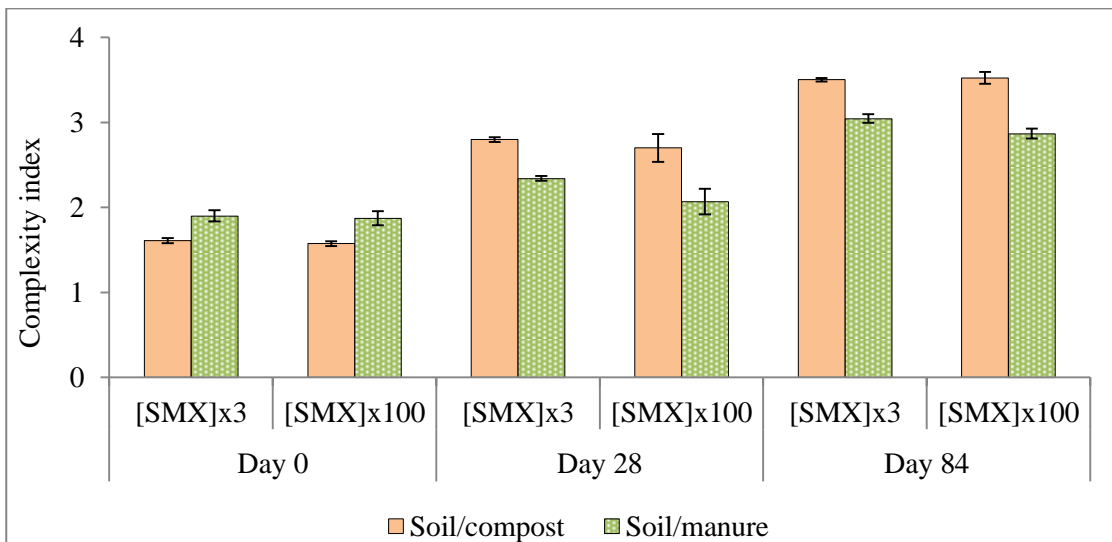


Figure IV.10. Evolution of the complexity index in CaCl_2 extracts from soil/compost and soil/manure mixtures over the incubation time, for $[\text{SMX}] \times 3$ and $[\text{SMX}] \times 100$ concentration levels.

soil/compost mixtures probably due to the lower stabilization of the OM in soil/manure mixtures compared to soil/compost mixtures after 84 d of incubation. As the complexity ratio depended on the fluorescence volumes of zones IV to VII (Figure IV.1), the higher the fluorescence volumes of zones IV, V and VI, the lower the measured concentration of SMX in CaCl₂ extracts from soil/compost mixtures ($r = -0.95$ to -0.97 , $p < 0.05$), i.e., when fulvic acid-like (IV), glycolated protein-like (V), melanoidin and lignocellulose-like (VI) organic matter were formed during the incubation time. In the same way, the measured SMX concentration decreased with the decrease of fluorescence volumes of zones I and II, i.e., the degradation of simple organic matter during the incubation time ($r = +0.96$ to $+0.98$, $p < 0.05$). Comparatively in soil/manure mixtures, the measured SMX concentration decreased with the increase of complex OM of zones IV, VI and VII ($r = -0.83$ to -0.99 , $p < 0.05$), i.e., with the formation of fulvic acid-like (V), melanoidin, lignocellulose-like (VI) and humic-acid like (VII) organic matter during the incubation time; while the simple organic matter of zones I and III decreased ($r = 0.87$ to 0.95 , $p < 0.05$) with respect to their degradation during the incubation.

These different correlations suggest that the SMX availability decreased over the incubation time according to the strong SMX binding to condensed and humified organic matter progressively formed through the incubation processes; and/or the biotransformation of SMX in parallel to the biodegradation of the organic matter. However, 1) the amounts of transformation products of SMX could be too low to detect them; 2) other compounds not selected in our analytical method were formed and/or 3) the compounds were sequestered in amended soil (e.g., aniline).

IV.4. Conclusion

The aqueous extraction with CaCl₂, EDTA or cyclodextrin solution was tested to assess the environmental availability of SMX and three transformation products in soils amended with sludge compost or manure. The conditions of the analytical method with online SPE-UHPLC-MS-MS were first developed and characterized to quantify the compounds in the aqueous extracts. The cyclodextrin (HPCD) extract had to be purified (QuEChERS method) due to complete ion suppression.

The freeze-storage of soil samples strongly impacted the SMX extractability. Therefore, the aqueous extraction of EOM/soil mixtures has to be performed on fresh soil samples. This is a

crucial step in particular for field situations where expected amounts of contaminant in available fractions are low.

The aqueous extraction and the analytical methods allowed us to follow the SMX environmental availability in amended soils over a three-month incubation. The aqueous solutions extracted different available fractions of SMX from soil/compost and soil/manure mixtures, according to the co-extraction of different types of organic matter.

The characterization of the co-extracted organic matter (OM) in the aqueous extracts enabled us to link: 1) the complexity of co-extracted OM in CaCl₂, EDTA and HPCD extracts to the quantity of free-dissolved SMX quantified in extracts; 2) the strong decrease of SMX availability to the organic matter humification during the incubation.

Consequently, the sorption of SMX to amended soils could be responsible for its low biodegradation and the poor quantification of transformation products: AcSMX was quantified at the incubation beginning and its available fraction decreased over the incubation, whereas 3A5MI was quantified after 84 d for the highest initial SMX concentration levels. The further work could be to test the potential toxicity of the aqueous extracts in bioassays, in order to determine if only the free-dissolved SMX or if both free-dissolved and associated SMX to DOM are bioactive.

Acknowledgments

The authors would like to thank Olivier Crouzet for the design and realization of the incubation experiment; Ghislaine Delarue and Valentin Serre for their technical support, and Myriam Germain for her help in statistical analyses. This work was supported by grants from Region Ile-de-France (DIM ASTREA 2013-16) and ANR – French National Research Agency (CEMABS project, ANR-13-CESA-0016-01). Environmental samples came from the QualiAgro field experiment financially supported by Veolia Research and Innovation.

IV. Supplementary material



Figure IV.S1. Fluorescence (%) measured in the different zones in CaCl₂, EDTA and HPCD extracts of A] soil/compost mixtures and B] soil/manure mixtures at day 0.

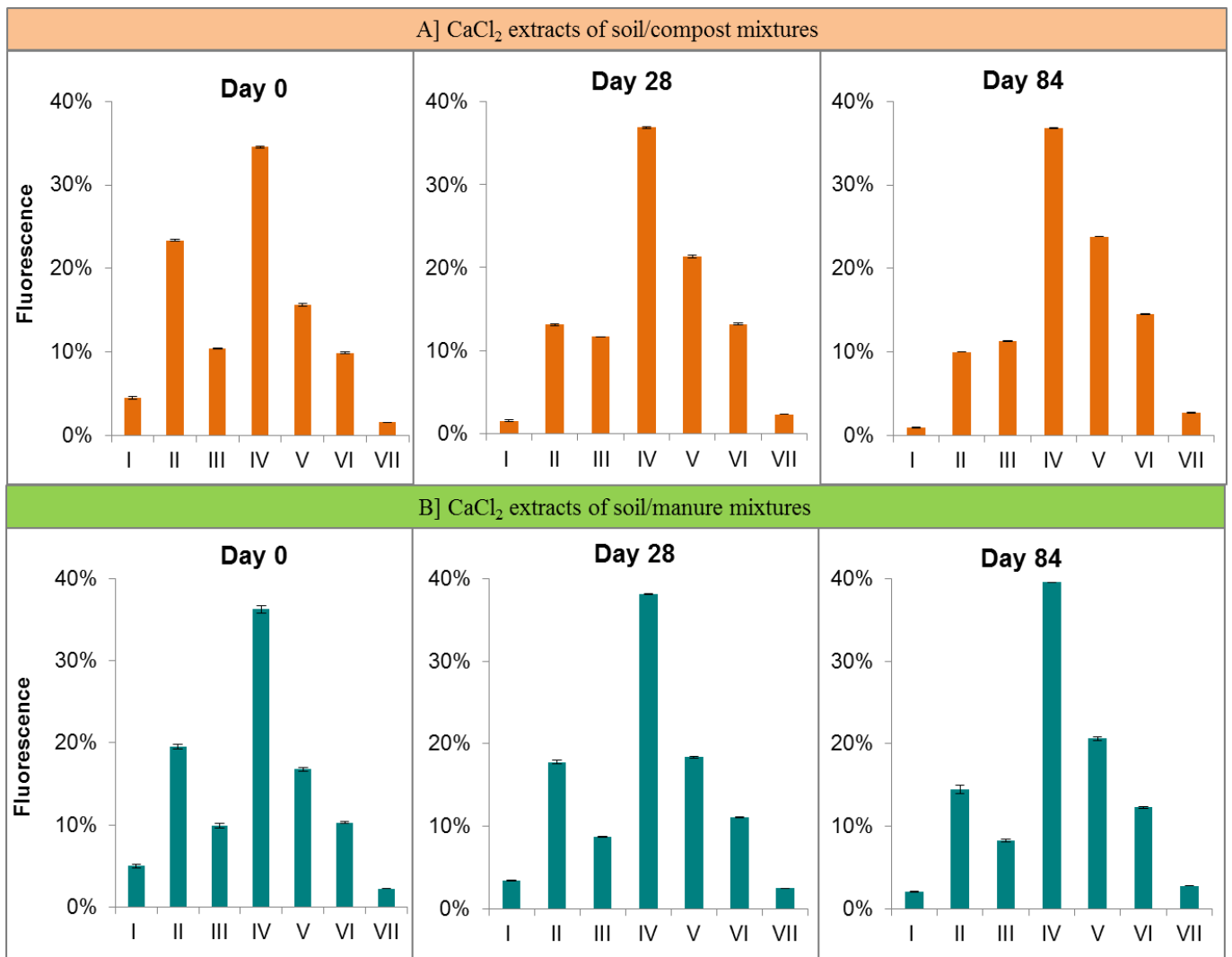


Figure IV.S2. Fluorescence (%) measured in the different zones in CaCl₂ extracts of A] soil/compost mixtures and B] soil/manure mixtures after day 0, 28 and 84 days of incubation.

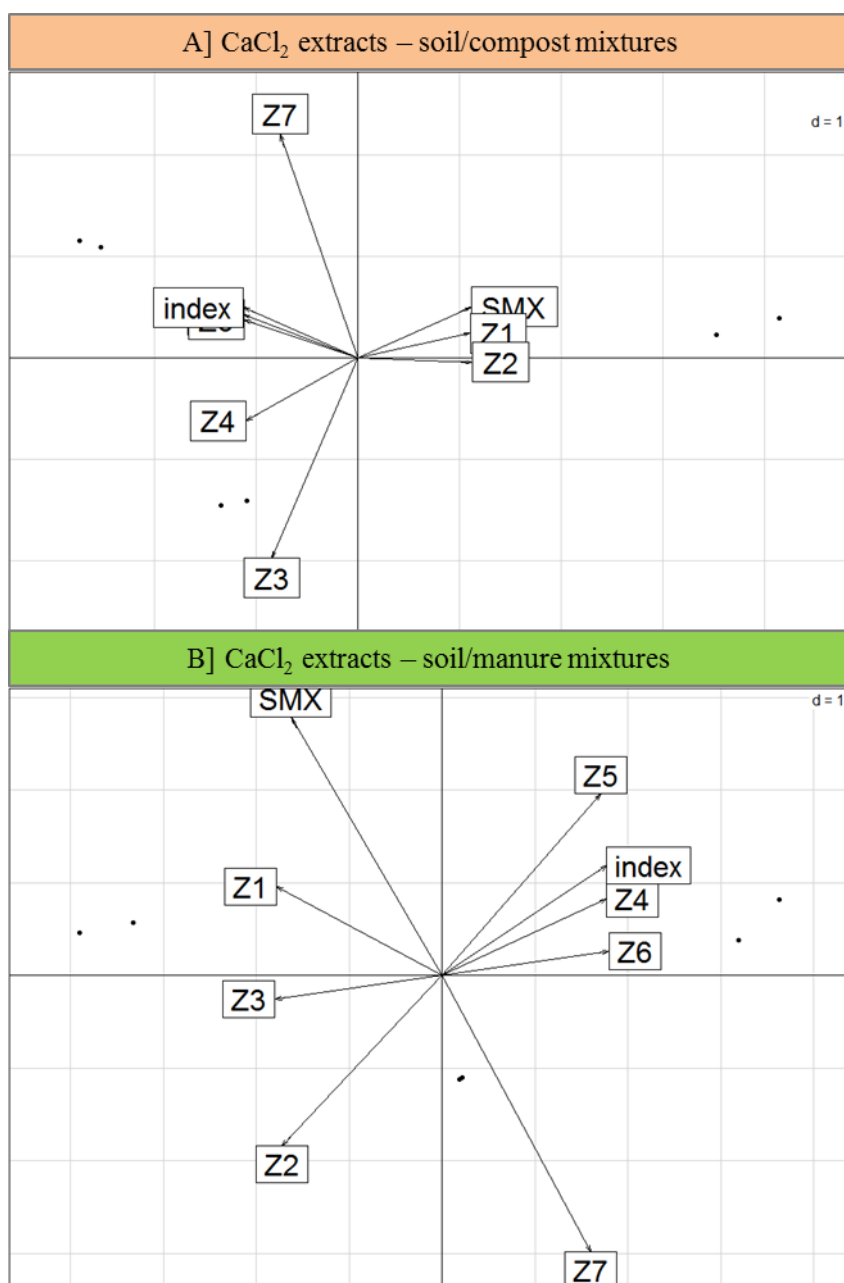


Figure IV.S3. Principal component analysis (PCA): SMX concentration measured over the incubation time (0, 28 and 84 days) in CaCl₂ extracts in function of the fluorescence zones (Z1 to Z7) and the complexity index, in A] soil/compost mixtures and B] soil/manure mixtures, initially spiked with the lowest SMX concentration. For both PCA, 99% of the dataset variability was explained on the first plan (in A] axe 1: 86% and axe 2: 13%; in B] axe 1: 92% and axe 2: 7%).

La méthode analytique utilisant la SPE couplée à l'UHPLC-MS-MS a été développée afin de quantifier le sulfaméthoxazole et trois produits de transformation dans les extraits aqueux (CaCl₂, EDTA, cyclodextrine) et d'estimer leurs fractions disponibles dans les sols amendés par les MAFOR (Chapitre IV). Cependant, la disponibilité des antibiotiques dans les sols dépend aussi des propriétés des sols, notamment de la teneur en matière organique, de la texture, du pH et de la capacité d'échange cationique.

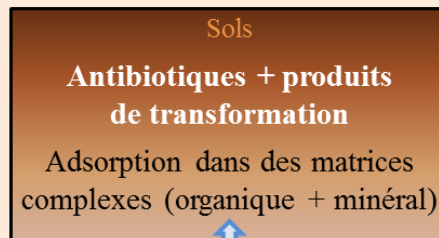
Etant donné qu'il n'existe pas de méthode universelle pour estimer la biodisponibilité des antibiotiques dans les sols, il est essentiel de comprendre quelles propriétés des sols influencent le plus la fraction disponible mesurée par les différents extractants.

L'objectif est d'utiliser la méthode d'extraction et d'analyse pour évaluer la disponibilité des antibiotiques dans différents contextes pédoclimatiques. Dans ce but, la méthode développée précédemment a été appliquée dans le cas de sols contaminés directement par le sulfaméthoxazole et présentant des propriétés physico-chimiques différentes. Ces sols (témoins) étaient issus de différents sites expérimentaux situés à Feucherolles (QualiAgro, Yvelines, France), Epoisses (INRA de Dijon, Bourgogne, France) et London (Agriculture and AgriFood, Ontario, Canada).

Dans le Chapitre V, les questions suivantes ont été traitées :

- 1) La méthode d'extraction et d'analyse permet-elle d'estimer la disponibilité du sulfaméthoxazole dans des sols présentant différentes propriétés physico-chimiques ?
- 2) Quelles propriétés des sols influencent le plus la disponibilité du sulfaméthoxazole ?

Chapitre V



Extractions aqueuses



Disponibilité du sulfaméthoxazole dans différents sols



- 1) Utilisation de la méthode d'extraction et d'analyse pour estimer la disponibilité du sulfaméthoxazole dans des sols de différentes origines?
- 2) Quelles sont les propriétés des sols les plus influentes?

Chapter V: Influence of soil properties on the assessment of short-term environmental availability of sulfamethoxazole by soft extraction

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Abstract

The fate of antibiotics in soils depends on their environmental availability which is influenced by the physicochemical properties of both antibiotics and soils. Sulfonamide antibiotics are generally mobile in soils as a result of their low sorption capacity. However, soil properties such as organic matter content and pH can influence the adsorption of sulfonamides on soils. In this work, different aqueous solutions (CaCl₂, EDTA, cyclodextrin), previously selected to target the environmental availability of sulfamethoxazole (SMX) in soils amended with exogenous organic matter, were used here to assess the environmental availability of SMX in three unamended soils with different pedoclimatic properties.

Sulfamethoxazole diluted in water was directly added on soil samples at two concentrations, including an environmentally relevant one. The soft extraction with each aqueous solution was performed less than one hour after the antibiotic application on soils, in order to maximize the availability and better identify the soil properties influencing the most the SMX availability.

Depending on the SMX concentration and the extraction mechanism of the aqueous solution, the antibiotic availability varied between 2% and 94% of the initial SMX concentration. At soil pH, SMX was mostly anionic in the three soils and its availability mainly depended on the soil cationic exchange capacity, the organic matter and clay contents. However, the correlations with the soil properties depended on the aqueous solution used, confirming different extraction mechanisms between the three aqueous solutions.

Keywords

Antibiotics; aqueous extractions; soils; texture; organic carbon

V.1. Introduction

The recycling of organic waste and treated wastewaters in agriculture can lead to the contamination of agricultural soils by antibiotics such as sulfonamides (Kemper, 2008; Du and Liu, 2012). The presence of antibiotics in the environment can induce the acquisition and the dissemination of microbial resistances to antibiotics (Sarmah et al., 2006). The antibiotics that are available in soils can be transferred to groundwater and/or impact the soil microorganisms (Harmsen, 2007). Consequently, assessing the environmental availability of antibiotics in soils is of prime importance in the purpose of risk assessment. Considering their physicochemical properties, the sulfonamides are small and polar molecules with amphoteric properties (Baran et al., 2011). The reported sorption coefficients of sulfonamides in soils (K_d) are comprised between 0.6 and 7.4 L kg⁻¹ (Sarmah et al., 2006); consequently, the sulfonamides are considered mobile in soils and they can be found in groundwater (Blackwell et al., 2009; García-Galán et al., 2010). However, the sorption of sulfonamides in soils depends on the environmental conditions and soil properties (Thiele-Bruhn et al., 2004; Wegst-Uhrich et al., 2014). Several mechanisms of sorption are described for sulfonamides in soils. The organic matter plays a major role in the sorption strength of sulfonamides by nonbonding Van der Waals interactions and hydrogen bonding (Białk-Bielińska et al., 2014). The sorption of sulfonamides increases with the organic matter content (Figueroa-Diva et al., 2010), and clays can be also sorbates for sulfonamides (Boxall et al., 2002; Thiele-Bruhn et al., 2004; Ter Laak et al., 2006). Hydrophobic and polar interactions are important in sulfonamide adsorption on organic and/or mineral surfaces (Białk-Bielińska et al., 2014). Moreover, the soil pH is a factor influencing the sulfonamide adsorption, which globally decreases with increasing pH, due to the electrostatic repulsion between anionic sulfonamides at pH above pK_a values and negative charges on solid phases (Ter Laak et al., 2006; Białk-Bielińska et al., 2014). The weak forces involved in all these interactions can lead to the sulfonamide desorption, but the formation of non-extractable residues can also occur in the 24 h following the soil contamination (Höltge and Kreuzig, 2007; Müller et al., 2013). The sorption/desorption equilibrium is an important process affecting the fate of sulfonamides in soils and their environmental availability.

Consequently, the study of the environmental availability of sulfonamides in soil has to take into account the diversity of both soil physicochemical properties and pedoclimatic contexts. Since the detection frequency of sulfamethoxazole (SMX) is high in soils and waters (García-Galán et al., 2010; Baran et al., 2011), we selected this antibiotic and assessed its availability by measuring its extractability with different aqueous extractants (Chapter IV). Extraction and analytical methods were previously developed to assess the environmental availability of SMX in soils amended by manure and sludge compost. We highlighted that the SMX availability depended on the inherent

properties of organic amendments such as pH and organic matter quality. We assumed the extraction and analytical methods could be also applied on soils without amendment to determine which soil properties could influence the SMX availability.

The influence of soil properties on the recoveries of pharmaceutical compounds was studied by Salvia et al. (2013) by using a solvent-based extraction method. The present study aimed at testing the soft extraction method with aqueous solutions. Three soils with different textures and organic matter contents were selected. The SMX availability was assessed at very short-term (after 30-60 min of contact with soil) in order to obtain a maximum SMX availability from the soft extraction carried out overnight (17 h). Indeed, the loss of sulfonamide extractability was instantaneous on a time scale in minutes and the sequestration occurred within the 24 h following addition (Müller et al., 2013). Since the effects of both the soil moisture and the type of matrix in which the sulfonamide was brought to soil (water or liquid manure) were studied for sulfamethazine by Müller et al. (2013), we studied here the effects of soil-solute interactions on the short-term extractability of SMX by varying the soil properties and the initial SMX concentration, i.e., from environmentally relevant concentration ($22 \mu\text{g kg}^{-1}$ dry soil; Toth et al., 2011; DeVries et al., 2015) up to high concentration (1.32 mg kg^{-1} dry soil, i.e., 60 times the environmentally relevant concentration).

V.2. Material and methods

V.2.1. Chemicals

Analytical standards (purity > 99%) and sulfamethoxazole (SMX) were purchased from Dr Ehrenstorfer GmbH (Ausburg, Germany). Deuterated labelled analytical standard sulfamethoxazole- d_4 (benzene- d_4) was obtained from CDN isotopes (Pointe-Claire, Canada). The stock solutions were prepared in methanol (Carlo Erba, Val-de-Rueil, France) at 242 mg L^{-1} for SMX and 285 mg L^{-1} for SMX- d_4 . The stock solutions were stored in the dark at -20°C . Spiking solutions of SMX were prepared by dilution of the stock solution in Milli-Q water (Millipore, France) at two different concentrations: 1.1 and 66.6 mg L^{-1} .

The extracting solutions were prepared by dissolution in Milli-Q water of respective chemical products (purity > 99%): calcium chloride (CaCl_2 ; VWR, Fontenay-sous-Bois, France), disodium ethylenediaminetetraacetic acid (Na_2EDTA ; Sigma-Aldrich, Saint-Quentin Fallavier, France) and 2-hydroxypropyl- β -cyclodextrin (HPCD; Sigma-Aldrich). Sodium hydroxide (NaOH ;

Sigma-Aldrich) was used to adjust the pH of solutions. Anhydrous sodium sulfate (Na_2SO_4), anhydrous sodium acetate (NaOAc) and Na_2EDTA composed the salts for quick, easy, cheap, effective, rugged and safe extraction (QuEChERS; Bourdat-Deschamps et al., 2014). HPLC and LC-MS grade acetonitrile and LC-MS grade methanol were purchased from Carlo Erba and HPLC grade trifluoroacetic acid (TFA) from Sigma-Aldrich.

V.2.2. Soils

Three long-term field experiments located in Feucherolles (Yvelines, France), Epoisses (Bourgogne, France) and London (Ontario, Canada) were selected for the study. Soils from Feucherolles and London are cultivated soils and soil from Epoisses is grassland soil. In Feucherolles, the plots are amended with different types of exogenous organic matter to follow the beneficial effects of amendment on soil quality and the negative effects of soil contamination (Obriot et al., 2016). In London, the plots receive various antibiotics mixtures, at different concentrations in solution, without organic matter, to determine the antibiotic effects on soil microorganisms (Topp et al., 2013). In Epoisses, the plots receive solutions of antibiotics and metals at different concentrations, or effluents from wastewater treatment plant (unpublished information).

Topsoil horizons of untreated plots (no antibiotic, no amendment) were sampled from each site. The soil samples were sieved to 5 mm and stored at 4 °C. The physicochemical compositions of the three soils are detailed in Table V.1. Analyses of control samples using a solvent-based extraction (Ferhi et al., 2016) were realized to confirm the absence of SMX in soils from Epoisses and Feucherolles; the absence of SMX in soils from London was confirmed only by using the soft extraction method.

Table V.1

Key properties of the three different soils.

	Epoisses (France)	Feucherolles (France)	London (Canada)
Texture	Clay loam	Silt	Silt loam
% sand/silt/clay	9/49/42	6/79/15	18/67/15
Organic carbon (g kg ⁻¹)	45.5	10.5	19.8
Total N (g kg ⁻¹)	4.1	1.0	Unknown
% organic matter	7.9	1.9	3.4
pH	7.8	6.6	7.5
CEC cmol+ kg ⁻¹	25.1	7.9	13.2
CaCO ₃ g kg ⁻¹	102	0	Unknown

V.2.3. Soft extraction method

Soil samples were prepared in Falcon[®] tubes (50 mL, Corning, NY, USA) by weighing 6-g fresh soil and spiking soil with 100 μ L of SMX solution to reach two concentration levels: 22 μ g kg⁻¹ ([SMX] \times 1) and 1.32 mg kg⁻¹ dry soil ([SMX] \times 60). Each treatment was prepared in triplicate. By adding SMX in water, the soil water content was adjusted to 15% (g water/100 g dry soil) in soil samples from London and 25% in soil samples from Epoisses and Feucherolles. The soil samples from London were less wet compared to the soil samples from Feucherolles and Epoisses; however, we supposed that this difference was too low, compared to the contrasted conditions studied by Müller et al. (2013), i.e., moist vs air-dried, to have a real impact on the short-term extractability of the sulfonamide, mostly because the soft extractant was rapidly added. Indeed, a contact of 30-60 min was let between SMX and soils. Then, 15 mL of aqueous solution, i.e., CaCl₂ (0.01 M, pH 4.5), EDTA (0.1 M, pH 7) or HPCD (0.05 M, pH 7), were added to the soil sample to assess the SMX availability according to different extraction mechanisms (Chapter IV). Briefly, the soft extraction was performed by shaking overnight the mixture (~17 h, 15 rpm; Heidolphe[®] Reax 20, Schwabach, Germany). The supernatant was collected after centrifugation (11 000 x g for 10 min; Sorvall RC6+, ThermoFisher Scientific, Waltham, MA, USA).

For CaCl₂ and EDTA extracts of soils from Epoisses and Feucherolles, another centrifugation was performed on 1.5 mL extract (10 min at 12100 x g; MiniSpin, Eppendorf, Hamburg, Germany) to ensure the absence of particles before the injection. For the same extracts for the London soil, aliquots were filtered to 0.22 μ m after confirming that no loss of compounds

occurred within the filtration step (Supplementary material, Figure V.S1). This filtration was necessary to authorize the exit of soil extracts from the laboratory where extractions were performed (handling constraints for foreign soils). For the HPCD extracts of all soils, a QuEChERS purification was performed (Chapter IV). The use of organic solvent during this step allowed the exit of extracts of foreign soils. The internal standard SMX-d4 was added to an aliquot of each extract ($71 \mu\text{g L}^{-1}$) before centrifugation, filtration or purification.

The extracts were injected into online solid phase extraction – ultrahigh performance liquid chromatography – tandem mass spectrometry (SPE-UHPLC-MS-MS; Acquity UPLC system and triple-quadrupole mass spectrometer TQD, Waters, Milford, MA, USA). The chromatographic column was composed of a BEH C_{18} stationary phase (2.1 x 100 mm, $1.7 \mu\text{m}$ particle size; Waters). The analytical and MS-MS conditions were the same as those described in Chapter IV.

V.2.4. Analysis of dissolved organic carbon

The dissolved organic carbon (DOC) was measured in the CaCl_2 extracts of soil samples by using the total organic carbon (TOC) analyzer (Shimadzu, Marne-la-Vallée, France) after the filtration of extracts to $0.45 \mu\text{m}$. The DOC value (mg L^{-1}) was obtained from the difference between the total carbon value (TC, mg L^{-1}) and the inorganic carbon value (IC, mg L^{-1}).

V.3. Results and discussion

V.3.1. Availability of SMX in the different soils

The SMX availability depended on the initial spiking concentration, the soil and the aqueous solution used for extraction (Figure V.1). At the lowest SMX concentration ($22 \mu\text{g SMX kg}^{-1}$ dry soil), 2 to 79% of SMX (% of initial concentration) was available in soils, depending on the soft extractant used. The available fraction of SMX increased with increasing the initial SMX concentration to reach 11-94%. Depending on the soft extractant used, SMX was 1.2 to 5.6 times more available in soils spiked at the highest SMX concentration ($1.32 \text{ mg SMX kg}^{-1}$ dry soil) than in soils spiked at the lowest SMX concentration. In fact the adsorption of sulfonamides is nonlinear when the sulfonamide concentration increases; the concentration at the adsorbate surface increases resulting in a decrease of the sorption energy of active surface sites (Tolls, 2001; Maszkowska et

al., 2015). Consequently, the SMX adsorption decreased and the environmental availability was higher during the soft extraction, whatever the soft extractant used.

Considering the different soils, SMX was more available in soil from Feucherolles (15-79%) than in soils from Epoisses (8-49%) and London (2-40%) at the lowest SMX concentration (Figure V.1) whereas at the highest SMX concentration, the antibiotic was globally more available in soils from London (11-94%) or Feucherolles (33-91%) than in soils from Epoisses (15-73%).

Furthermore, *N*-acetylsulfamethoxazole (AcSMX), one of the main transformation products of SMX (Majewski et al., 2014), was also quantified in all aqueous extracts (0.3-0.5%), but only in soils from Feucherolles and London spiked at the highest SMX concentration. Two hypotheses can be made: 1) AcSMX was also formed in soils from Epoisses, but its adsorption on grassland soil (e.g., Epoisses) was higher than on arable soils (e.g., London, Feucherolles) as reported by Höltge and Kreuzig (2007), resulting in the absence of AcSMX quantification in the corresponding aqueous extracts; or 2) the SMX availability was too low in soils from Epoisses to be transformed into AcSMX; consequently, the soil properties were a key driver of the SMX availability for microbial transformation.

For both SMX concentrations and in all soil samples, the EDTA solution extracted the lowest available fraction of SMX. In both soils from Epoisses and Feucherolles, the highest available fraction of SMX was extracted with the HPCD solution, while in soils from London the CaCl₂ and HPCD solutions extracted similar available fractions of SMX.

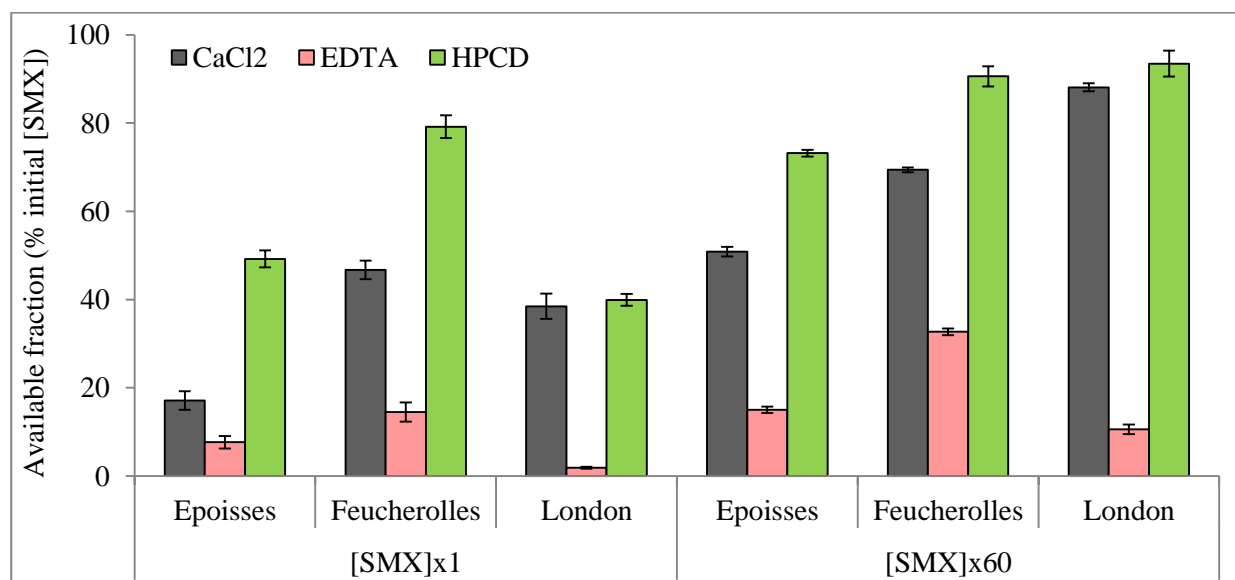


Figure V.1. SMX availability in soils from Epoisses (France), Feucherolles (France) and London (Canada), in function of the initial SMX concentration and the aqueous extractant used (n = 3). Initial [SMX] ×1 and [SMX] ×60 concentrations corresponded to 22 µg kg⁻¹ and 1.32 mg kg⁻¹ dry soil, respectively. The soft extraction was performed after a 30-60 min contact between SMX and soil.

V.3.2. SMX availability as a function of soil properties and extraction mechanisms

The soil pH is a determinant factor in the sorption of sulfonamides (Boxall et al., 2002). According to soil analyses (Table V.1), the soil pH was comprised between 6.6 and 7.8. With a pK_a around 5.6 (Qiang and Adams, 2004), sulfamethoxazole was mainly as anionic form in all soils and soil extracts since the pH values were above the pK_a (Figure V.2). Consequently, we assumed that in our study, the soil pH did not explain the difference of SMX extractability between the soil samples. The adsorption of anionic sulfonamide mainly occurs by electrostatic interactions with soil cations particularly abundant in the clay fraction, and by hydrophobic exclusion and/or polar interactions with organic matter (Thiele-Bruhn et al., 2004; Figueroa-Diva et al., 2010).

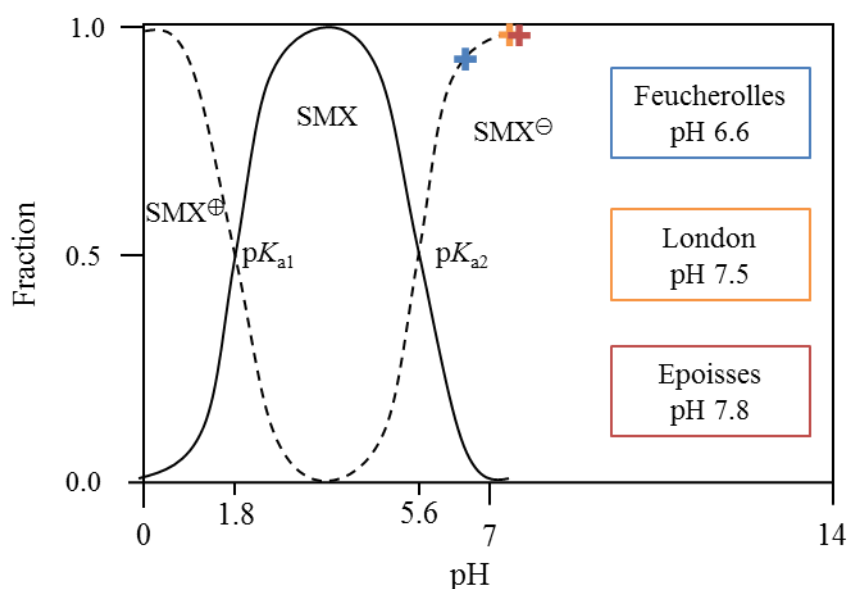


Figure V.2. Speciation of sulfamethoxazole (SMX) according to the pH value with pK_a values from Qiang and Adams (2004). Cross-marks indicate the main form of SMX at pH of soils from Feucherolles (in blue), London (in orange) and Epoisses (in red).

Considering the soil texture (Table V.1), the main difference was the clay content, which was the highest in soil from Epoisses (42%) compared to the two other soils (15%). With respect to the adsorption of sulfonamides on clays and particularly on humic acid-clay complexes (Thiele-Bruhn et al., 2004; Gao and Pedersen, 2010), these antibiotics tend to be more adsorbed on clay loam soil than on silt loam soil (Wegst-Uhrich et al., 2014). The effect of clays on sorption was evidenced with the CaCl_2 -based extraction which gave significantly lower recoveries of SMX in clay loam soil from Epoisses than in the silt and silt loam soils from Feucherolles and London (Figure V.1). The soil content in metallic oxides and hydroxides can also influence the sulfonamide

sorption; but the sorption to inorganic sorbents plays a minor role compared to the organic sorbent (Kahle and Stamm, 2007a; Leal et al., 2013).

Considering the organic matter, sulfonamides in soils can adsorb on organic matter and particularly on humic substances by covalent bonding (Gulkowska et al., 2012; Müller et al., 2013), but the effect of organic matter content (1.9 to 7.9%, Table V.1) on SMX availability was not clear in the three soils considered. As a consequence, the environmental availability of SMX depended on the extraction mechanisms of solutions of CaCl₂ (section V.3.2.1), HPCD (section V.3.2.2) and EDTA (section V.3.2.3).

Principal component analyses (Figure V.3) and correlation tests (Pearson test, Table V.S1) underlined that the different available fractions of SMX in soils were due to both soil properties and extraction mechanisms of aqueous solutions.

V.3.2.1. CaCl₂-based extraction: mimesis of the soil solution

For both SMX concentrations, the antibiotic availability was the lowest in soils from Epoisses compared to soils from Feucherolles and London. Indeed, the available fraction decreased when the clay content increased ($r = -0.87$ to -0.95 , $p < 0.05$). As interactions between clay and organic matter (humic acid-clay complex) contribute to the soil cationic exchange capacity (CEC), the organic matter content, the clay content and the CEC were strongly correlated (Figure V.3). Consequently at the two SMX concentrations, the SMX availability was negatively correlated to organic matter and clay contents but also to the CEC (Figure V.3A and D, Table V.S1). The sorption mechanism of SMX can involve a cation bridging (e.g., Ca²⁺) sorbed primarily to negatively charged surface of SMX (Maszkowska et al., 2015).

Moreover, at low SMX concentration, the SMX availability was positively correlated with the silt content ($r = +0.98$, $p < 0.05$) while at higher SMX concentration, the available fraction increased with the sand content ($r = +0.72$, $p < 0.05$). These two correlations could be due to the preferential binding sites of SMX on clays compared to silts and sands (Thiele-Bruhn et al., 2004).

The dissolved organic carbon (DOC) concentrations were measured only in CaCl₂ extracts since the high carbon content in the initial EDTA and HPCD solutions interfered with the DOC measures in EDTA and HPCD extracts. Even after subtracting the initial carbon content in the extracting solutions, the correction of the DOC content in extracts was not sufficient. We assumed that the DOC concentration measured in CaCl₂ extracts was the most representative of the soil solution. The measured DOC concentrations were equal to 7.4, 13.6 and 17.4 mg L⁻¹ in CaCl₂ extracts of soils from London, Feucherolles and Epoisses, respectively. The highest DOC

concentration measured in soil extracts from Epoisses was consistent with the highest organic carbon contents in this soil (Table V.1). Given the possible association of antibiotics with dissolved organic matter (Tolls, 2001), the measured DOC concentrations were consistent with the measured fractions of SMX, i.e., the higher the DOC concentration, the lower the free-dissolved SMX in the CaCl₂ extract and the lower available fraction (Figure V.3).

V.3.2.2. HPCD-based extraction: encapsulation of compound

At low SMX concentration, the SMX availability was the highest in soils from Feucherolles and the lowest in soils from London. By increasing the initial SMX concentration, similar available fractions were extracted in soils from Feucherolles and London, which were higher than in soils from Epoisses (Figure V.1).

For the lowest SMX concentration (Figure V.3C), the SMX availability was also negatively correlated with the sand content ($r = -0.83$, $p < 0.05$), while at the highest SMX concentration (Figure V.3F), the SMX availability was positively correlated with the silt content ($r = +0.85$, $p < 0.05$) and as for the CaCl₂-based extraction, the SMX availability was negatively correlated to the clay and the organic matter contents ($r = -0.97$ and -0.92 , $p < 0.05$).

Statistical evaluation of the influence of soil properties on recoveries and matrix effects by using an exhaustive extraction method was carried out by Salvia et al. (2013). Soils with different physicochemical properties were spiked with a mixture of 23 pharmaceuticals including SMX (50 $\mu\text{g kg}^{-1}$ dry soil). The main result was the negative correlation of recoveries with clay, organic matter contents and CEC, as for the soft extractions with CaCl₂ and HPCD solutions. Considering SMX, the lowest recovery (51%) was obtained in a loam soil with a high CEC, high organic matter and clay contents compared to another loam soil for which the highest recovery (75%) was obtained. Moreover, since the solvent extracts were submitted to QuEChERS purification like our HPCD extracts, we concluded that the purification step had decreased matrix effects to better highlight the soil properties that really influenced the SMX extractability.

V.3.2.3. EDTA-based extraction: chelation of cations

With respect to the mechanism of extraction of EDTA, the cations of humic acid-clay complexes were chelated by EDTA that could promote the extraction of SMX-organic matter complexes in the same time. Consequently, with respect to the clay content 2.8 times higher in soils from Epoisses than in soils from London and Feucherolles (Table V.1), the SMX availability was

higher in soils from Epoisses than in soils from London (Figure V.1). However, the available fraction was still higher in soils from Feucherolles probably due to the lowest organic matter content (1.9%) and consequently a low SMX adsorption compared to the two other soils.

For both SMX concentrations (Figure V.3B and E), the SMX availability was negatively correlated with the sand content ($r = -0.81$ to -0.91 , $p < 0.05$), and at the highest SMX concentration, the SMX availability also decreased with the decrease of silt content ($r = +0.68$, $p < 0.05$). Contrary to CaCl_2 - and HPCD-based extractions, the SMX extractability was not correlated with the organic matter content or clay content or CEC, reflecting different extraction mechanisms between solutions. One possible explanation could be the co-extraction of colloids and organic matter by EDTA. We have evidenced that the potential co-extraction of organic matter such as humic substances by the EDTA solution led to the lowest concentrations of freely-dissolved SMX in EDTA extracts quantified by online SPE-UHPLC-MS-MS compared to CaCl_2 and HPCD extracts (Chapter IV). As proposed by Wegst-Uhrich et al. (2014), the co-extraction of organic matter by the aqueous solutions can lead to the association of SMX with dissolved or colloidal organic matter. In conclusion, the extraction mechanism of EDTA solution was less specific of free-dissolved SMX in soil solution and easily-desorbed SMX from soil surfaces, compared to CaCl_2 or HPCD solutions.

Ter Laak et al. (2006) used different aqueous solutions in the sorption study of antibiotics, by varying the salt in the solution, the pH and the ionic strength of solutions. In the same way, some other aqueous solutions could be used in the availability assessment of antibiotics in soils, i.e., within the desorption equilibrium, to understand the interaction mechanisms between antibiotics and soil surfaces.

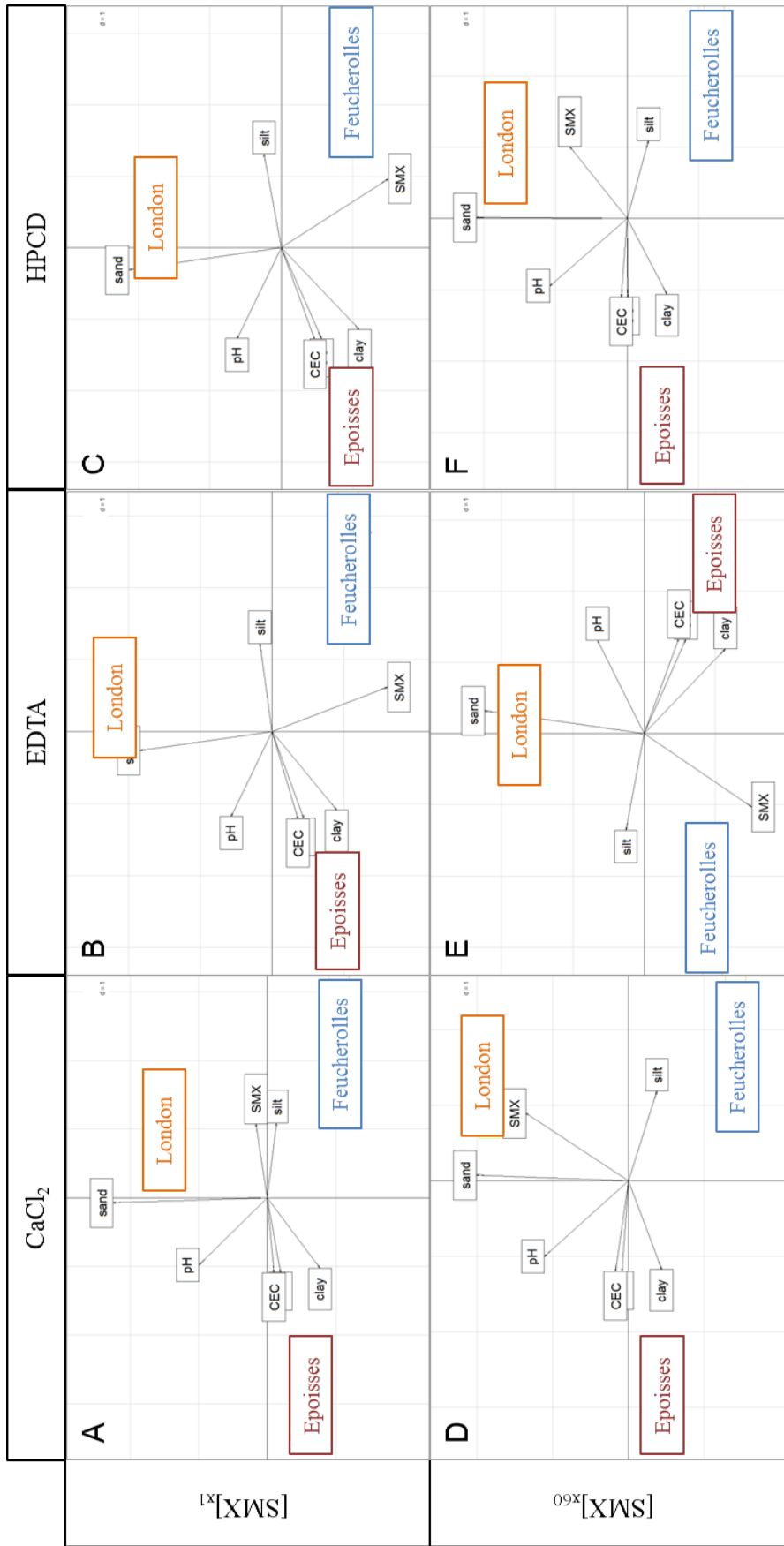


Figure V.3. Principal component analysis (PCA) of available fractions of SMX (%) in function of selected soil properties: % sand/silt/clay, CEC, pH and % organic carbon (OC) in soils from Feucherolles, London and Epoisses. The SMX availability was assessed by soft extraction with CaCl₂, EDTA and HPCD solutions, at two SMX concentration levels ([SMX]_{x,1} = 22 μg kg⁻¹ dry soil and [SMX]_{x,60} = 1.32 mg kg⁻¹ dry soil). The OC vector is behind the CEC vector. For the six PCA, 99.4-100% of the dataset variability was explained by the first PCA plan: A) axe1 = 80.8%, axe 2 = 18.9%; B) axe 1 = 69.7%, axe 2 = 29.7%; C) axe 1 = 73.4%, axe 2 = 26.5%; D) axe 1 = 73.6%, axe 2 = 26.4%; E) axe 1 = 73.9%, axe 2 = 26%; F) axe 1 = 78.2%, axe 2 = 21.3%.

V.4. Conclusion

Depending on the initial SMX concentration, a fraction of SMX was not environmentally available in soils, even after a short contact between the antibiotic and the soil sample (30-60 min). The SMX availability varied in function of both soil properties and extraction mechanisms of CaCl₂, cyclodextrin and EDTA solutions. According to the SMX adsorption on clay fraction and organic matter, the SMX availability was globally negatively correlated to cationic exchange capacity, organic matter and clay contents when it was assessed by CaCl₂- and cyclodextrin-based extractions. On the contrary, this correlation was not established for the EDTA-based extraction, evidencing a clear difference in the extraction mechanism of EDTA solution that could extract SMX less specifically than CaCl₂ and cyclodextrin solutions.

Further work would be to compare the environmental availability of antibiotics between a larger number of soils with more contrasted soil properties, taken into account a larger number of soil characteristics including the pH. Indeed, these studies are necessary to prioritize the factors influencing the most the antibiotic availability. The factors correlated between themselves, like organic matter, clay content and cationic exchange capacity, should not be considered as separated factors. In the objective of risk assessment, it is important to determine threshold values for each soil characteristics above/under which the antibiotic availability would not anymore depend on this factor.

Acknowledgments

The authors would like to thank Géraldine Depret for the soil sampling and analysis in Epoisses (France), Edward Topp and Lyne Sabourin for the soil sampling and analysis in London (Canada), and Julie Hagberg for her help in the TOC analyses. The authors also thank Nathalie Bernet, Nathalie Cheviron and Virginie Grondin for their assistance in the laboratory where the soil samples from London were received and stored, and where the extractions were performed to respect the legislation concerning the experimentation with foreign soils. This work was supported by grants from Region Ile-de-France (DIM ASTREA 2013-16) and ANR – French National Research Agency (CEMABS project, ANR-13-CESA-0016-01).

V. Supplementary material

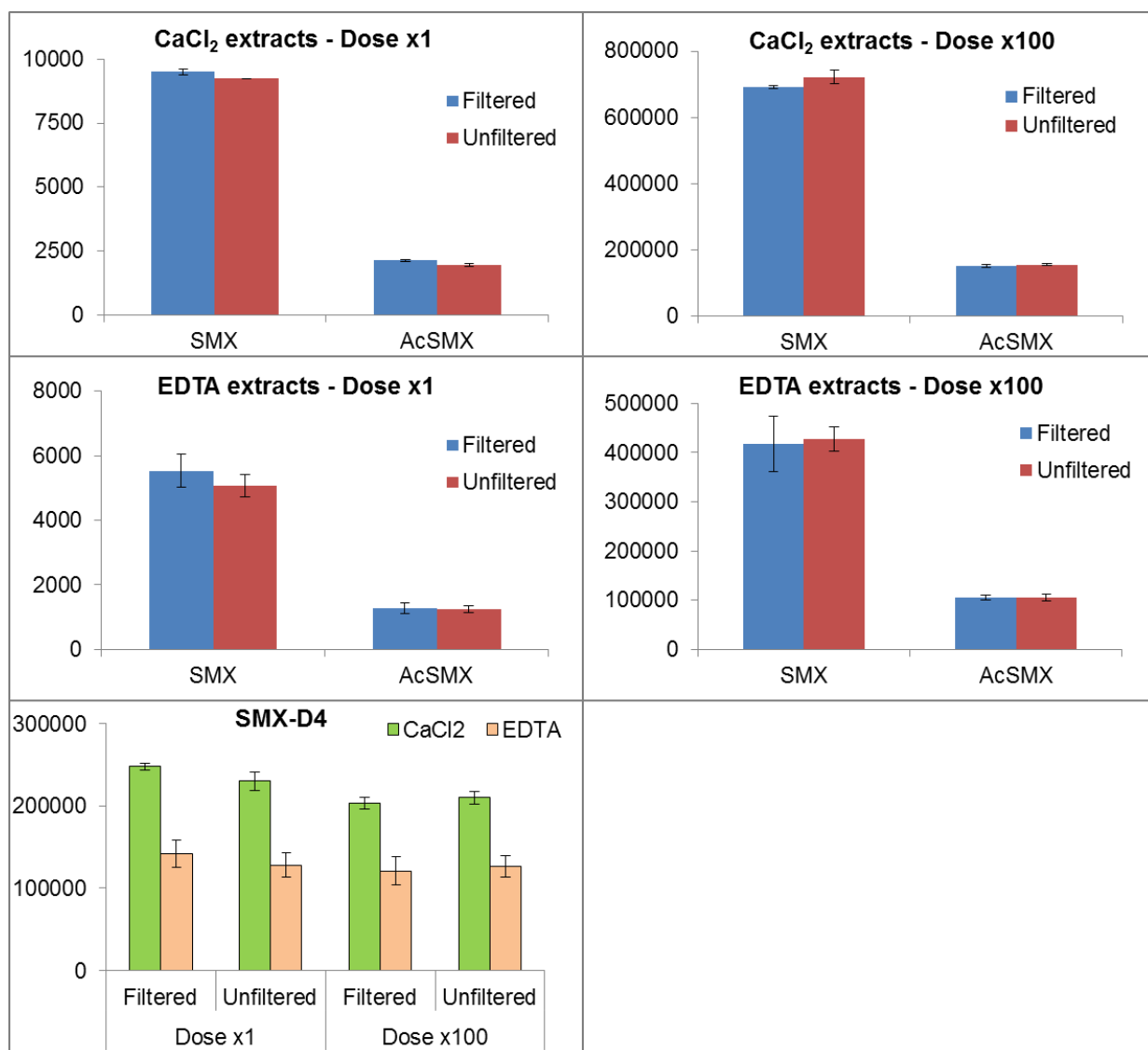


Figure V.S1. Graphical representation of SMX, AcSMX, SMX-D4 surface areas in filtered and unfiltered extracts. The extracts were spiked at two concentration levels: $2 \mu\text{g L}^{-1}$ (dose x1) and $200 \mu\text{g L}^{-1}$ (dose x100). The concentration of internal standard SMX-D4 was $71 \mu\text{g L}^{-1}$ in all extracts. The filtration test was to determine if the SMX, AcSMX and SMX-D4 compounds were lost within the filtration step ($0.22 \mu\text{m}$) of CaCl₂ and EDTA extracts. No loss occurred.

Table V.S1

Significant results of correlation tests (Pearson) between SMX availability and soil properties selected from the principal component analysis.

Initial SMX concentration	Soil component	Soft extracting solution		
		CaCl ₂	EDTA	HPCD
[SMX] ×1	% sand		r = -0.91 (p = 6x10 ⁻⁴)	r = -0.83 (p = 0.006)
	% silt	r = +0.98 (p = 4.4x10 ⁻⁶)		
	% clay	r = -0.95 (p = 8.6x10 ⁻⁵)		
	% OC and CEC	r = -0.99 (p = 7.9x10 ⁻⁷)		
[SMX] ×60	% sand	r = +0.72 (p = 0.03)	r = -0.81 (p = 0.008)	
	% silt		r = +0.68 (p = 0.046)	r = +0.85 (p = 0.004)
	% clay	r = -0.87 (p = 0.002)		r = -0.97 (p = 9.8x10 ⁻⁶)
	% OC and CEC	r = -0.72 (p = 0.03)		r = -0.92 (p = 0.0005)

Partie 2. Estimation de la biodisponibilité

Les méthodes d'extraction douces ont permis de mettre en évidence des fractions disponibles dans les sols en fonction de l'antibiotique, des types de sol et de MAFOR. Les efficacités d'extraction sont fortement influencées par une dynamique temporelle, en grande partie liée à des interactions modifiées par la décomposition et l'humification des matières organiques et des processus de transformation des antibiotiques (Chapitres II à V). Afin de déterminer si la méthode d'extraction permet d'estimer la biodisponibilité des antibiotiques, la fraction extraite doit être mise au regard d'effets biologiques des antibiotiques.

Etant donnée l'activité antibactérienne des antibiotiques, les micro-organismes du sol sont particulièrement ciblés et plusieurs types d'effets peuvent être étudiés (Chapitre I). En effet, les antibiotiques peuvent impacter les micro-organismes à court ou moyen terme, en entraînant des effets sur les communautés et les fonctions microbiennes ; et/ou à long terme, avec notamment, le développement d'antibiorésistances. Une forme de résistance peut être l'adaptation de micro-organismes, qui deviennent alors capables de biodégrader les antibiotiques dans les sols. Ce phénomène a particulièrement été observé dans les sols issus des parcelles expérimentales de London (Ontario, Canada), exposés de façon chronique à des doses d'antibiotiques relativement fortes. Sur ce site, les antibiotiques sont appliqués directement en solutions sur les sols, sans apports de MAFOR, afin d'évaluer directement les impacts sur les micro-organismes du sol. La biodégradation de certains antibiotiques a été mise en évidence et elle a été accélérée en comparaison aux sols dans lesquels les micro-organismes ont peu ou n'ont pas été exposés aux antibiotiques.

En collaboration avec le laboratoire d'Edward Topp (Agriculture and AgriFood) à London (Ontario, Canada), la méthode d'extraction douce a été testée sur ces sols pour estimer les fractions disponibles d'antibiotiques de familles différentes : la ciprofloxacine (fluoroquinolone), la sulfaméthazine (sulfonamide) et l'érythromycine (macrolide), au regard de leur biodégradation. Etant donné que les solutions de Borax et d'EDTA avaient été présélectionnées pour estimer la disponibilité de la ciprofloxacine (Chapitre II), ces deux solutions ont été utilisées pour les trois antibiotiques, afin de répondre aux questions suivantes dans le Chapitre VI :

- 1) Quelle est la disponibilité environnementale des trois antibiotiques dans les sols recevant régulièrement les antibiotiques ?
- 2) Les antibiotiques disponibles dans les sols sont-ils biodégradés ?
- 3) L'apport des antibiotiques par l'application d'un fumier sur le sol influence-t-il la disponibilité et la biodégradation des antibiotiques ?

Partie 2. Estimation de la biodisponibilité

Approche analytique : radiomarquage (^{14}C -antibiotique)

Chapitre VI

Sols agricoles

Antibiotiques

Adsorption dans des matrices complexes (organique + minéral)



Extractions aqueuses



(Bio)disponibilité de la ciprofloxacine, de la sulfaméthazine et de l'érythromycine



Biodégradation des antibiotiques par des micro-organismes adaptés



- 1) Disponibilité environnementale des différents antibiotiques dans les sols recevant de façon chronique et directement les antibiotiques?
- 2) Biodégradation accélérée pour les antibiotiques disponibles?
- 3) Quelles différences lorsque les antibiotiques sont introduits dans les sols via l'application de fumier?

Chapter VI: Environmental availability and biodegradation of ciprofloxacin, sulfamethazine and erythromycin in agricultural soils in a context of long-term exposure to antibiotics

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Abstract

Antibiotics are chronically introduced in agricultural soils as a result of the organic waste or wastewater application. The long-term exposure of soil microorganisms to antibiotics can lead to a selection pressure and the adaptation of microorganisms to biodegrade the antibiotics. Control soils and soils treated with high concentrations of different antimicrobial mixtures (10 mg kg⁻¹ soil) were sampled from the long-term experimental field device located in London (Ontario, Canada) and incubated with sulfamethazine, erythromycin or ciprofloxacin, three antibiotics of different classes. The environmental availability of these antibiotics in soils was assessed by performing non-exhaustive extractions with Borax or EDTA solutions and their biodegradation was followed during seven-day incubation experiments.

As a result, sulfamethazine and erythromycin were initially available in soils (60% and 36% of the initial content, respectively, when applied in solution) and were biodegraded more rapidly in long-term exposed soils (20% and 65%, respectively) than in control soils (0.4% and 3%, respectively). Ciprofloxacin was poorly available (8%) and not biodegraded in both untreated soils and soils treated with high concentrations of antibiotics. The soil microorganisms were adapted to degrade specifically an environmentally-available antimicrobial compound and this, in soils receiving either the antibiotic in solution or via the manure application.

Keywords

Antibiotic class; environmental behavior; soft extraction; adaptation; soil microorganisms

VI.1. Introduction

The use of antibiotics in human and veterinary medicines has led to their chronic release in the environment. The contamination of ground, surface and drinking waters, of sediments and soils by antibiotics residues has been widely recognized (Sarmah et al., 2006; Kemper, 2008). Following the application of farmyard manure and sewage sludge, antibiotics can be found in agricultural soils at concentrations ranging from $0.1 \mu\text{g kg}^{-1}$ to 1.5mg kg^{-1} soil (Toth et al., 2011; Du and Liu, 2012; DeVries et al., 2016).

Several antibiotic classes exist, e.g., sulfonamides, fluoroquinolones, macrolides, and the environmental behavior of antibiotics is generally class-dependent since the compounds within a same structural class exhibit similar physicochemical properties (Thiele-Bruhn, 2003). The antibiotic behavior in soils also depends on the composition and the physicochemical properties of the soil. Besides, as most of antibiotic molecules are ionizable, the environmental conditions govern their speciation, and therefore the mechanisms of adsorption on soil since neutral forms, zwitterion, anion or cation do not have equivalent chemical properties. For example, the sorption of sulfonamides in soils is highly influenced by the soil pH, the properties of soil organic matter and the particle size fractions. Sulfonamides can be highly mobile in some soils (Boxall et al., 2002; Thiele-Bruhn et al., 2004). Fluoroquinolones can strongly adsorb on soils by several mechanisms and particularly via the cation bridging of the negative charge (anion or zwitterion) to clay minerals (Thiele-Bruhn, 2003). Fluoroquinolones are not mobile and can persist in soils (Uslu et al., 2008). Considering macrolides, their adsorption is considered lower than that of fluoroquinolones but higher than the adsorption of sulfonamides (Wegst-Uhrich et al., 2014; Pan and Chu, 2016).

The environmental availability of antibiotics in soils, represented by the antibiotics that could be transferred in the environment or interact with living organisms, can also depend on the physicochemical and composition of the matrix in which the compounds are introduced into soils. For example, the pH of organic waste and the quality of the organic matter brought to soil were shown to be determinant in the ciprofloxacin availability in amended soils (Chapter II). In the same way, the sorption of the sulfachloropyridazine sulfonamide was lower in manure-amended soils mainly due to the alkaline pH of manure that decreased the sorption of sulfonamides negatively charged at this pH (Boxall et al., 2002). Moreover, the mineralization of the sulfamethoxazole sulfonamide was inhibited in soils amended with previously-contaminated biosolids, compost or sludge, compared to its mineralization in soils without amendment (Höltge and Kreuzig, 2007; Li et al., 2015); the degradation of sulfamethazine and sulfachloropyridazine decreased also in soils amended with liquid slurry (Accinelli et al., 2007). The interactions that may pre-exist inside the matrix according to the contact time with antibiotics may further limit their availability.

Depending on the environmental availability of antibiotics in soils, the frequency and duration of the exposition to concentrations, antibiotics can impact the soil microorganisms (Ding and He, 2010), including the acquisition of antibiotic resistance (Jechalke et al., 2014). In particular, the long-term exposure of soil microorganisms can lead to their adaptation to antimicrobial degradation (Topp et al., 2013; Topp et al., 2016). Thereby the biodegradation of sulfamethazine and tylosin was accelerated in soils historically exposed to a mixture of sulfamethazine, tylosin (macrolide) and chlortetracycline (tetracycline) with individual concentrations of 10 mg per kg of soil; as a consequence, the two antibiotics were removed more rapidly in these soils highly treated with antibiotics than in untreated control soils (Topp et al., 2013). In the same way, the accelerated biodegradation of erythromycin, clarithromycin and azithromycin was evidenced in soils highly treated with these macrolides (Topp et al., 2016). The accelerated biodegradation of antibiotics could imply a lower persistence of the compounds in soils and a lower exposure of soil microorganisms to the antibiotics. Bacteria of the genus *Microbacterium* degrading sulfamethazine and sulfadiazine were isolated from soils (Tappe et al., 2013; Topp et al., 2013) and sludge (Bouju et al., 2012). However the biodegradation was not accelerated for chlortetracycline despite the soil treatment at the same concentration level, probably due to the rapid dissipation of this antibiotic by adsorption on soil matrix (Topp et al., 2013). Furthermore, the exposure scenario of long-term plots to antibiotics could be considered as artificial since the antibiotics are directly applied in solutions on soils (Topp et al., 2013), while the antibiotic fate in soils can depend on the matrix in which the antibiotics are brought to the soils (Accinelli et al., 2007; Strauss et al., 2011).

Consequently, several hypotheses can be made: 1) the environmental availability of antibiotics could be determinant for their biodegradation in soils; 2) the adaptation of soil microorganisms to antibiotic biodegradation could be antibiotic-specific; 3) the soil microorganisms could be adapted to biodegrade different compounds within a same antimicrobial class; and 4) the biodegradation of antibiotics could be modified when the antibiotics are introduced in soils via the manure application, compared to the direct application of antibiotics in solution.

Related to these hypotheses, our objectives were to assess the environmental availability and the biodegradation of antibiotics in agricultural soils in a context of long-term exposure to antibiotics (hypothesis 1); to study, when an accelerated biodegradation was evidenced, the environmental availability and the biodegradation of antibiotics in soils treated with antibiotics belonging to the same class of antibiotics or to a different class (hypotheses 2 and 3); and finally, to compare the environmental availability and the biodegradation of antibiotics in soils directly contaminated with the antibiotic or via the manure application. Three antibiotics belonging to three different antimicrobial classes were selected: sulfamethazine (SMZ, sulfonamide), ciprofloxacin (CIP, fluoroquinolone) and erythromycin (ERY, macrolide). The soft extraction with aqueous

solutions was used as an indirect chemical method to assess the environmental availability of antibiotics (Chapter II). Different incubation experiments were conducted the most often within seven-day periods since 50% of SMZ and ERY were mineralized in about 1 day (Topp et al., 2013; Topp et al., 2016). The incubation experiment with the CIP antibiotic was performed during 21 days since its environmental availability was known to be low (Chapter II). The experiments were carried out with control and highly treated (10 mg antibiotic kg⁻¹) soils. Soil samples were initially spiked with each antibiotic at 1 mg kg⁻¹ dry soil and the use of ¹⁴C-labeled antibiotics allowed us to completely follow the ¹⁴C-activity distribution in the available, solvent-extractable, non-extractable and mineralized fractions.

VI.2. Materials and methods

VI.2.1. Chemicals and reagents

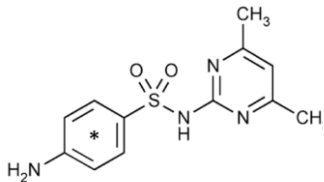
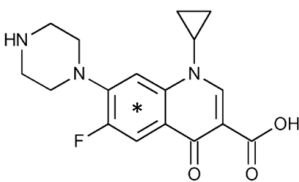
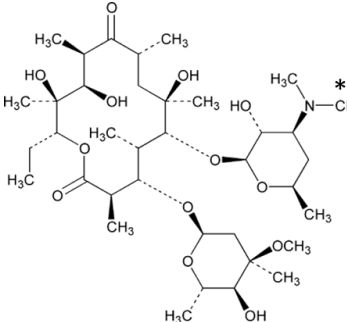
Radiolabeled [phenyl-¹⁴C] sulfamethazine (SMZ, specific activity 2.04 GBq mmol⁻¹, radioactive purity 99%), [phenyl-¹⁴C] ciprofloxacin (CIP, specific activity 0.56 GBq mmol⁻¹, radioactive purity 99%) and [methyl-¹⁴C] erythromycin (ERY, specific activity 1.48 GBq mmol⁻¹, radioactive purity > 97%) were purchased from American Radiolabeled Chemicals, Inc. (Saint-Louis, MO, US). Unlabeled antibiotics were purchased from AK Scientific (Union City, CA, US).

Structures and physicochemical properties of antibiotics are described in Table VI.1 and speciation diagrams of antibiotics as a function of pH are presented in Figure VI.1.

Sodium ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, Oakville, ON, Canada) and sodium tetraborate (Borax; Sigma-Aldrich) were dissolved in Milli-Q water (prepared on site) to prepare the soft extracting solutions at 0.1 M and 0.2 M, respectively. The pH of EDTA solution was adjusted to 7 with NaOH. These two types of aqueous solutions gave satisfactory results to assess available fractions of ciprofloxacin in soils amended with compost or manure (Chapter II). Acetonitrile (HPLC quality, VWR; Mississauga, ON, Canada) and phosphoric acid (H₂PO₄, VWR) were used for solvent-based extraction.

Table VI.1

Structures and physicochemical properties of antibiotics. The asterisk on the molecule structures indicates the location of ^{14}C atoms.

Molecule	Sulfamethazine (SMZ)	Ciprofloxacin (CIP)	Erythromycin (ERY)
Antibiotic family	Sulfonamide	Fluoroquinolone	Macrolide
Structure			
Molecular weight (g mol ⁻¹)	278.3	331.3	733.9
pK _a *	2.0 – 7.5	3.01 – 6.1 – 8.7 – 10.6	8.9
Log K _{ow} *	0.89	0.28	3.06

* The acid dissociation constants (pK_a) were taken from Qiang and Adams (2004) and the coefficients octanol-water distribution (Log K_{ow}) from DeVries and Zhang (2016).

VI.2.2. Agricultural soils and manure

Soil samples were taken from the experimental farm in London, Canada. The soil is a silt loam composed of 18% sand, 67% silt, 15% clay. The main properties are: organic matter content 3.4%, pH 7.5, cation exchange capacity (CEC) 13.2 cmol₊ kg⁻¹.

The experimental plots annually receive different mixtures of antibiotics and untreated plots are considered as control. Each treatment is applied in triplicate. Three different mixtures of antibiotics are applied on different plots. The first mixture is composed of sulfamethazine, tylosin and chlortetracycline (STC) and is applied on soils to achieve individual concentrations of 0.01, 0.1 and 1 mg kg⁻¹ soil from 1999 to 2004; 0.1, 1 and 10 mg kg⁻¹ soil since 2005 (Topp et al., 2013). The second mixture is composed of fluoroquinolones (FQ; ciprofloxacin and norfloxacin) and is applied on soils since 2010 to achieve concentrations of 0.1 and 10 mg kg⁻¹ soil. Finally, the third mixture is

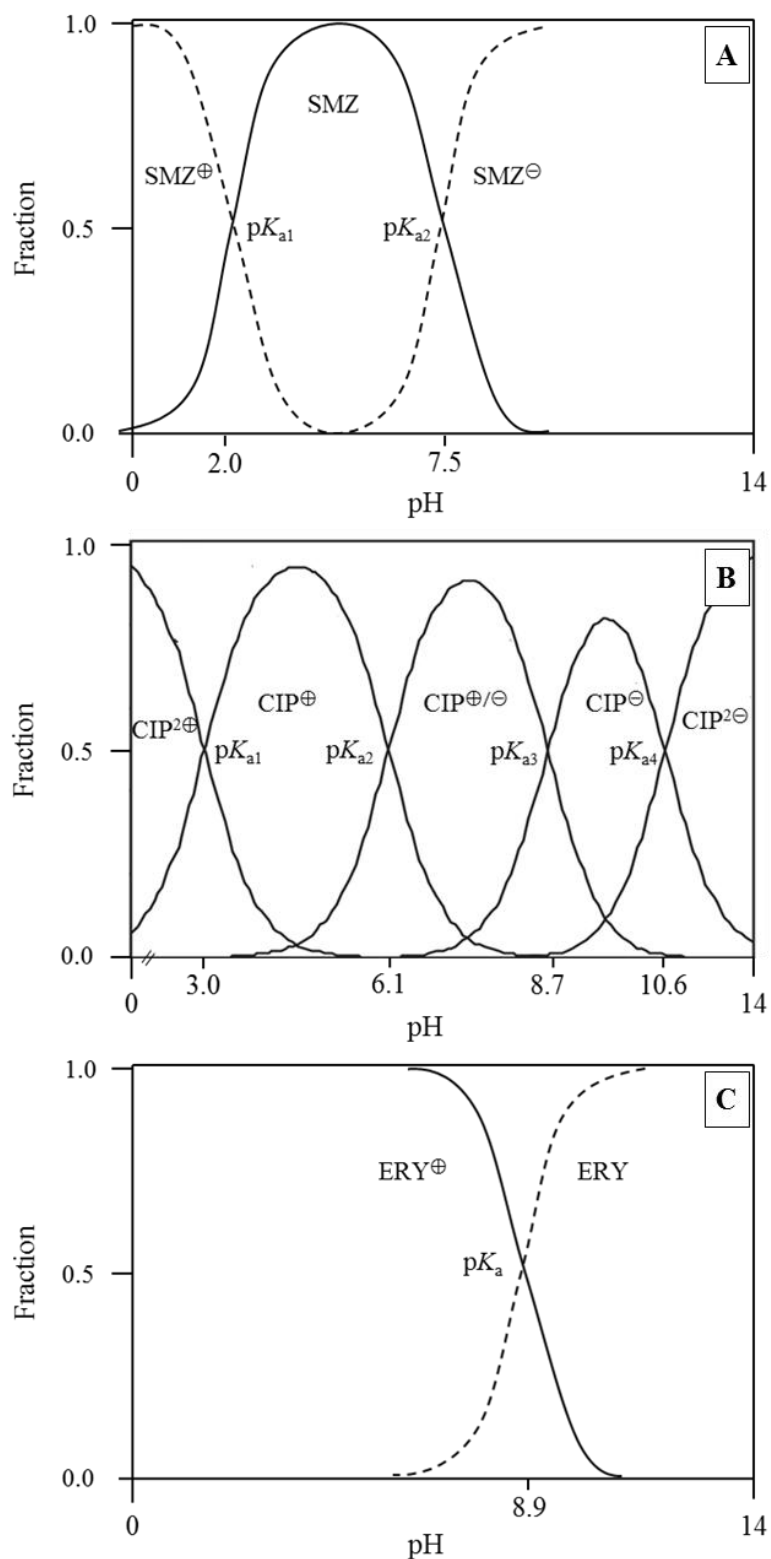


Figure VI.1. Speciation diagrams of antibiotics A) sulfamethazine (SMZ), B) ciprofloxacin (CIP) and C) erythromycin (ERY) as a function of pH. pK_a values were taken from Qiang and Adams (2004).

composed of macrolides (MC; erythromycin, clarithromycin, azithromycin) and is applied on soils since 2010 to achieve concentrations of 0.1 and 10 mg kg⁻¹ soil (Topp et al., 2016).

The soils used in our study were sampled 30 days after the last application. Soil samples were sieved to 2 mm and stored at -7°C.

The manure on which the antibiotic was applied before its incorporation into soil samples (section VI.2.3.1) was a composted dairy manure with the main following properties: pH 8.0, 6.5% dry matter and 5% organic matter.

VI.2.3. Incubation experiment

VI.2.3.1. Preparation of microcosms

Two series of incubation experiments in microcosms were carried out with first the antibiotic brought initially to soil directly in solution and then with manure.

In the first series, control soils and highly treated soils (10 mg antibiotics kg⁻¹) were selected. The soil samples were prepared by weighing 20 g of soil (dry weight equivalent) in 150 mL baby-food jars and adjusting the moisture content at 15%; for the incubation experiment with the CIP antibiotic, 25 g of soil were weighed. One-gram portion of soil was removed and spiked with 100 µL of antibiotic solution containing both the unlabeled compound (SMZ, CIP or ERY; 200 mg L⁻¹) and the radiolabeled compound (¹⁴C-CIP: 16.8 MBq L⁻¹; ¹⁴C-SMZ: 17.7 MBq L⁻¹; or ¹⁴C-ERY: 24.7 MBq L⁻¹). Thereby, the concentration brought reached 1 mg antibiotic kg⁻¹ dry soil. For the highly treated soils, each antibiotic was added both on the soil receiving at field the antibiotic mixture containing the compound and on the soils receiving another antibiotic mixture in order to determine if cross-enhancement of biodegradation could occur. As the antibiotic solution was in methanol, the solvent was evaporated before incorporating the one-gram portion to the rest of the soil sample. Then, the soil samples were thoroughly mixed with a spatula and baby-food jars were placed in sealable glass 1 L Mason jars. Four replicates were prepared for each treatment. A scintillation vial was filled with water (10 mL) to maintain the moisture conditions, and another one was filled with NaOH solution (1 N, 5 mL) to trap the released ¹⁴C-CO₂ during the incubation period. All jars were placed in the dark at 30 °C and incubated for 7 days (SMZ, ERY) or 21 days (CIP). The jars were opened on days 3, 7, 14 and 21 (unless otherwise specified) to renew the jar headspace and change the NaOH solution.

In the second series of incubation experiments, the antibiotic was brought to the soil via the manure application (and not directly in contact with soil) in order to simulate the field situation of manure recycling. The soils used in this experiment were the soils treated with 10 mg STC or 10 mg MC kg⁻¹ soil, and the corresponding untreated soils as controls. To respect the mass ratio between manure and soil (3% w/w, in dry weight; Chapter II) and also the moisture conditions previously described, the dairy manure was slightly dried to 35% dry mass. Then, 1.5 g of manure was weighted and the antibiotic (SMZ or ERY) was added on the manure, as previously described for soil. After a contact of approximatively 30 min, the spiked manure was incorporated into 20-g soil sample (control, treated with STC or MC mixture) to reach a final concentration equal to 1 mg antibiotic kg⁻¹ dry soil. Four replicates were prepared for the treatments with ERY and three replicates for the treatments with SMZ. The jars were filled and incubated as described for the first series of incubation experiments. The jars were opened on days 3, 7, 14 and 18 days to renew the jar headspace and change the NaOH solution.

VI.2.3.2. Determination of the ¹⁴C-activity distribution

The mineralization of the ¹⁴C-antibiotic was followed over the incubation through the ¹⁴C-CO₂ trapping in the NaOH solution. Extractants of increasing efficiency were used to extract the less and less available antibiotics from soil samples. The successive extractions were performed in scintillation vials on a 2-gram soil sample taken in each microcosm at specific dates. The ¹⁴C-activity distribution in soils was determined after 0, 3 and 7 days for SMZ and ERY and after 0, 7 and 21 days for CIP.

First, the environmental availability of ¹⁴C-antibiotics was assessed by soft extractions with two different aqueous solutions: EDTA (0.1 M, pH 7) and Borax (0.02 M, pH 9). The EDTA solution was preselected for its chelating power that could be interesting given the speciation of antibiotics as a function of pH. The Borax solution was preselected for its properties of surfactant and alkaline buffer (Chapter II). In a first step, the extraction efficiency of EDTA and Borax solutions was compared for the soil directly contaminated by each antibiotic; the extraction was performed after 30-60 min of contact between the antibiotic and soil, i.e., when the environmental availability should be the highest. This preliminary study aimed at identifying the most suitable extractant to be selected for the two series of incubation experiments. In this preliminary study and in the incubation experiments, a volume of 6 mL of soft extractant was added to the soil sample and the mixture was shaken end-over-end for one night (17 h) at ambient temperature. The aqueous

extract was obtained after centrifuging the mixture at $1380 \times g$ for 10 min (HL-4 swinging bucket rotor, Sorvall GLC-1 centrifuge; Fisher Scientific; Ottawa, ON, Canada).

Then, the fraction of molecules more adsorbed on soils or on soil/manure mixtures was recovered thanks to a solvent-based extraction with a mixture of acetonitrile/water/10% H_2PO_4 (6/3/1, in volumes). A 6 mL volume of this solvent was added to the soil samples three times. Each time, the mixture was shaken for 30 min using a wrist action shaker and a centrifugation was performed at the end of each period. For each soil sample, the three organic extracts were collected in a scintillation vial.

After the last extraction, the non-extractable fraction and the mass balance recoveries were determined by combusting the dried and ground soil sample in an oxidizer (Biological Oxidizer OX 700; R.J. Harvey, Tappan, NY, US). The ^{14}C - CO_2 produced during the combustion was trapped in scintillation cocktail (R.J. Harvey, Tappan, NY, US).

VI.2.3.3. Analytical measurements

The radioactivity in aqueous and organic extracts, in NaOH solutions and in cocktail after the combustion of dried soils was counted by liquid scintillation counting using a Beckman Coulter Model LA 6500 instrument. Aliquots of extracts (1 mL) and NaOH solution (5 mL) were diluted in 10 mL scintillation cocktail (UniverSol, ICN; Costa Mesa, CA, US).

High performance liquid chromatography (HPLC) analyses were carried out to determine the main compounds present in the available fraction. The aqueous extracts were freeze-dried and the residue was dissolved in 750 μL (unless otherwise specified) of mobile phase. Standards of SMZ, ERY and CIP were injected in HPLC (Agilent; Mississauga, ON, Canada) coupled to ultraviolet (UV) detection (Agilent). An Agilent Zorbax Eclipse XDB-C18 column (4.6 mm x 100 mm, 5- μm pore size) was used and the mobile phase consisted of A) water + 0.01% trifluoroacetic acid (TFA) and B) acetonitrile + 0.01% TFA with a flow rate of 1 mL min^{-1} and a gradient of 5% B to 100% B in 10 min. Given the potentially-low concentrations of compounds in extracts, the UV response was expected too low to detect the compounds and identify the parent compound or the transformation products. Therefore, the column effluent was fractionated every minute in a HPLC vial with a fraction collector (Agilent) placed after the UV detector. Then, the radioactivity was measured in each vial by liquid scintillation counting after adding 10 mL of scintillation cocktail. Thus we could determine the approximate retention times of ^{14}C -compounds. The mass balance of radioactivity between parent compound and potential transformation products was determined by injecting a known volume of extract. The injection volume was adapted according to the

radioactivity of the sample. The injections of ^{14}C -SMZ and ^{14}C -ERY standards resulted in highest radioactivity levels counted in the fractions of HPLC effluent collected at 5-6 min and 8-10 min, respectively.

VI.2.4. Statistical analyses

The ^{14}C -fractions were expressed as percentages of the total ^{14}C -activity recovered in each 2-gram portion if more than 80% of initial ^{14}C -activity was recovered. This allowed us to compare the available, solvent-extractable, non-extractable and mineralized ^{14}C -fractions between antibiotics and soil treatments during the incubation. Statistical analyses were realized with RStudio software (version 0.98.501, RStudio Inc.) to compare the fractions between 1) the different antibiotics, CIP, ERY, SMZ, and 2) the soil treatments, control soils and soils receiving 10 mg antibiotics kg^{-1} soil. Pairwise differences were determined using parametric linear models or non-parametric Kruskal-Wallis tests.

VI.3. Results and discussion

VI.3.1. Selection of the soft extractant from the preliminary study

The soft extraction performed on soil samples directly contaminated with a solution led to different extractabilities of ^{14}C -antibiotics with the Borax or the EDTA solution after 30-60 min of contact with soil (Figure VI.2). The highest extractability was obtained for ^{14}C -SMZ with the Borax solution ($78 \pm 3\%$) and the lowest one for ^{14}C -CIP with the same solution ($5 \pm 1\%$); comparatively $31 \pm 1\%$ of ^{14}C -ERY was extracted with the Borax solution. The order of antibiotics was kept with the EDTA solution, i.e., $\text{SMZ} > \text{ERY} > \text{CIP}$ considering the extracted fractions, but with smaller differences between the molecules.

Some differences of extractability between the two extracting solutions were highlighted. As the adsorption of ionizable molecules depends on their pK_a and on soil pH (Thiele-Bruhn, 2003), the pH of the aqueous solution is a key factor for their extractability.

According to the antibiotic pK_a (Table VI.1, Figure VI.1) and to the soil pH of 7.5, SMZ was both neutral and anionic. The Borax solution (pH 9) extracted more than two times more ^{14}C -SMZ ($78 \pm 3\%$) than the EDTA solution ($33 \pm 1\%$). The SMZ recovery was higher at pH 9 than at pH 7

($p < 0.05$). Indeed, SMZ was anionic in Borax solution which could decrease its adsorption on soil (Strauss et al., 2011). The EDTA solution could chelate the soil cations and extract anionic SMZ whereas the neutral SMZ could remain adsorbed on soil by several interactions, including hydrophobic interactions with the soil organic matter (Wegst-Uhrich et al., 2014).

ERY was mostly cationic at soil pH with the protonation of the tertiary amine group of the molecule (Table VI.1, Figure VI.1). ERY was rapidly adsorbed on clay particles by cationic exchange and the hydrated cations can adsorb on the negatively charged soil constituents such as organic matter by hydrogen bonding (Kim et al., 2004; Wegst-Uhrich et al., 2014). The pH difference between EDTA and Borax solutions had little impact on the ^{14}C -ERY extractability with fractions reaching $24 \pm 1\%$ and $31 \pm 1\%$, respectively ($p < 0.05$). The EDTA solution probably chelated the soil cations and co-extracted organic matter on which ERY was adsorbed, whereas the competition between the negative charges of the soil and the Borax solution could enhance the desorption of cationic ERY from soil surfaces.

CIP was mostly as the zwitterion in the soil (Table VI.1, Figure VI.1). The EDTA solution (pH 7) extracted the highest fraction ($12 \pm 2\%$) of ^{14}C -CIP ($p < 0.05$). It was already shown that in compost-amended soil (neutral pH) the EDTA solution extracted a highest fraction of ^{14}C -CIP than the Borax solution (Chapter II). The chelation of soil cations by EDTA could release CIP residues by reducing cation bridging and consequently improving the extraction.

The Borax and EDTA solutions extracted different fractions of antibiotics in soils that could be environmentally-available (Harmsen, 2007). Antibiotic molecules in soil solution and molecules easily-desorbed from soil matrix could be available for microbial degradation. Consequently, in the following sections (VI.3.2 and VI.3.3), both extractants were used. For soils contaminated with ^{14}C -ERY and ^{14}C -SMZ, two of the four replicates were used for each soft extraction (Borax or EDTA); for ^{14}C -CIP, only the extraction with EDTA was performed (four replicates). Consequently we compared the results from the EDTA extraction for the soils directly contaminated with antibiotics. In the last section (VI.3.4), we selected the Borax solution to extract the available antibiotics in manure-amended soils. Assuming the molecule adsorption could be stronger on manure-amended soils, the Borax solution could extract more efficiently the antibiotics (SMZ and ERY) compared to the EDTA solution. Moreover we previously recommended to use an aqueous solution with a pH close to the manure pH.

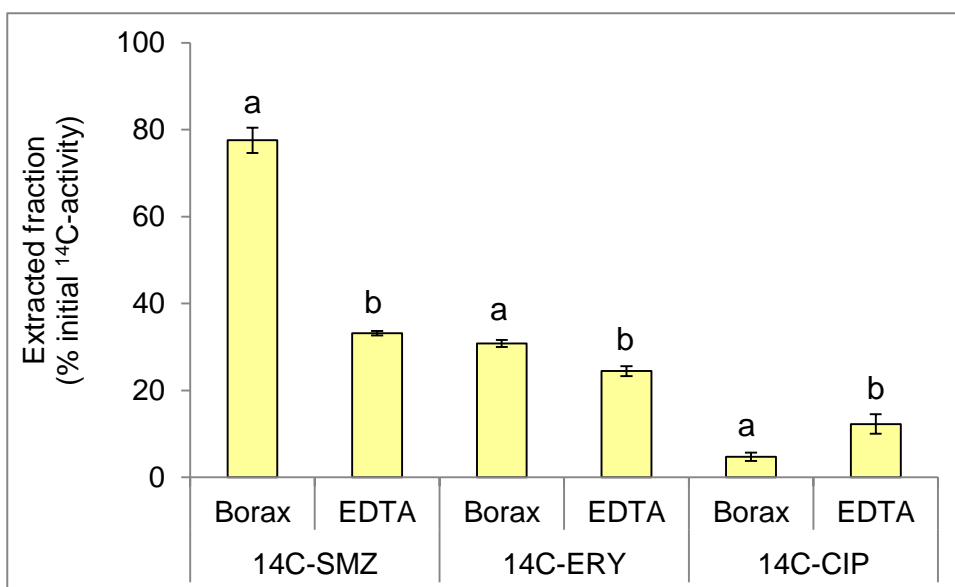


Figure VI.2. Fractions of ¹⁴C-antibiotics (% initial ¹⁴C-activity) extracted from soils with aqueous solutions of Borax or EDTA, after 30-60 min of contact (n = 3). The different letters represent the differences between the extracted fractions for a same antibiotic (linear model, p < 0.05).

VI.3.2. Environmental availability of antibiotics in soils long-term exposed to antibiotics

The distribution of the ¹⁴C-activity in microcosms at days 0 and 7 is presented in Figure VI.3 for the three antibiotics applied to control soils and soils receiving 10 mg kg⁻¹ of FQ, STC or MC mixtures, respectively. Since the mass balance recoveries reached 80-115% of the theoretical ¹⁴C-activity, all ¹⁴C-fractions were referred to the total ¹⁴C-activity recovered in each sample according to section VI.2.4.

VI.3.2.1. Ciprofloxacin

At day 0, the ¹⁴C-CIP availability assessed through the EDTA extraction was already very low with 6.6-10% of the total ¹⁴C-activity recovered. At initial time, ¹⁴C-CIP was more available in soils receiving fluoroquinolones at 10 mg FQ kg⁻¹ soil than in control soils (p < 0.05). The available fractions slightly decreased in the two soils between 0 and 7 days (p < 0.05).

The solvent-based extraction enabled to desorb a high fraction of ¹⁴C-CIP. The available and solvent-extractable fractions together reached 66-76% of the total ¹⁴C-recovered, whatever the soil treatment (p > 0.05) and the date (p > 0.05). Non-extractable residues (NER) were initially present in both soils with fractions similar between soil treatments (24-31%; p > 0.05). The NER fractions remained stable over the incubation time (p > 0.05). However, at day 7 the NER fraction was higher

in control soils than in soils receiving a high dose of FQ ($p < 0.05$). The higher fraction of available ^{14}C -CIP and the lower NER fraction in highly treated soils could be due to a saturation of binding sites as fluoroquinolones can persist in soils (Uslu et al., 2008; Picó and Andreu, 2007).

Finally, the mineralized fraction reached 0.1% after 7 days with no difference between treatments ($p > 0.05$). The mineralization of impurities in solution instead of ^{14}C -CIP, pure at 99%, could not be excluded. The low availability of ^{14}C -CIP may have limited the mineralization. ^{14}C -CIP mineralization was previously shown to be very low in soils (Girardi et al., 2011; Chapter II). The incubation period was extended to 21 days to see if more ^{14}C -CIP would be mineralized in the highly treated soils. However, after 21 days, the mineralized fractions at days 7 and 21 were similar ($p > 0.05$) and there was still a higher ^{14}C -CIP availability in soils receiving 10 mg FQ kg^{-1} than in control soil ($p < 0.05$). The radioactivity level in aqueous extracts was too low to carry out HPLC analyses.

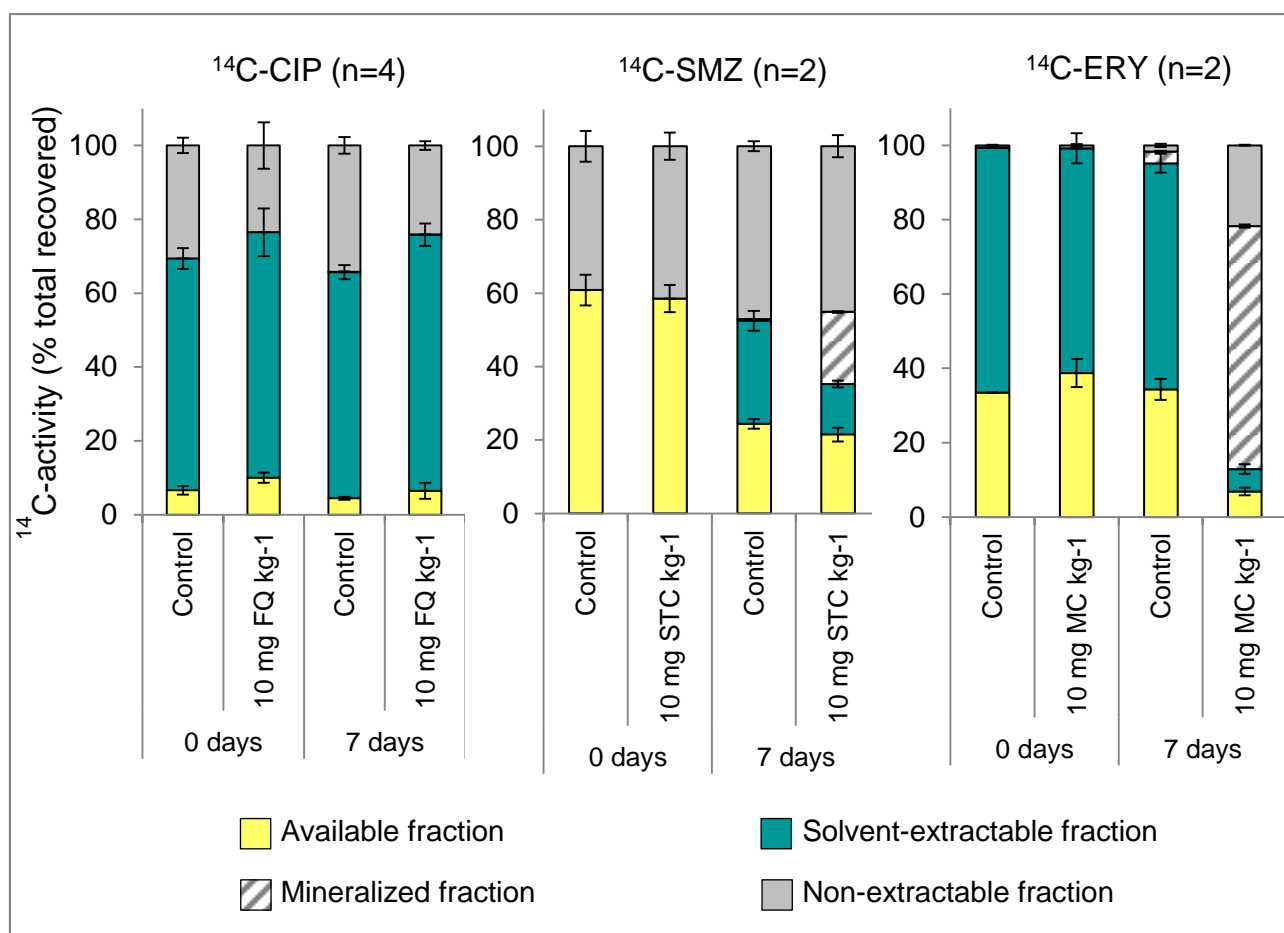


Figure VI.3. Distribution of the ^{14}C -activity in fractions (% total recovered) in soils spiked with ^{14}C -antibiotics. Soil samples were taken from plots receiving 0 (control) or 10 mg antibiotics per kg of dry soil. These plots were respectively FQ, STC and MC for the incubation concerning CIP, SMZ, and ERY biodegradation. The EDTA solution was used to extract the available fraction.

VI.3.2.2. Sulfamethazine

At day 0, 59-61% of ^{14}C -SMZ was available in control soil and in the highly treated soil from the STC plot. The availability decreased to reach 22-25% after 7 days ($p < 0.05$). The available fractions were similar to those given by Vittoria Pinna et al. (2012) who assessed the SMZ availability with a CaCl_2 -based extraction. If the solvent-based extraction did not release more ^{14}C -SMZ at day 0, the fractions of available and solvent-extractable ^{14}C -SMZ reached together 35% in the STC soils and 52% in control soils at day 7. The NER fraction initially equaled 39-41% and stayed stable over the incubation in both soil samples ($p > 0.05$). Consequently the lower cumulative fraction in the STC soil (available + solvent-extractable) was mainly explained by the higher fraction of ^{14}C -SMZ mineralized in soils receiving the antibiotic mixture (Figure VI.3; $p < 0.05$). Indeed, after 7 days, 20% of ^{14}C -SMZ was mineralized compared to only 0.4% in control soils. This confirmed that the ^{14}C -SMZ mineralization was accelerated in soils receiving annually the antibiotic mixture (Topp et al., 2013).

HPLC analyses were carried out on aqueous extracts for both soils and the radioactivity in the different HPLC fractions collected was counted. At day 0, the aqueous extracts were mostly composed of ^{14}C -SMZ since the highest radioactivity level was observed for the fractions collected between 5 and 7 min, like for the ^{14}C -SMZ standard. After 3 and 7 days of incubation, a second high radioactivity level was counted in the fractions collected between 1 and 2 min, only in extracts belonging to soils where ^{14}C -SMZ was biodegraded; this polar compound could correspond to a transformation product from SMZ biodegradation.

VI.3.2.3. Erythromycin

Initially, 34-36% of ^{14}C -ERY was available (Figure VI.3). The solvent-based extraction enabled to recover all the ^{14}C -ERY in both control soil and highly treated soil from the MC plot. Consequently the NER fraction was very low (0.6-0.8%). The available fraction remained stable in control soil during 7 days (34%, $p < 0.05$) but the cumulative fraction of available and solvent-extractable ^{14}C -ERY slightly decreased over the same period ($p < 0.05$). The NER fraction was only 1.6% and the mineralized fraction 3.2% after 7 days. Comparatively, the available and the cumulative fractions in soils receiving the antibiotic mixture decreased to reach respectively 7% ($p < 0.05$) and 13% ($p < 0.05$). Most of the ^{14}C -activity recovered was found in the mineralized fraction, i.e., 65% of ^{14}C -ERY after 7 days, and the NER fraction represented 22% (Figure VI.3). As very few NER were formed in control soils, we supposed that the NER formed in the highly

treated soil could be mostly transformation products and/or biogenic non-extractable residues (Kästner et al., 2014). We should keep in mind that the mineralization of the antibiotic was not necessarily complete. The ^{14}C -radiolabelling was on the C atom of a methyl group and not of a phenyl group like in the SMZ molecule (Table VI.1). As a consequence, the ^{14}C -methyl could be more bioavailable for microbial degradation and removed at quite early stages of biodegradation pathways with no further information about the unlabeled residues that could be still present in soil. The result however confirmed that the biodegradation of ^{14}C -ERY was accelerated in the MC soil compared to control soils (Topp et al., 2016).

According to the HPLC analyses, at day 0, the aqueous extracts were mostly composed of ^{14}C -ERY since the highest radioactivity level was in the fractions collected between 8 and 10 min (corresponding to ^{14}C -ERY standard). After 3 and 7 days of incubation, a second high radioactivity level was counted in fractions collected between 1 and 2 min, only in extracts from soils where ^{14}C -ERY was biodegraded. Therefore, we concluded that non-identified transformation products were formed in these soils over the incubation time.

VI.3.2.4. Comparison between antibiotics

The environmental availability of the three antibiotics was consistent with the adsorption coefficients (K_d) of sulfamethazine, erythromycin and norfloxacin (another fluoroquinolone) in agricultural soils (Pan and Chu, 2016) in the following order: fluoroquinolone > macrolide > sulfonamide. Indeed the environmental availability was the lowest for ciprofloxacin and the highest for sulfamethazine. Furthermore, it was shown that the nature of substituted groups in fluoroquinolone antibiotics has little effect on the sorption interactions according to their sorption mechanisms in soils, contrary to those in sulfonamide antibiotics (Figuroa-Diva et al., 2010).

Considering the possible link between the environmental availability and the biodegradation of ^{14}C -antibiotics, soil microorganisms were adapted to biodegrade ^{14}C -SMZ and ^{14}C -ERY that were both available in soils contrary to ^{14}C -CIP. As a result, when antibiotics are tightly associated with soil (e.g., CIP), they could be less available for the possible selection of new catabolic pathways and thus, enrichment of biodegradation capacity through metabolism and growth. Several hypotheses could explain the absence of a specific biodegradation for ^{14}C -CIP: 1) the environmental availability of the antibiotic was so low that it was not available for microbial degradation; 2) the compound was not selected as a nutrient by soil microorganisms; and 3) microorganisms were not adapted in these soils to metabolize ciprofloxacin.

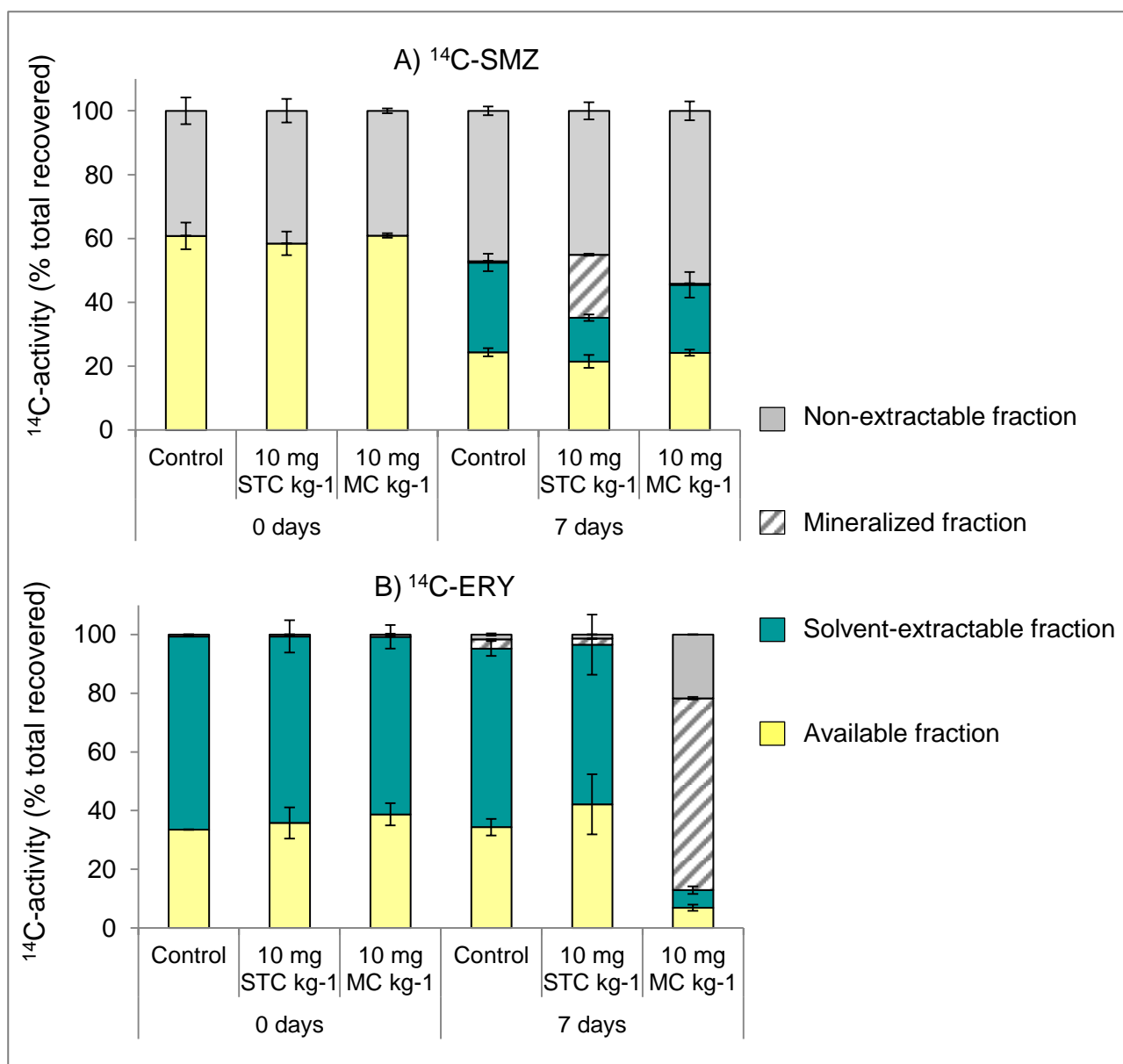


Figure VI.4. Distribution of ^{14}C -activity (% total recovered, $n = 2$) in fractions for **A)** ^{14}C -SMZ and **B)** ^{14}C -ERY in soils. Soils were taken from plots receiving 0 (control), 10 mg kg⁻¹ of mixture of sulfamethazine, tylosin and chlortetracycline (STC) or mixture of macrolides (MC). Soft extraction to assess the available fraction was performed with the EDTA solution.

When accelerated biodegradation was evidenced for ^{14}C -ERY and ^{14}C -SMZ, the decrease of the antibiotic availability was explained by the increase of the mineralized fraction ($p < 0.05$). For ^{14}C -ERY, a part of the solvent-extractable compounds, i.e., more sorbed to soil, could be also available for microbial degradation since this fraction also decreased when the mineralized and the NER fractions increased.

VI.3.3. Potential cross-enhancement of antibiotic biodegradation

The fate of ^{14}C -SMZ and ^{14}C -ERY was studied in control soils, in soils receiving 10 mg kg^{-1} of the STC mixture or 10 mg kg^{-1} of macrolides MC (Figure VI.4). With respect to the results, no accelerated biodegradation was observed for ^{14}C -SMZ in soils receiving 10 mg kg^{-1} of macrolides (Figure VI.4-A) and for ^{14}C -ERY in soils receiving 10 mg kg^{-1} of the STC mixture (Figure VI.4-B) since there was no difference with control soils ($p > 0.05$). Besides the available fractions (EDTA extraction) were similar (no difference with control soils, $p > 0.05$) and HPLC analyses did not reveal any difference with HPLC results in section VI.3.2.

Since the soil microorganisms adapted to biodegrade the SMZ antibiotic were not able to biodegrade ERY, the antibiotic biodegradation was confirmed to be class-specific. We could have expected an accelerated mineralization of ^{14}C -ERY in soils receiving the STC mixture. Indeed tylosin present in the mixture STC is a macrolide like erythromycin and the accelerated biodegradation of tylosin was highlighted by Topp et al. (2013). However our results evidenced that the antibiotic biodegradation was compound-specific. Although the toxicity mechanisms of both macrolides are the same, the chemical structures were sufficiently different for biodegradation not to be cross-enhanced.

VI.3.4. Biodegradation of sulfamethazine and erythromycin in manure-amended soils

For both ^{14}C -SMZ and ^{14}C -ERY compounds, the ^{14}C -distribution in manure-amended soils was compared with the distribution in soils without manure (Figure VI.5).

The retention of ^{14}C -SMZ was higher in manure-amended soil (Figure VI.5A) with smaller available (Borax extraction) and solvent-extractable fractions and a higher NER fraction than in soil without manure ($p < 0.05$). Actually, the sorption of SMZ was higher on manure-amended soils compared to unamended soils, depending on the pH and the organic carbon content (Strauss et al., 2011; Vittoria Pinna et al., 2012). For the highly treated soils, the fraction of mineralized ^{14}C -SMZ after 3 days was higher in manure-amended soil (9.7%) than in soil alone (3.6%; $p < 0.05$).

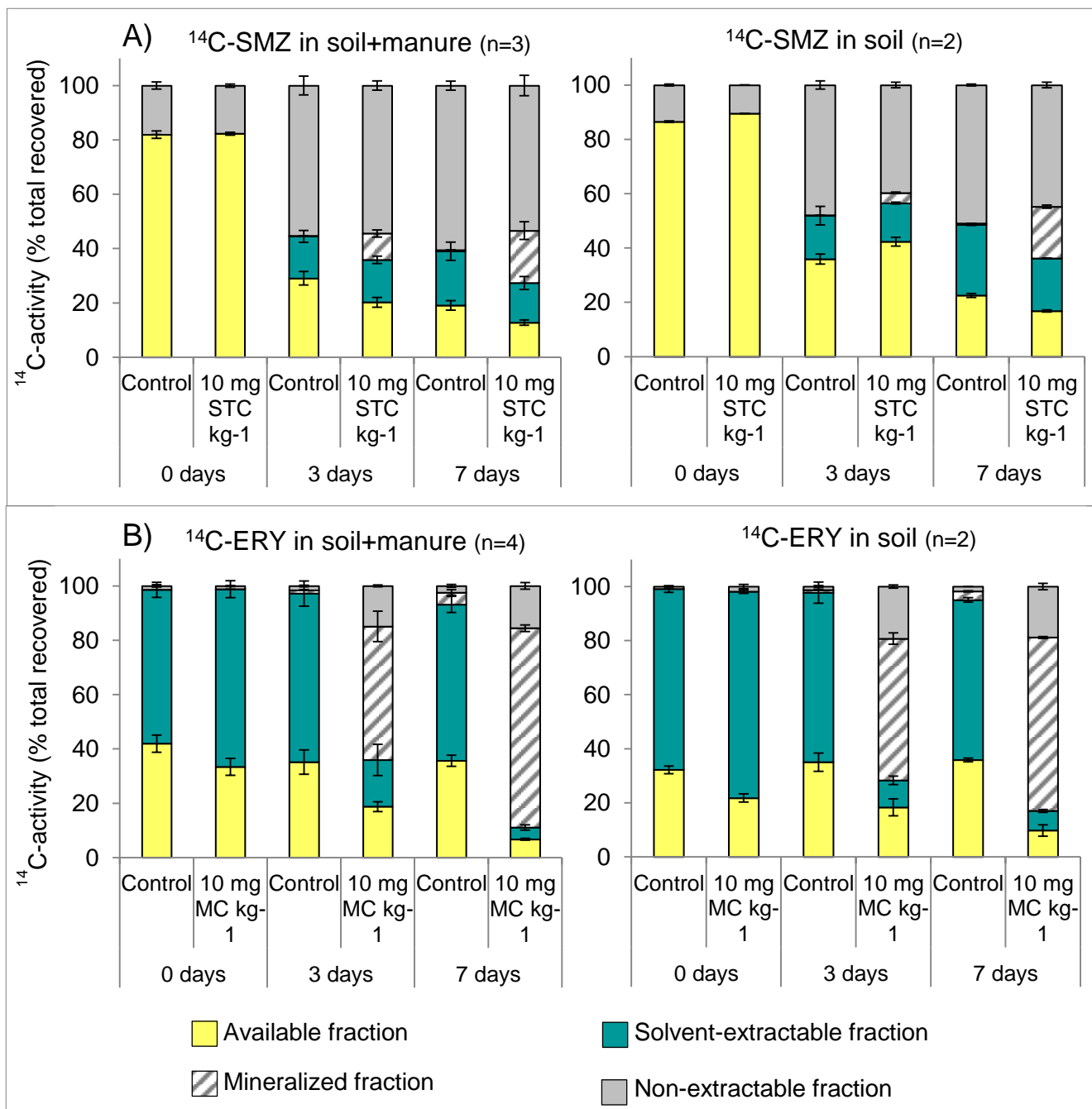


Figure VI.5. Distribution of ¹⁴C-activity (% total recovered) in fractions for A) ¹⁴C-SMZ and B) ¹⁴C-ERY in soil supplemented with the antibiotic directly, or added via supplemented manure. Soils were taken from plots receiving 0 (control) and 10 mg antibiotics kg⁻¹ soil. These plots were respectively STC and MC for the incubation concerning SMZ and ERY biodegradation, respectively. Soft extraction to assess the available fraction was performed with the Borax solution.

However the mineralized fractions were globally similar after 7 days ($p > 0.05$) and even after 18 days of incubation, they reached 23-24% of initial ^{14}C -activity in both soils ($p > 0.05$, data not shown).

The antibiotic ^{14}C -ERY was initially more available in manure-amended soils (33-42%) than in unamended soils (22-32%, $p < 0.05$; %; Figure VI.5B). Since the antibiotic was added on the manure whose pH was close to the pK_a of ERY, ERY was either present as cation or as neutral forms (Figure VI.1). The neutral ERY is less adsorbed on soils (Kim et al., 2004; Wegst-Uhrich et al., 2014); consequently, the extractability with the Borax solution was higher at day 0. However, after 3 days of incubation, the available fractions of ^{14}C -ERY were similar in manure-amended soils and soils without manure ($p > 0.05$). Slight differences were obtained in the ^{14}C -activity distribution according to the soil treatment and the day of incubation. Concerning the ^{14}C -ERY mineralization, the fraction was slightly higher in manure-amended control soil (1.1%) than in non-amended soil (0.9%; $p < 0.05$). After 18 days of incubation, the mineralized fraction equaled 26% in manure-amended control soil vs 15% in control soil without manure ($p < 0.05$). For the highly treated soils, the mineralized fractions were globally similar through the incubation ($p > 0.05$) and even after 18 days when the ^{14}C -ERY mineralization represented 61-63% of initial ^{14}C -activity (data not shown). HPLC analyses did not reveal any differences with HPLC results for antibiotics directly brought to soils.

By adding manure on soil, nutrients (e.g., carbon, nitrogen) were brought to soil as well as microorganisms and we could expect a higher microbial activity and possibly a higher biodegradation of antibiotics in manure-amended soils compared to non-amended soils (Accinelli et al., 2007). The manure addition could also increase the microbial biomass in soils (Topp et al., 1996) and bring microorganisms potentially competent for biodegradation that were enriched in the animal and/or during manure storage (Jacobsen et al., 2005). In the same way, the application of triclocarban and triclosan on soils via liquid municipal biosolids or dewatered municipal biosolids led to the biodegradation of both compounds more rapidly and more slowly, respectively (Al-Rajab et al., 2009). The effect of manure on the mineralization rates of ^{14}C -SMZ and ^{14}C -ERY in STC and MC soils were relatively small but different according to the antibiotic. Indeed, the ^{14}C -SMZ biodegradation was more accelerated in manure-amended soils (9.7% at day 3) than in soil without manure (3.6% at day 3). For ^{14}C -ERY, manure had no significant effects on the acceleration of the biodegradation. The potentially-higher microbial biomass in manure-amended soil did not result in a more rapid co-metabolic transformation of the antibiotics (Topp et al., 1996). The available fraction was higher at day 0 in manure-amended soils and so was the mineralized fraction (slightly higher) in manure-amended soil than in soil without manure after 3 and also after 18 days of

incubation. However, this difference was only highlighted in control soils where mineralization was low. Otherwise, we could suppose that the ^{14}C -methyl of ERY antibiotic was so rapidly mineralized in high-rate treated soils (i.e., in less than seven days) that any attenuation or enhancement of biodegradation was difficult to detect when manure was added in soil. In conclusion, the manure addition did not change the mineralization rate of antibiotics in soils through their adsorption; this result comforted that the artificial field exposure scenario (i.e., antibiotic application in solutions at field) is not unrealistic.

VI.4. Conclusion

Sulfamethazine and erythromycin were two environmentally-available antibiotics in soils contrary to ciprofloxacin when soft extractions were performed. Consequently, in context of long-term exposure of soil microorganisms to antibiotics, accelerated biodegradation was evidenced for sulfamethazine and erythromycin in less than 7 days while ciprofloxacin was not biodegraded even after 21 days of incubation. Moreover in our experiments, the antibiotic biodegradation was shown to be specific to the antibiotic class and also to the antibiotic compound. Further work could be the selection of an antibiotic class for which there is a primed biodegradation capacity, and then to challenge it with a range of molecules in the same class that vary in sorption capacity (e.g., sulfonamides).

The application of manure containing sulfamethazine or erythromycin on soils did not show high difference in terms of environmental availability and biodegradation compared to soils directly receiving the antibiotics. This result comforted that the artificial exposure scenario of soils to antibiotics was not unrealistic.

In perspectives, we have to consider and study the environmental availability and the potential toxic effects of transformation products that could be more toxic than the parent compound. Furthermore, it is not excluded that transformation products could contribute to the selection pressure for antimicrobial resistances.

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Dans le Chapitre VI, les résultats ont mis en évidence que seuls les antibiotiques disponibles dans le sol ont pu être biodégradés de façon accélérée par les micro-organismes. L'adaptation des micro-organismes du sol aux antibiotiques résulte d'une exposition à long terme, sur plusieurs années. Cependant, les antibiotiques peuvent aussi impacter les communautés et les fonctions microbiennes à plus court terme, d'autant plus si les antibiotiques sont initialement disponibles dans les sols après l'apport de MAFOR.

Dans le cadre d'une collaboration avec le Pôle Ecotoxicologie de l'UMR ECOSYS, la méthode d'extraction a été appliquée pour estimer la disponibilité des antibiotiques au regard de leurs effets sur les communautés et les fonctions microbiennes associées. L'activité de nitrification représente un indicateur potentiel de l'exposition des micro-organismes aux antibiotiques dans le sol (biodisponibilité toxicologique). L'objectif majeur traité dans ce chapitre est de déterminer si les molécules extraites par les solutions aqueuses sont représentatives de la biodisponibilité toxicologique des antibiotiques dans les sols amendés par les MAFOR.

Dans le Chapitre VII, les fractions disponibles de la ^{14}C -ciprofloxacine et du ^{14}C -sulfaméthoxazole (présentées dans les Chapitres II et III) ont été mises au regard des effets sur l'activité nitrifiante, selon un gradient de concentrations en antibiotiques, afin de répondre aux questions suivantes :

- 1) L'adsorption de l'antibiotique peut-elle jouer sur sa toxicité ?
- 2) La diminution de la disponibilité de l'antibiotique au cours du temps entraîne-t-elle une diminution de la toxicité ?

Dans un second temps, le sulfaméthoxazole a été sélectionné pour établir des relations dose-réponse de l'activité de nitrification en fonction des concentrations en sulfaméthoxazole mesurées dans les sols amendés (présentées dans le Chapitre IV) par différents extractants (CaCl_2 , EDTA, cyclodextrine ou solvant), afin de répondre à la question suivante :

- 3) Quelles fractions de molécules extraites sont réellement biodisponibles pour les communautés nitrifiantes ?

Partie 2. Estimation de la biodisponibilité

Approche analytique : SPE-UHPLC-MS-MS

Chapitre VII

Sols amendés par les MAFOR
**Antibiotiques + produits
de transformation**
Adsorption dans des matrices
complexes (organique + minéral)



Extractions aqueuses



(Bio)disponibilité de la ciprofloxacine et du sulfaméthoxazole



Toxicité des antibiotiques sur l'activité des communautés
nitrifiantes du sol



- 1) Antibiotique moins toxique si adsorbé?
 - 2) Diminution de la disponibilité :
diminution de l'effet toxique?
 - 3) Quelles fractions de molécules extraites sont réellement
biodisponibles dans le sol?
- } Fractions disponibles des ¹⁴C-antibiotiques (Chapitres II et III)
- } Fractions disponibles du ¹²C-sulfaméthoxazole (Chapitre IV)

Chapter VII: Environmental availability of sulfamethoxazole and ciprofloxacin and effects on soil N-cycle microbial processes in agricultural soils

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Keywords

Antibiotics; bioavailability; aqueous extraction; dose-response; nitrification

VII.1. Introduction

Human and veterinary antibiotics can be excreted as much as 30–90% of the administrated doses, as parent compounds or metabolites in urine and feces. These residues of antibiotics can be present in sewage sludge and manure, at levels up to several mg kg⁻¹ (Zhang and Li, 2011; Song and Guo, 2014). The agricultural recycling of exogenous organic matter (EOM; e.g., manure, sludge compost) as fertilizers and/or amendments in cropped soils has been identified as a main pathway for introducing antibiotic residues in soils and disseminating antibiotic resistant bacteria and genes, with potential effects on the soil microbial communities and functions (Golet et al., 2003; Sarmah et al. 2006; Kemper, 2008; Brandt et al., 2015). Consequently, there is a growing concern about the potential risks resulting from exposure to antibiotic residues in soils. Many investigations have reported the contamination of agricultural soils by antibiotics at concentrations ranging from few µg to several hundred µg kg⁻¹ soil. In long-term amended soils, tetracyclines were the most abundant (from 100-500 µg kg⁻¹ up to several mg kg⁻¹ dry soil; Hamscher et al., 2005) while sulfonamides and fluoroquinolones were present at lower concentrations (10-100 µg kg⁻¹ soil; Kemper, 2008; Song et al., 2010; Ji et al., 2012; DeVries and Zhang, 2016). Usually, the concentration levels of sulfonamides or fluoroquinolones residues do not exceed the recommended trigger value of 100 µg kg⁻¹ soil in amended cropping soils with considerations based on predicted environmental concentrations (PEC; EMEA, 2008). Sulfamethoxazole and ciprofloxacin antibiotics are among the sulfonamides and fluoroquinolones the most frequently quantified in EOM and soils (Baran et al., 2011; DeVries and Zhang, 2016).

In agroecosystems, soil microbial communities are the key players in biogeochemical cycles of carbon and nutrients through their control of organic matter mineralization, and therefore in the carbon fluxes and nutrient recycling (i.e., nitrogen or phosphorus) and availability for plants (Falkowski et al., 2008). Soil contamination could disturb feedbacks between microbial communities and ecosystem functions, owing to their exposure and sensitivity to the contaminants and their control of critical biogeochemical processes. Antibiotics can interact with soil microorganisms in various ways ranging from toxic effects (bacteriostatic or bactericide) limiting the microbial activities, up to microbial adaptation by exerting selective pressure, leading to the promotion of genetic resistances (Thiele-Bruhn and Beck, 2005; Schauss et al., 2009a; Heuer et al., 2011; Brandt et al., 2015). Several studies highlighted the toxic effects of different antibiotics on soil microbial communities, with activity decreases and changes in community composition (Thiele-Bruhn and Beck, 2005; Liu et al., 2009; Toth et al., 2011; Kotzerke et al., 2011a). Among microbial processes, the N-cycling related activities (e.g., nitrification) were more sensitive than broad scale processes provided by overall microbial communities (e.g., respiration, glucose mineralization) (Kotzerke et al., 2008; Toth et al., 2011; Hammesfahr et al., 2011a). However, only scarce studies experienced realistic total concentrations of antibiotics in soil (Toth et al., 2011; Rosendahl et al., 2012; Ollivier et al., 2013). Most of studies investigated the impacts of antibiotics directly applied on soil without EOM amendments and/or at irrelevant concentrations; they did not show any effect of antibiotics below concentrations of 10 mg kg⁻¹ dry soil (e.g., Kotzerke et al., 2008; Hammesfahr et al., 2008; Liu et al., 2009).

The effects of antibiotics on soil microorganisms, at relevant environmental concentrations, is essentially influenced by the metabolic activity of microorganisms, the time exposure and also the antibiotic bioavailability (Thiele-Bruhn, 2003; Schauss et al., 2009a; Jechalke et al., 2014). Nowadays, it has been established that the “total” concentrations of an organic contaminant in soil environment can be above concentrations at which the organic contaminant is available to interact with soil organisms and more particularly to affect microorganisms (Alexander, 2000; Lanno et al., 2004). The sorption – desorption kinetic of an organic compound in the soil governs the solid – liquid distribution (sequestration in soil matrix vs. molecules in the soil solution) and it influences in a large extend the extractability and the bioavailability of the contaminant (Hatzinger and Alexander, 1997; Pignatello, 2000; Lueking et al., 2000). Because of the high diversity of functional and ionizable groups in antibiotic molecules, the sorption of sulfonamides and fluoroquinolones in soils is governed by several mechanisms related to surface complexation (organic matter and clay), cation exchange or bridging interactions, polar and hydrophobic interactions (Tolls, 2001; Figueroa-Diva et al., 2010).

It is difficult to provide an interacting system in solid phase (i.e., bioassay) with endogenous microorganisms and contaminants, retaining the ability to measure both the toxicity and the exposure. In many cases, contaminants are first extracted from soils (pore water, aqueous or solvent-based extractions) before being brought into contact in solution with bioassays. Such works improve the understanding of the soil parameters influencing chemical availability of antibiotic residues (Schwarzenbach et al., 2003; Lin and Gan, 2011; Kong et al., 2012). Nevertheless, the current knowledge on the microbial bioavailability of antibiotics in soils is still limited. The antibiotic bioavailability could be not only limited to antibiotics in soil solution, since sorbed compounds on soil particles can still have antimicrobial effects probably as result of their bioavailability (Chander et al., 2005; Goetsch et al., 2012; Peng et al., 2014).

Consequently, soft extractions, allowing weakly sorbed molecules to be extracted in addition to those in soil solution, could be relevant as chemical indicator of the microbial exposure. The bioavailability of different organic contaminants (e.g., pesticides, PAHs) evidencing through their microbial biodegradation has been previously investigated by using extractions with CaCl_2 /methanol or 2-hydroxypropyl- β -cyclodextrin (HPCD) solutions (Barriuso et al., 2004; Harmsen et al., 2005; Semple et al., 2006). More recently, the CaCl_2 -based extraction was arbitrarily considered to explain the effects of antibiotics on soil microbial activity (Schauss et al., 2009b, Kotzerke et al., 2008, 2011a; Hammesfahr et al., 2008, 2011a, 2011b; Rosendahl et al., 2012). However, considering the diversity of mechanisms involved in antibiotic sorption in soils and the capacity of antibiotic soil-bound residues to keep an antimicrobial activity, the CaCl_2 -based extraction should not be so easily considered as a reference chemical method to estimate the available fractions for soil microorganisms.

The unrealistic exposure pathways (i.e., bioassays based on soil solution, soil microcosms without contamination via EOM application) has failed to improve our understanding in the relationships between microbial exposure and the dynamic of microbial responses, in connection with the behavior of antibiotic residues in soils at environmentally relevant concentrations. So far, understanding how the antibiotics released following EOM amendments in soils are available and affect the microbial communities in soils have been emerging as a critical question, regarding the growing interest for EOM recycling and its impact on the biogeochemical cycling of nutrients in agricultural soils. In addition, only very few studies have been addressed the challenge of estimating an actual bioavailability of antibiotics in soils which induces a microbial response (toxicity or resistance). Yet it is crucial to establish thresholds for safety contamination levels, related to human and environmental health issues (antibiotic resistance, disrupting biogeochemical cycles) by taking into account the antibiotic bioavailability.

The objective of this work was a comprehensive assessment of the antibiotic bioavailability inducing toxic effects on non-target soil microorganisms, by studying the relationships between chemical extraction (as a proxy of exposure) and effects on soil microorganisms. The present study had two requirements: 1) working at environmentally relevant concentrations; and 2) using EOM (sludge compost or farmyard manure) as vector for soil contamination by antibiotics. Two antibiotics (sulfamethoxazole, SMX and ciprofloxacin, CIP) were selected considering their frequent quantification in environmental matrices and their highly-different physicochemical properties. The potential nitrification activity was selected as the ecotoxicological endpoint.

Previously, we assessed the environmental availability of CIP (Chapter II) and SMX (Chapters III and IV) with several soft extractions. In the present study, we confronted the environmental availability of both antibiotics with microbial responses supposing that:

i) the adsorption of antibiotic could reduce its availability and consequently its toxicity for soil microbial activity;

ii) the initial toxicity of antibiotics could decrease over the incubation time thanks to the decrease of antibiotic availability related to their adsorption on EOM-amended soils;

iii) the antibiotic molecules dissolved in soil solution and easily-desorbed from soils could be bioavailable for soil microorganisms.

Consequently, two different incubation experiments were performed with manure or compost-amended soils to test the hypotheses. The first one (Part A) was carried out to compare SMX and CIP impacts of potential nitrification activity over a one-month incubation (hypothesis i) and to see how the impacts evolve with regard to the antibiotic availability (hypothesis ii). The results of Chapters II and III (^{14}C -antibiotics) were used for this Part A.

Then, the second incubation (Part B) was conducted to test the third hypothesis, by establishing dose-response relationships between antibiotic impacts and concentrations of extracted antibiotic from soil samples. The antibiotic impacts were assessed following a concentration gradient, in the short term (7 days following the soil amendment) and in longer term (28 days and 84 days following the soil amendment). The antibiotic concentrations were measured after aqueous or solvent-based extraction in order to obtain a gradient of antibiotic extractability with respect to the extraction mechanisms of solutions engaged. The antibiotic quantification in extracts was carried out by online SPE-UHPLC-MS-MS (Chapter IV).

Part VII.A. Environmental availability of ¹⁴C-ciprofloxacin and ¹⁴C-sulfamethoxazole in soils amended with exogenous organic matter: impacts of ¹²C-antibiotics on microbial activities during one-month incubation

VII.A.2. Materials and methods

VII.A.2.1. Incubation experiments

As described in Chapters II and III, the microcosms were composed of soil amended with exogenous organic matter (EOM; farmyard manure or compost of sewage sludge and green waste) at a dose equivalent to the field rate application (Obriot et al., 2016). The antibiotic was applied on the EOM and a contact time of 30-60 min was let between the spiking step and the step of EOM incorporation into soil by thoroughly mixing with an Inox[®] spatula. Thereby, although the antibiotics did not undergo the EOM treatment processes, their transfer in soils was simulated by the EOM application as in field conditions. The final moisture content of each soil/EOM mixture was 80% of maximum water holding capacity (WHC), i.e., around 25% (g water/100g dry soil).

The nominal concentrations of SMX and CIP ([SMX] ×1 and [CIP] ×1, respectively) were established as mean environmental concentrations in various amended soils, from literature data (e.g., DeVries and Zhang, 2016). The [SMX] ×1 and [CIP] ×1 concentrations were equal to 22 µg SMX and 146 µg CIP kg⁻¹ dry soil, respectively.

For chemical determinations, the moist soil and moist EOM weights were equivalent to 5 g and 0.15 g dry matter, respectively. Briefly, the EOM was contaminated by 100 µL solution in Milli-Q water (Millipore, France) of ¹⁴C-ciprofloxacin (¹⁴C-CIP; 12 MBq L⁻¹) or ¹⁴C-sulfamethoxazole (¹⁴C-SMX; 24 MBq L⁻¹) to reach final concentrations of 146 µg ¹⁴C-CIP and 44 µg ¹⁴C-SMX kg⁻¹ dry soil, respectively. Each treatment was prepared in triplicates (n = 3). It is worth noting that the SMX concentration was doubled ([SMX] ×2) in this incubation experiment with the aim of detecting the transformation products within the incubation period (not discussed here).

For microbiological determinations, a similar incubation experiment was concomitantly carried out with 50 g of soil and 1.5 g of EOM. Here, the EOM was spiked at three different concentrations of unlabeled CIP or SMX (Sigma-Aldrich, Saint-Quentin Fallavier, France) in Milli-Q water, to reach final concentrations in soil/EOM mixtures of 22, 220 and 1100 µg kg⁻¹ dry soil ([SMX] ×1, [SMX] ×10, [SMX] ×50, respectively); and 146, 1460 and 7300 µg CIP kg⁻¹ dry soil ([CIP] ×1, [CIP] ×10, [CIP] ×50, respectively). In a control set the EOM was spiked with only

Milli-Q water. Each treatment was prepared in triplicates ($n = 3$) or in six replicates for the control set ($n = 6$).

For both incubation experiments, all microcosms in closed jars were placed at 28 °C in the dark for 28 days. During the incubation, the oxygen renewal in the jars was realized each week with their opening and the moisture was adjusted if necessary by weighing the microcosms. After 0, 7 and 28 days of incubation, different chemical and microbiological analyses were realized to assess the antibiotic availability and their potential effects on soil microorganisms.

VII.A.2.2. Chemical determinations

Referring to the Chapters II and III, the ^{14}C -activity distribution was followed over the incubation time by measuring the radioactivity in available fraction, solvent-extractable fraction, mineralized fraction and non-extractable fraction, after 0, 7 and 28 days.

Briefly, the soft extraction was performed to assess the environmental availability of antibiotics, by adding 15 mL of aqueous solution to the soil sample (6 g moist soil) and shaking the solid/liquid mixture overnight with an overhead shaker (~17 h, 15 rpm, Heidolph® Reax 20, Schwabach, Germany). Two used aqueous solutions were common for ^{14}C -CIP and ^{14}C -SMX: EDTA (0.1 M, pH7) and HPCD (0.05 M), and an additional aqueous solution was used for each antibiotic, CaCl_2 (0.01 M) for ^{14}C -SMX and Borax (0.2 M) for ^{14}C -CIP.

Then, a solvent-based extraction was performed on centrifugation pellets after the soft extraction. As described in Chapters II and III, this method consisted in extracting in several successive steps the ^{14}C -molecules with different acetonitrile/McIlvaine buffer mixtures (pH 9 or pH 2; Ferhi et al., 2016). Supernatants from extraction steps at pH 9 and pH 2 were mixed together and the solvent-extractable fraction corresponded to the fraction of antibiotic residues more adsorbed on soil/EOM mixtures.

The mineralization of ^{14}C -antibiotics was followed over the incubation period by measuring ^{14}C - CO_2 trapped in a NaOH solution (1 N, 6 mL). The non-extractable fractions and mass balances were determined by combusting the dried soil/EOM mixtures in the oxidizer (Biological Oxidizer, OX 700, Zinsser Analytic, Frankfurt, Germany). The ^{14}C - CO_2 produced during the combustion was trapped in Oxysolve 400 (Zinsser Analytic).

The ^{14}C -activity was measured by liquid scintillation counting (Tri-Carb 2100 TR, Packard Instruments, Meriden, CT, USA) in extracts (1 mL) and NaOH solution (1 mL) diluted in Ultima Gold XR scintillation cocktail (Perkin Elmer, Waltham, MA, USA) and in Oxysolve. All counting results were expressed as % initial ^{14}C -activity.

VII.A.2.3. Microbiological determinations

The effects on soil microbial activity were assessed on the potential nitrification activity (PNA), in all microcosms with unlabeled SMX or CIP, after 7, and 28 days of incubation. PNA was measured in fresh soil samples, using a modified version of ISO 15685 (2012). For each sample, moist soil (3 g equivalent dry soil) was incubated in 50 mL Falcon® tubes with 30 mL of 0.6 mM N-NH₄⁺ solution (diammonium sulfate salt diluted in distilled water). Three analytical replicates were analyzed for each experimental sample. Chlorate inhibition method was not used to avoid possible side mixture effect with antibiotics. The soil slurries were aerobically incubated in an orbital shaker (150 rpm; 25 °C). After 2 h, 24 h and 48 h of incubation, 1 mL aliquot was sampled in tubes and transferred into Eppendorf tubes, centrifuged for 5 min at 13000 × g and at 4°C to stop ammonia oxidation. Then, the supernatant was stored in microtiter 96 deep-well plates, at -20 °C until analyses of NO₃⁻ and NO₂⁻ productions into a microtiter plate by Griess reaction (0.5 M HCl; 1 g L⁻¹ vanadium chloride; 2.5 g L⁻¹ sulfanilamide; and 0.25 g L⁻¹ N-(1-naphthyl)-ethylenediamine dihydrochloride; Sigma-Aldrich; incubated 1 h 30 min at 60°C). The absorbance (λ = 530 nm) was measured using a microtiter plate reader (Xenius, SAFAS, Monaco). Potential nitrification activity (PNA) was calculated as the production rate of N-NO₃⁻ + N-NO₂⁻ between 2 h and 24 h following the equation Eq. (1):

$$PNA = \frac{[NO_3^-]_{final} - [NO_3^-]_{initial}}{T_{final} - T_{initial}} \times V_s \div W \quad (1),$$

where V_s is the volume of solution, W is the weight of dry soil and T the time of incubation.

VII.A.2.4. Statistical analyses

Statistical analyses were realized with RStudio software (version 0.98.501, RStudio Inc.) to compare the ¹⁴C-fractions during the incubation and also to compare the microbial activities. Variations in the extractable fractions of ¹⁴C-antibiotics (% initial ¹⁴C-activity) were tested between different incubation times. The effects on nitrification activities were tested by comparing the control and the treatments at a given incubation time. This was done by using linear mixed-effects models (ANOVA, p < 0.05) and post-hoc pairwise comparisons. In addition, for a given antibiotic dose, the magnitudes of effects on PNA between different incubation times were compared with a Wilcoxon-test (p < 0.05).

VII.A.3. Results

VII.A.3.1. Fate of ^{14}C -SMX and ^{14}C -CIP in soil/EOM mixtures

According to the results of incubation experiments with radiolabeled antibiotics, described in Chapters II (^{14}C -CIP) and III (^{14}C -SMX), the available fractions of both antibiotics were highly contrasted in particular at day 0 (Figure VII.1).

Briefly, the available fraction of ^{14}C -SMX depended on the soft extractant (CaCl_2 , EDTA or HPCD); it decreased from 31-60% at day 0 to 10-32% at day 7 and to 7-32% at day 28 in soil/compost mixtures (Figure VII.1). In soil/manure mixtures, the available fraction of ^{14}C -SMX decreased in the same way, even if it was 1.4-1.8 times higher than in soil/compost mixtures at day 7, depending on the soft extractant.

Contrary to ^{14}C -SMX, the available fraction of ^{14}C -CIP was initially very low, i.e., 0.7-6.3% at day 0 in soil/compost mixtures and 2.0-7.2% in soil/manure mixtures (Figure VII.1). The EDTA-based extractability of ^{14}C -CIP remained stable over the incubation time ($p > 0.05$), while the HPCD- and Borax-based extractabilities of ^{14}C -CIP slightly decreased over the incubation in soil/compost mixtures (0.3-1.0% at day 28). For both soft extractants, the ^{14}C -CIP availability increased at day 7 in soil/manure mixtures to reach 10-16%. The increase of available CIP in soil/manure mixtures between 0 and 7 days of incubation could be due to the biodegradation of manure organic matter, leading to the CIP release in soil/manure mixture (Chapter II). The CaCl_2 -based extraction was not performed to avoid the fluoroquinolone sorption by cationic complexation (i.e., Ca^{2+} ; Carrasquillo et al., 2008) that could alter its extraction. However, a soft extraction performed with KCl led to 0.5% and 1.7% ^{14}C -CIP availability in soil/compost and soil/manure mixtures, respectively, after 24 h of contact between the fluoroquinolone and the EOM (Chapter II).

The pH measured (in water) in soil microcosms was globally stable over the incubation time: 6.5-6.9 in soil/compost mixtures and 7.5-7.6 in soil/manure mixtures. The pH values measured in aqueous extracts were consistent with the pH measured in soil/EOM mixtures, i.e., slightly higher in extracts from soil/manure than from soil/compost mixtures (Chapters II and III). Consequently, we assumed that the evolution of the available fractions within the incubation time was not due to an evolution of microcosm pH.

Concerning the solvent-based extraction, the sum of available and solvent-extractable fractions was higher for ^{14}C -SMX than for ^{14}C -CIP (Table VII.S1), whatever the soft extractant previously used (EDTA and HPCD as shared extractants). Indeed, ^{14}C -SMX was globally 2 times

more extractable than ^{14}C -CIP in soil/compost mixtures throughout the incubation, and in soil/manure mixtures at day 0, with both HPCD and EDTA extracting solutions.

The non-extractable fraction was already high at day 0 in soil/EOM mixtures incubated with ^{14}C -CIP (64-67%, Table VII.S1). Non-extractable ^{14}C -SMX residues represented only 12-15% in both soil/EOM mixtures at day 0. The non-extractable fraction of ^{14}C -SMX increased from 12-29% to 36-62% after 7 days of incubation, according to the decrease of the cumulative fraction. Mass balances reached 81 to 103% of initially-applied ^{14}C -activity for both antibiotics. Finally, 2% of ^{14}C -SMX was mineralized after 28 days of incubation against 0.3% for ^{14}C -CIP (Table VII.S1). Even if the mineralization rate was low for SMX, we assumed that the sulfonamide was more available than CIP for microbial degradation.

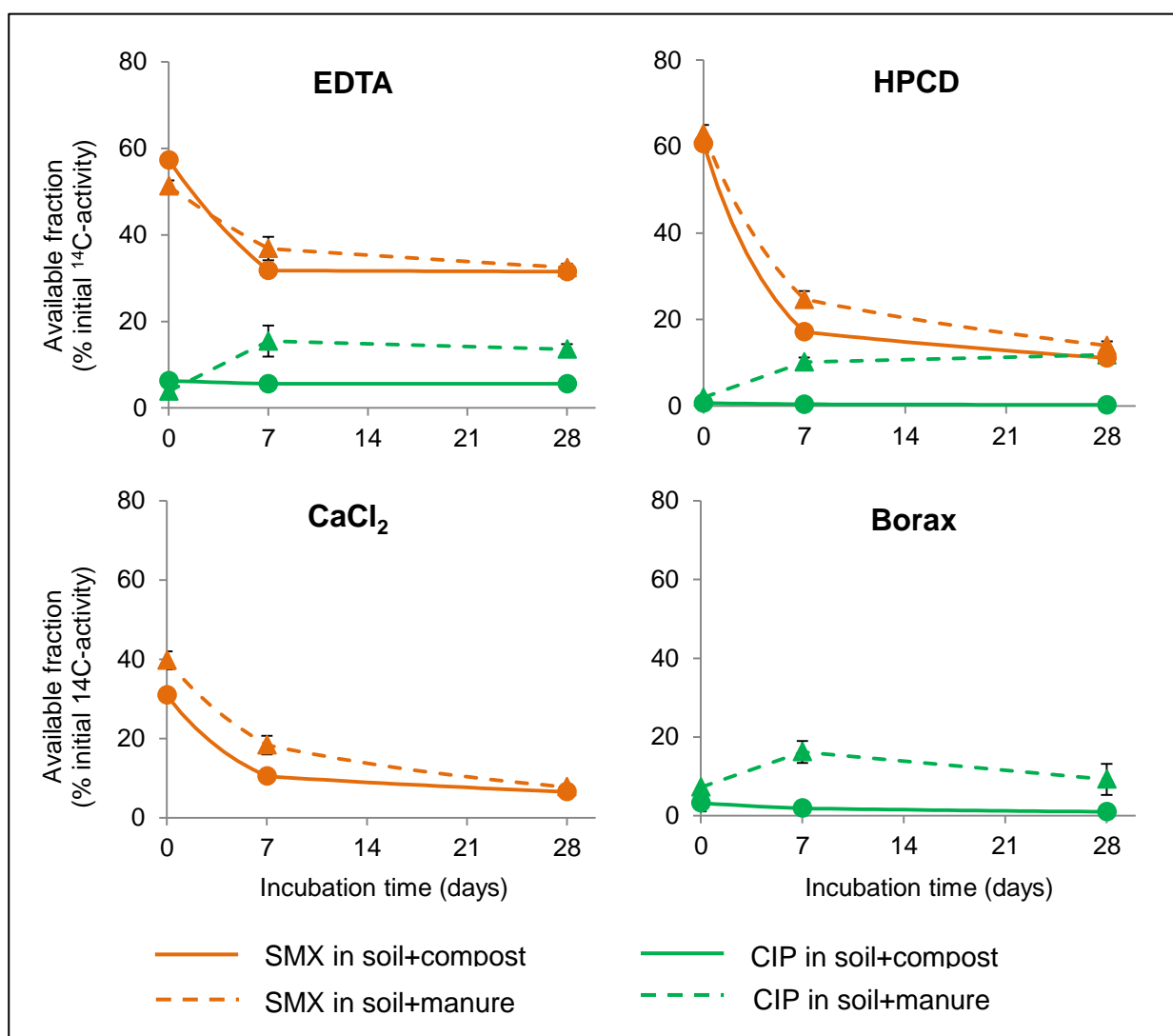


Figure VII.1. Available fractions of ^{14}C -SMX ($44 \mu\text{g kg}^{-1}$ dry soil) and ^{14}C -CIP ($146 \mu\text{g kg}^{-1}$ dry soil) in soil/compost (solid line) and soil/manure mixtures (dashed line) assessed by CaCl_2 -, EDTA-, Borax- or HPCD-based extraction. Data points and error bars represent the mean of three replicates \pm standard error. Solid and dashed lines represent hypothetic dissipation curves. CaCl_2 -based extraction was only performed for ^{14}C -SMX while the Borax-based extraction was only performed for ^{14}C -CIP.

VII.A.3.2. *Effects of SMX and CIP on potential nitrification activity (PNA)*

The high ammonium (NH_4^+) content in compost sludge (6.4 g kg^{-1} dry matter) was related to a high PNA in the corresponding soil/compost mixtures (PNA = 4.05 and $4.55 \mu\text{g N-NO}_3^- \text{ g}^{-1}$ dry soil h^{-1} at days 7 and 28, respectively, in control soil/compost mixture without antibiotic), while the lower NH_4^+ content in fresh manure (0.5 g kg^{-1} dry matter) lead to a very low PNA in the corresponding soil/manure mixtures (PNA = 0.21 and $0.93 \mu\text{g N-NO}_3^- \text{ g}^{-1}$ dry soil h^{-1} at days 7 and 28, respectively, in control soil/manure mixture without antibiotic).

In soil/compost mixtures (Figure VII.2A), CIP had no effect on PNA even at the highest concentration, while SMX significantly inhibited the PNA throughout the incubation period. Indeed, in soil/compost mixtures, at day 7, SMX induced a significant decrease in PNA by 20% and 41% compared to the control at 0.22 mg kg^{-1} ([SMX] $\times 10$) and 1.1 mg kg^{-1} dry soil ([SMX] $\times 50$), respectively (Figure VII.2A). The PNA inhibitions induced by SMX were lower at day 28, with 10% and 26% inhibition for [SMX] $\times 10$ and [SMX] $\times 50$ concentrations, respectively. The time related decreases in the magnitude of SMX effects between days 7 and 28 was not significant at $\alpha = 0.05$ (at SMX $\times 10$, $p = 0.082$; at SMX $\times 50$, $p = 0.065$, Wilcoxon-test).

In soil/manure mixtures (Figure VII.2B), both the CIP and SMX surprisingly induced an increase in PNA rates. SMX impacted PNA only at the highest concentration ([SMX] $\times 50 = 1.1 \text{ mg kg}^{-1}$) with a significant increase by 46%, at day 7 and 29% at day 28, compared to the manure control soils. Also, CIP induced a PNA increase at both the concentrations of 1.46 mg kg^{-1} ([CIP] $\times 10$) and 7.3 mg kg^{-1} dry soil ([CIP] $\times 50$) by + 42% and + 83% at day 7, respectively; and by + 24% and + 59% at day 28, respectively, compared to the manure control soils. The time related decrease in the magnitude of SMX or CIP effects for a given dose, between days 7 and 28 was not significant at $\alpha = 0.05$ (for [SMX] $\times 50$, $p = 0.109$, for [CIP] $\times 10$, $p = 0.109$ and for [CIP] $\times 50$, $p = 0.281$; Wilcoxon-test).

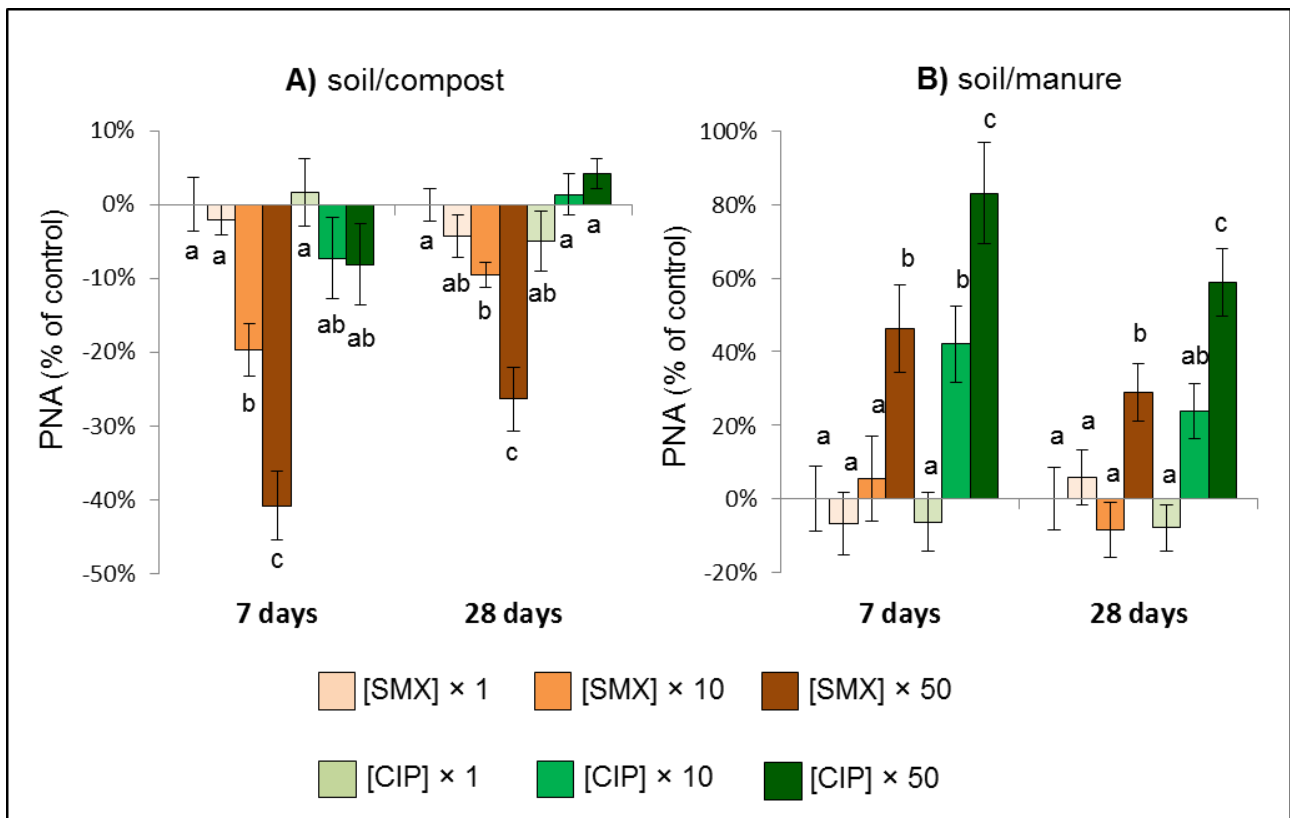


Figure VII.2. Response of potential nitrification activities (PNA) in microcosms containing: **A)** soil/compost mixtures; or **B)** soil/manure mixtures, contaminated by SMX and CIP, after 7 and 28 days of incubation. The concentrations [SMX] ×1, ×10 and ×50 corresponded to 0.022, 0.22 and 1.1 mg kg⁻¹ dry soil, respectively; and the concentrations [CIP] ×1, ×10 and ×50 corresponded to 0.15, 1.5, and 7.5 mg kg⁻¹ dry soil, respectively. Different letters indicated significant differences between treatments at a given incubation time (ANOVA, pairwise comparison, p < 0.05).

VII.A.4. Discussion

VII.A.4.1. Effects of SMX and CIP on soil microbial communities

Amendments with the sludge compost, rich in NH₄⁺, enhanced the soil nitrification activity, as previously observed for other organic amendments (Hammesfahr et al., 2011a; Cui et al., 2014) while the lower NH₄⁺ content in fresh manure did not. Ammonium ions are the substrate of nitrifying microorganisms. Theoretically, the CIP and the SMX antibiotic have a bacteriostatic action, inhibiting growth and multiplication of bacteria, but do not kill them. Therefore, in the environment, the bacteriostatic effect of the sulfonamides or fluoroquinolones on microbial communities strongly depends on the growth conditions of the microorganisms (active or in starvation) and then substrate availability (Backhaus et al., 2000; Thiele-Bruhn and Beck, 2005). Thus, in soil/compost mixtures, the growth promotion of nitrifying communities by this NH₄⁺ rich

amendment highlighted strong bacteriostatic effects of the SMX from 0.22 mg kg⁻¹ soil (Figure VII.2A). In the same soil/compost mixture, the absence of CIP effect on PNA was previously observed for other fluoroquinolone (difloxacin; Rosendahl et al., 2012; Kotzerke et al., 2011a) or for a β -lactam (amoxicillin, Kotzerke et al., 2011b).

However, the difference of NH₄⁺ availability only partially explains the different response of nitrification in the presence of SMX, between soil/compost and soil/manure mixtures. The strong PNA increase only in soil/manure mixtures treated with high level of CIP and SMX, is still an unexplained result: the SMX availability was closely similar in the two soil/EOM mixtures and the CIP availability was higher in soil/manure mixtures than in soil/compost mixtures. Some previous works also highlighted similar stimulations of nitrification and some other soil microbial activities in microcosm experiments by fluoroquinolones (Liu et al., 2009; Cui et al., 2014), sulfonamides (Hammesfahr et al., 2011a) or other antibiotics (Konopka et al., 2015). Such stimulations of the nitrification by sulfonamides were also observed in sewage sludge (Halling-Sørensen, 2001). Overall, the cause of such stimulations remained unexplained. Hormesis effect can be excluded here due to the magnitude of the observed effect since this classical ecotoxicological response to low doses of contaminant only slightly increases the cell activity (non-specific stress response). Also, the improvement of soil nitrogen cycling by N release from the degradation of antibiotic molecules, hypothesized by Konopka et al (2015), is unlikely in our experiment: the mineralization of the antibiotic is low (Table VII.S1) and the N input due to antibiotic is very small compared to the N content in soil/EOM mixtures. Actually, to explain these opposite responses between the two soil/EOM mixtures, one hypothesis could be that some archaea functionally redundant to nitrifying bacteria could take over nitrification in large proportions, inducing an indirect improvement of nitrification by antibiotics. Indeed, owing to their differences in organic matter and nutrient quality, the two EOMs differently modified the composition of soil microbial communities. Manure amendment can promote some archaeal populations that are in soils and insensitive to these antibiotics (Schauss et al., 2009b; Konopka et al., 2015). However, the contribution of nitrifying archaea to soil nitrification process is still discussed in neutral soils (Prosser and Nicol, 2012) and such an indirect effect would likely only counteract the negative effect on bacterial nitrifiers, resulting in few or weak effects on soil nitrification, and none such as strong increases.

VII.A.4.2. SMX and CIP bioavailability for soil microbial communities

According to the available fractions measured by soft extractions in a concomitant experiment (section VII.A.3.1, Figure VII.1), the effects of the antibiotics on PNA (stimulation or

inhibition) occurred only when antibiotics were chemically available in soil/EOM mixtures. As examples, ^{14}C -SMX was available (31-63%) whatever the soft extractant used (CaCl_2 , EDTA or HPCD) and the SMX availability strongly decreased after 7 days in both soil/EOM mixtures. The available fraction was 1.4-1.8 times higher in soil/manure than in soil/compost mixtures at day 7, depending on the soft extractant used. The dissipation rate can indeed depend on the soil properties and the sorption affinity of the antibiotic to soils (Kodešová et al., 2016). The decrease of ^{14}C -SMX extractability by the CaCl_2 solution was consistent with the decrease of sulfadiazine extractability, another sulfonamide, in manure-amended soils (Rosendahl et al., 2011), and also with the strong decrease of SMX extractability in amended soils even after solvent-based extraction (Höltge and Kreuzig, 2007). In parallel to the SMX availability, PNA was affected in both soil/EOM mixtures, from 0.22 mg SMX kg^{-1} soil. Concerning the fluoroquinolone, ^{14}C -CIP was chemically available mainly in soil/manure mixtures (10-18% of the initial amount at day 7 compared to less than 5% in compost/soil mixtures) where PNA was disturbed from 1.46 mg CIP kg^{-1} soil.

In soil/compost mixtures, only SMX exerted antibacterial potency on the nitrification while CIP did not have any effect. Similar results have been previously reported by comparing microbial impacts of sulfonamides to those of other antibiotics exhibiting strong sorption in soil, i.e., fluoroquinolones or tetracyclines (Thiele-Bruhn and Beck 2005; Kotzerke et al., 2008, 2011a; Rosendahl et al., 2012; Liu et al., 2011). Sulfonamides and fluoroquinolones had different mechanisms and strength of sorption on soil: this resulted in a weak to moderate sorption of SMX compared to the stronger sorption of CIP and consequently in different environmental availabilities (Figure VII.1).

From these results, among all extractants, the HPCD extraction could be the most suitable to estimate the CIP bioavailability and also the SMX one (as well as CaCl_2) in soil/compost mixtures. In opposite the solvent-based extraction was the least adapted to assess the antibiotic bioavailability since 23-33% of initial ^{14}C -CIP was extracted in soil/compost mixtures (Table VII.S1), without any effect on microbial activity over the incubation time.

In addition, the decreases of the magnitude of SMX effects in soil/compost mixtures (at 0.22 and 2.2 mg kg^{-1} soil, $p < 0.1$) over the 7-28 day period highlighted a partial recovery of the soil nitrification. The tendency to a temporal decrease of the initial PNA impact was not supported by statistical differences in soil/manure mixtures, for CIP or SMX. Similar short-term impacts of sulfonamides or fluoroquinolones on various soil microbial activities, including nitrification, were previously observed (Thiele-Bruhn and Beck, 2005; Schauss et al., 2009b; Kotzerke et al., 2008; Liu et al., 2011). Actually, due to the bacteriostatic action, the recorded antibiotic effect on microbial activity 7 days after treatment could be mainly the outcome of the initial exposure from

day 0 to day 7, and it could be similar for the effect at day 28 as result of the exposure between days 7 and 28. Hence, the strong initial dissipation of available SMX until day 7 and then slowed until day 28 (Figure VII.1) contributed to the temporal attenuation of the effect on PNA from day 7 to day 28. Despite an important decrease in SMX environmental availability was not reported between day 7 and 28, the CaCl₂- and HPCD-based extractions showed a weak but significant decrease, unlike the EDTA solution that extracted the same amount of antibiotics within the same period. Therefore, CaCl₂ and HPCD solutions seemed to be the most suitable extractants to describe the PNA response, and then the toxicological bioavailability of SMX.

VII.A.5. Conclusion

SMX and CIP have different mechanisms of sorption on soil explaining a weak to moderate sorption for SMX and stronger sorption for CIP. This induced different environmental availabilities assessed by aqueous extractions. By using the different extraction mechanisms of CaCl₂, EDTA, Borax or cyclodextrin solutions, the available fractions were highly contrasted between CIP and SMX. In parallel, the results of the antimicrobial effects on potential nitrification activities (PNA) markedly differed between the two antibiotics and the two soil/EOM mixtures. The environmentally-available SMX inhibited the PNA in soil/compost mixtures while CIP was not available and had no effect on PNA. However, CIP was slightly available in soil/manure mixtures and stimulated the PNA (like SMX). These PNA stimulations by both antibiotics are still unexplained. Moreover, the time related decrease of the sulfamethoxazole availability in soil/EOM mixtures could result in an attenuation of PNA inhibition, over the incubation time.

From these first conclusions, we carried out a more detailed study relying on the antibiotic quantification in various extracts, by online SPE-UHPLC-MS-MS in parallel to the measure of effects on soil microbial communities, in order to select a soft extractant that would be the best to assess the antibiotic bioavailability for soil microorganisms (Part B).

Considering the contrasted behaviors between the two antibiotics, we selected sulfamethoxazole in a first place for several reasons: i) SMX was more easily extracted; ii) the analytical method was developed in online SPE-UHPLC-MS-MS (Chapter IV); and iii) its availability was more changing over time than ciprofloxacin (this Chapter, Part A). Concerning the soil amendments, both the sludge compost and manure were studied in the following incubation experiment performed over a longer period (84 days), but we decided to focus on the sludge compost for which dose-dependent inhibition effects were clearly evidenced in the first experiment

(Part A). Several microbial parameters were monitored (e.g., microbial biomass, extracellular enzyme activities, substrate-induced respiration, denitrification), but PNA, as the most sensitive to antibiotics, was selected as the microbial indicator to study the toxicological bioavailability of SMX.

Part VII.B. Dose-response relationships between extracted SMX fractions and impacts on microbial activity during a three-month incubation of compost-amended soils

VII.B.2. Materials and methods

VII.B.2.1. Incubation experiments

According to the protocol described in Chapter IV, control soil (equivalent to 170 g dry soil) was amended with compost of sewage sludge and green waste (equivalent to 5 g dry matter) to carry out a three-month incubation in glass jar microcosms. The EOM was spiked with the solution of SMX in Milli-Q water to obtain a gradient of concentrations from 0.022 mg kg⁻¹ ([SMX] ×1) up to 2.22 mg kg⁻¹ dry soil ([SMX] ×100). A control set was prepared by spiking EOM with only Milli-Q water. The final moisture of soil microcosms was equal to 80% of the maximum water holding capacity, i.e., 27% (g water/100 g dry soil). Four replicates were prepared for each treatment. All microcosms were incubated at 15 °C in the dark for 84 days. During the incubation, the oxygen renewal in the jars was realized each week with their opening and the moisture was adjusted if necessary by weighing the microcosms.

After 0, 7, 28 and 84 days of incubation, soil was sampled in each microcosm to realize different chemical and microbiological analyses, in particular to measure the SMX concentrations in aqueous and solvent-extractable fractions and to assess the impacts of SMX on soil microbial activities.

VII.B.2.2. Chemical analyses

VII.B.2.2.1. Soft extractions

As described in Chapter IV, three aqueous solutions composed of 0.01 M CaCl₂, 0.1 M EDTA (pH 7) and 0.05 M HPCD were used as soft extracting solutions to assess the environmental availability of SMX. The soft extraction was performed on 6-g fresh soil samples following the same protocol previously described in Part A. The extract aliquots of 1.5 mL were centrifuged (MiniSpin, 10 min at 12100 x g, Eppendorf, Hamburg, Germany) to ensure the absence of particles before the injection. CaCl₂ and EDTA extracts were directly injected in online SPE-UHPLC-MS-MS, while a QuEChERS purification was performed on HPCD extracts prior to injection. In order to correct matrix effects (CaCl₂ and EDTA extracts) and QuEChERS recoveries (HPCD extracts), an internal standard SMX-d4 (71 µg L⁻¹) was added to each extract aliquot (Chapter IV).

VII.B.2.2.2. Solvent-based extraction

The solvent-based extraction was carried out in order to measure the amount of SMX more strongly sorbed to soil/EOM mixtures and not immediately available. In Falcon[®] centrifuge tubes (50 mL), samples of 3-g wet soil were spiked with 200 µL internal SMX-d4 standard to reach the final concentration of 0.15 mg kg⁻¹ dry soil. According to Ferhi et al. (2016), the solvent-based extraction was based on two successive 15 min ultrasonic extractions, using first a 30/70 (v/v) mixture of McIlvaine buffer pH 9 / acetonitrile and then a 30/70 (v/v) mixture of McIlvaine buffer pH 2 containing 0.41 mol L⁻¹ Na₂EDTA / acetonitrile. Each extraction step was followed by a centrifugation (11000 x g for 10 min) and both supernatants were mixed in a new Falcon[®] tube (50 mL). QuEChERS salts (0.7 g of NaOAc, 1 g of Na₂EDTA, 0.28 g of Na₂SO₄) were added to the extract and the mixture was vortex shaken. After the centrifugation (11000 x g for 10 min), 500µL of the upper phase were placed in a HPLC vial and evaporated under nitrogen flow. The residual drop was dissolved in 500 µL of Milli-Q water by vortex shaking.

VII.B.2.4.3. Analytical conditions of online SPE-UHPLC-MS-MS

The analytical conditions were the same as previously described in Chapter IV. Briefly, the UHPLC device was composed of an Acquity UPLC system (Waters), an online SPE cartridge

(Oasis HLB, 2.1 x 20 mm, 25 µm particle size, Waters) and a BEH C₁₈ column (2.1 x 100 mm, 1.7 µm particle size, Waters). The mobile phase was composed of water + 0.01% TFA (A) and acetonitrile + 0.01% TFA (B) with a gradient profile starting at 100% A for 2.2 min and increasing to 100% B in 15 min, with a flow rate at 0.4 mL min⁻¹. The injected volume was set at 200 µL. The UHPLC effluent was introduced in the triple-quadrupole mass spectrometer (TQD, Waters, Milford, MA, USA). The quantification and confirmation transitions for SMX and the three transformation products were previously given in Chapter IV. Acquisition and treatment of data were respectively realized with MassLynx and QuanLynx software (Waters).

VII.B.2.3. Microbiological determinations

As previously described in Part A, the potential nitrification activity (PNA) was selected and measured in samples of soil microcosms as the most sensitive microbial indicator of the direct bacteriostatic effects of the SMX. Several other microbial indicators were assessed (activities and functional gene abundances), but they were not included in this study.

VII.B.2.4. Data analysis and statistics

Statistical analyses were realized as previously described in Part A, under the RStudio software (version 0.98.501, RStudio Inc.). Extraction efficiencies were tested between SMX doses at a given sampling date and between incubation times for a given dose, by using linear mixed-effect models (ANOVA, $p < 0.05$) and post-hoc pairwise comparisons. The effects of SMX on nitrification activities were tested by comparing each treatment to the control, at a given incubation time, by using the Dunnett test ($p < 0.05$), in order to determine the no-observed effect concentration (NOEC) and the low-observed effect concentration (LOEC). In addition, for a given dose of SMX, the magnitudes of effects on PNA were compared between different incubation times, with the Kruskal-Wallis test ($p < 0.05$).

To investigate SMX bioavailability for soil microorganisms, non-linear and linear relationships were tested between the SMX concentration or fraction (% initial SMX concentration) and the percentage of PNA inhibition at each sampling date. The SMX concentration or fraction was determined by soft extraction or solvent-based extraction. These relationships were compared to those performed with theoretical concentrations of initially added SMX.

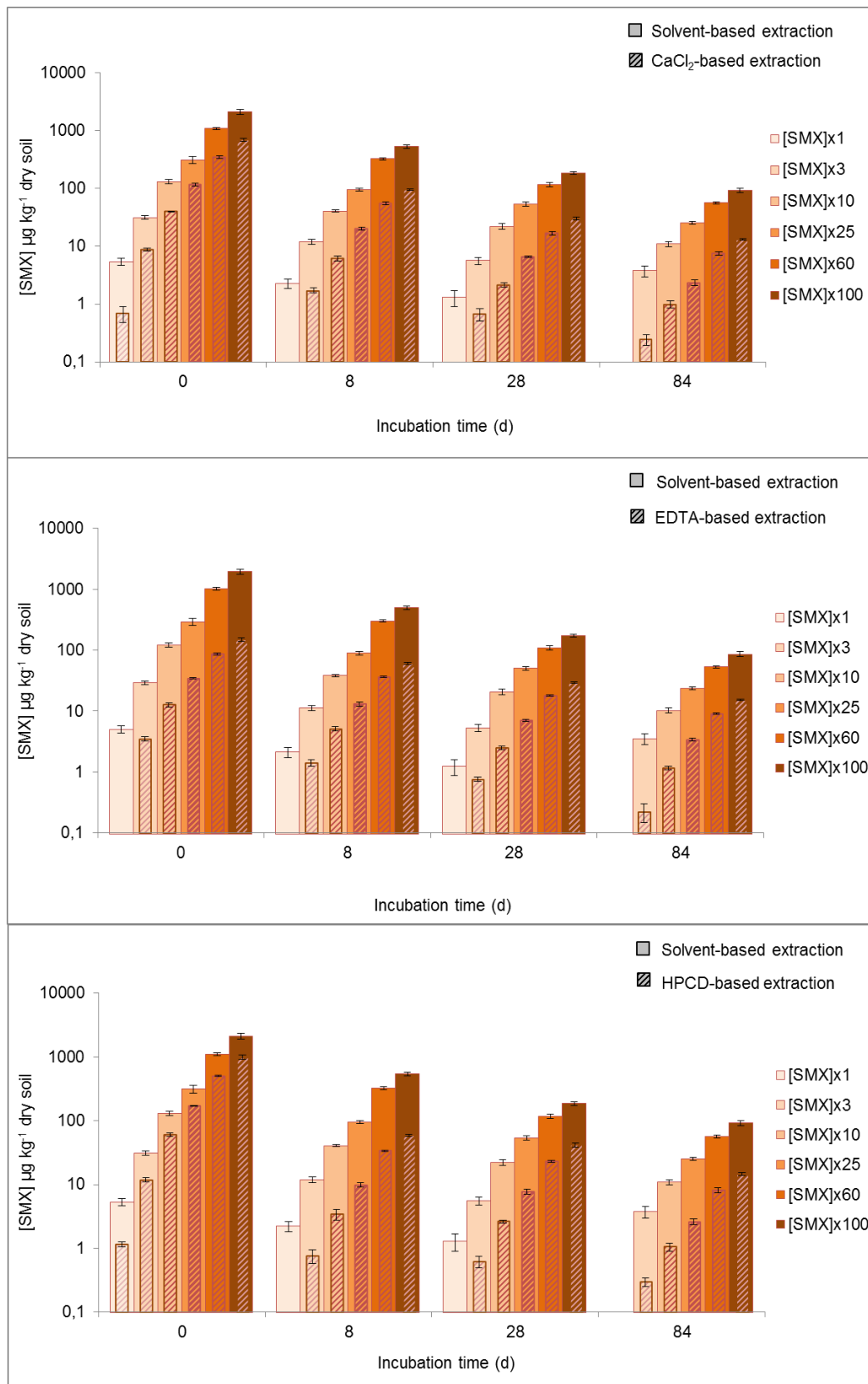


Figure VII.3. Concentrations of available and solvent-extractable SMX ($\mu\text{g kg}^{-1}$ dry soil, $n = 4$; \log_{10} scale) in soil/compost mixtures in function of the initial SMX concentrations and the incubation time. The available fraction of SMX was extracted by aqueous solution (CaCl_2 , EDTA or HPCD; striped squares) and the solvent-extractable fraction by acetonitrile/buffer mixtures (full squares). SMX was not detected at the lowest initial concentration at day 84. $[\text{SMX}] \times 1 = 22 \mu\text{g kg}^{-1}$ dry soil.

VII.B.3. Results

VII.B.3.1. SMX quantification by online SPE-UHPLC-MS-MS

At day 0, depending on the initial SMX concentration (from 22 to 2220 $\mu\text{g kg}^{-1}$ dry soil), the concentrations of available SMX ranged between 0.7 and 689 $\mu\text{g kg}^{-1}$ for CaCl_2 -based extraction; between 3.5 and 148 $\mu\text{g kg}^{-1}$ for EDTA-based extraction; and between 1.1 and 986 $\mu\text{g kg}^{-1}$ for HPCD-based extraction (Figure VII.3). As described in Chapter IV, the available fractions (% initial SMX concentration) initially varied between 3.0 and 42%. The soft extracting solutions extracted less and less available SMX over the incubation time and the SMX availability strongly decreased between 0 and 8 days. Indeed at day 8, the concentrations of available SMX ranged between 1.7 and 94 $\mu\text{g kg}^{-1}$ with the CaCl_2 -based extraction; between 1.4 and 61 $\mu\text{g kg}^{-1}$ with the EDTA-based extraction; and between 0.8 and 58 $\mu\text{g kg}^{-1}$ with the HPCD-based extraction. At day 8 and after, SMX was not anymore quantified in soil samples initially spiked with the lowest SMX concentration ($[\text{SMX}] \times 1 = 22 \mu\text{g kg}^{-1}$). The aqueous solutions extracted less and less SMX throughout the incubation since at day 84, the concentrations ranged between 0.2 and 15 $\mu\text{g kg}^{-1}$, depending on the initial SMX concentration.

For the lowest initial SMX concentration ($[\text{SMX}] \times 1$), the antibiotic was quantified at days 8 (2.3 $\mu\text{g kg}^{-1}$) and 28 (1.3 $\mu\text{g kg}^{-1}$), but not at day 84, by using the solvent-based extraction (Figure VII.3). The extraction recoveries reached 23-90% at day 0, depending on the initial SMX concentration, and decreased over the incubation time to reach 9.7-22.8% at day 8; 5.6-7.9% at day 28 and finally 4-5% at day 84. The decrease of the extraction recoveries was probably due to the rapid formation of non-extractable residues (Chapter III; Höltge and Kreuzig, 2007). Moreover, the extraction recoveries were highly influenced by the initial SMX dose and this, throughout the incubation time.

VII.B.3.2. Impacts of SMX on potential nitrification activity (PNA) in soil/compost mixtures

SMX impacted the soil microbial activity by inhibiting the PNA in soil/compost mixtures and the inhibition increased with the SMX concentration increasing (Figure VII.4). After 8 days of incubation, the lowest dose inducing a significant effect (LOEC) was 220 $\mu\text{g SMX kg}^{-1}$ ($[\text{SMX}] \times 10$), with a PNA inhibition of -37.3% compared to the control. The highest SMX concentration ($[\text{SMX}] \times 100$: 2200 $\mu\text{g SMX kg}^{-1}$) induced -75.5% of PNA inhibition. In the previous experiment (Part A), the equivalent SMX concentrations of $[\text{SMX}] \times 10$ less inhibited the PNA (-20%, see

section VII.A.3.2). We supposed that the different incubation conditions (e.g., temperature) could be responsible for the different magnitude of response. After 28 days, SMX always significantly inhibited the PNA from 220 $\mu\text{g SMX kg}^{-1}$ ($[\text{SMX}] \times 10$), with 29.6% of decrease compared to the control. After 84 days of incubation, the significant PNA inhibition by 10.5% for 220 $\mu\text{g SMX kg}^{-1}$ ($[\text{SMX}] \times 10$) was significantly lower than at day 8 (Figure VII.4; $p = 0.031$, Kruskal-Wallis test). A time related attenuation of the PNA inhibition by SMX at day 84 compared to days 8, was also observed for the intermediate $[\text{SMX}] \times 25$ and $\times 60$ concentrations (550 and 1320 $\mu\text{g SMX kg}^{-1}$) ($p = 0.031$, Kruskal-Wallis test); while the PNA inhibition (- 66,0%) by the highest SMX concentrations ($[\text{SMX}] \times 100 = 2200 \mu\text{g SMX kg}^{-1}$) did not significantly decrease from day 8 to day 84 ($p = 0.061$).

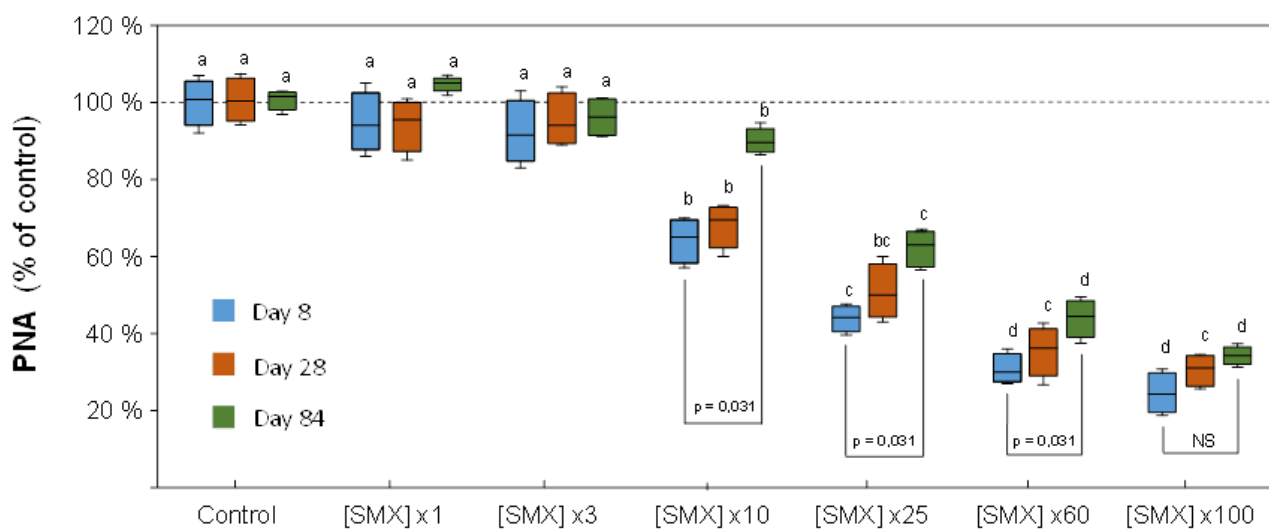


Figure VII.4. Effect of SMX on the potential nitrification activity (PNA) in function of the theoretical initial SMX concentrations, in the microcosms containing soil/compost mixtures, at days 8, 28 and 84. $[\text{SMX}] \times 1 = 0.022 \text{ mg kg}^{-1}$ dry soil. Different letters indicated statistical differences between SMX concentrations, for a given sampling date (ANOVA, pairwise comparisons, $p < 0.05$). Significant differences between sampling dates, at a given SMX concentration, were indicated (p-value, Kruskal-Wallis test).

VII.B.4. Discussion

VII.B.4.1. SMX toxicity on potential nitrification activity

Some ecotoxicological parameters can be deduced from a dose-response curve analysis, by using the theoretical concentrations and the initial available concentrations, at day 0 or day 8. The NOEC, i.e., the maximal SMX concentration without any significant effect on PNA, corresponded to the theoretical initial concentration of $66 \mu\text{g kg}^{-1}$ dry soil ($[\text{SMX}] \times 3$), for the three sampling dates of the incubation period. The corresponding SMX concentrations were 21 and $12 \mu\text{g kg}^{-1}$ dry soil the in solvent extraction at day 0 and 8, respectively.

The LOEC, i.e., the lowest concentration with significant effect was the nominal concentration of $220 \mu\text{g kg}^{-1}$ dry soil ($[\text{SMX}] \times 10$), corresponding to 89 and $40 \mu\text{g kg}^{-1}$ soil, in the organic extracts, at day 0 and 8, respectively. As previously described in Part A, these SMX concentrations inducing effects on nitrification were very low compared to the those in several studies on various soil microbial activities. Only Toth et al. (2011) found inhibitory concentrations of sulfonamides at similar low doses, while the most realistic studies where effects on nitrification were observed, had initial concentrations of sulfonamides (sulfadiazine) of at least $600 \mu\text{g kg}^{-1}$ to several mg kg^{-1} dry soil, working with contaminated pig manure (Hammesfahr et al., 2011b; Ollivier et al., 2013). Our results can be explained by the nutrient effect of the compost amendment that highly stimulated the nitrification in the soil experiment and thus, enhanced the bacteriostatic effect of the SMX.

The partial recovery of the microbial nitrification could be explained by the contribution of two concomitant processes: i) the observed SMX dissipation over the incubation time, in addition to ii) a shift in the composition of the microbial community with functionally redundant resistant microbial populations outcompeting the sensitive populations. Similar partial or total recoveries of microbial activities, after a short-term inhibition were already observed for higher doses of other sulfonamides (e.g. sulfadiazine) in amended soils (Part A, section VII.4). Actually, at the moderate SMX concentrations ($[\text{SMX}] \times 10, \times 25, \times 60$), the SMX dissipation over time mainly occurred by SMX adsorption on solid matrices, through the relatively rapid formation of non-extractable residues and to a lesser extent by degradation (Chapters III and IV). This prevented further antibiotic impacts by decreasing the exposure level and then by favoring the microbial recovery. The latter could be mediated by the emergence and selection of resistant microbial populations, functionally redundant of the sensitive such as the nitrifying archaea, outcompeting the sensitive

nitrifying bacteria, inside the nitrifying microbial community. Also, some nitrifying bacteria could acquire resistant genes to sulfonamides. At the highest SMX concentration, the initial impact on PNA or the SMX exposure remained too high to permit the functional recovery of the nitrifying microbial community.

The NOEC and LOEC as the initial SMX inputs in soils following the compost amendment were 66 and 220 $\mu\text{g SMX kg}^{-1}$ soil, respectively. These values encompassed the recommended trigger value of 100 $\mu\text{g kg}^{-1}$ soil defined on PEC-based consideration (EMEA, 2008). Otherwise, the environmentally relevant soil concentrations of SMX and other sulfonamides from the literature data are generally measured by using a solvent-based extraction with the aim at being the most exhaustive as possible. In this work, the solvent-extracted concentrations impacting the soil nitrification (at day 8: NOEC = 12 $\mu\text{g kg}^{-1}$ soil and LOEC = 40 $\mu\text{g kg}^{-1}$ soil) were below SMX concentrations quantified in other amended soils (DeVries and Zhang, 2016). Therefore, we could suppose that following the soil amendment with compost, soil antibiotic concentrations ranging from 12 to 40 $\mu\text{g kg}^{-1}$ soil might be at risk for soil microbial activities involved in nutrient cycling. Nevertheless, depending on the antibiotic dissipation, a recovery may occur in one month for the nutrient state and in three months for the activities.

VII.B.4.2. Selection of the chemical method suitable to assess the SMX bioavailability

First, we selected the SMX doses for which a linear regression could be tested regarding the dose-response curves generated with log-normal or log-logistic model (R software, example in Figure VII.5A), i.e., the concentrations for which SMX was quantified by our analytical approach. Consequently, at days 8 and 28, we selected measured concentrations in organic extracts for [SMX] $\times 1$ to $\times 100$; and in soft extracts for [SMX] $\times 3$ to $\times 100$. At day 84, we selected measured concentrations for both solvent and aqueous extracts for [SMX] $\times 3$ to $\times 100$.

Using the SMX concentrations measured in extracts ($\mu\text{g kg}^{-1}$ dry soil), the dose-response curves showed a threshold SMX concentration from which there was a PNA response (% inhibition; Figure VII.5B). A linear domain on the dose-response curves was obtained thanks to the log transformation of concentrations (Figure VII.5C). However, given that all measured concentrations were highly auto-correlated in such a dose-dependent experiment, all correlations made between PNA inhibition and the different measured concentrations were similar and strongly positive ($R^2 > 0.87$). Overall, it evidenced that the PNA inhibition increased with the SMX concentration, whatever the incubation time and the extractant used. In the same way, it highlighted that the

microbial exposure, and consequently the bioavailable fraction, would also increase with the SMX dose.

To overcome this effect of dose, we decided to consider the ratio between the SMX concentration measured in CaCl₂, EDTA, HPCD or organic extract and the initial theoretical SMX dose as the explaining variable of the PNA response (Figure VII.5D, Figure VII.6). In fact, this ratio represented the efficiency of each extractant to desorb SMX from the soil/compost mixtures. It was used in order to select the chemical extraction that could better describe the variability in PNA response in terms of toxicological bioavailability.

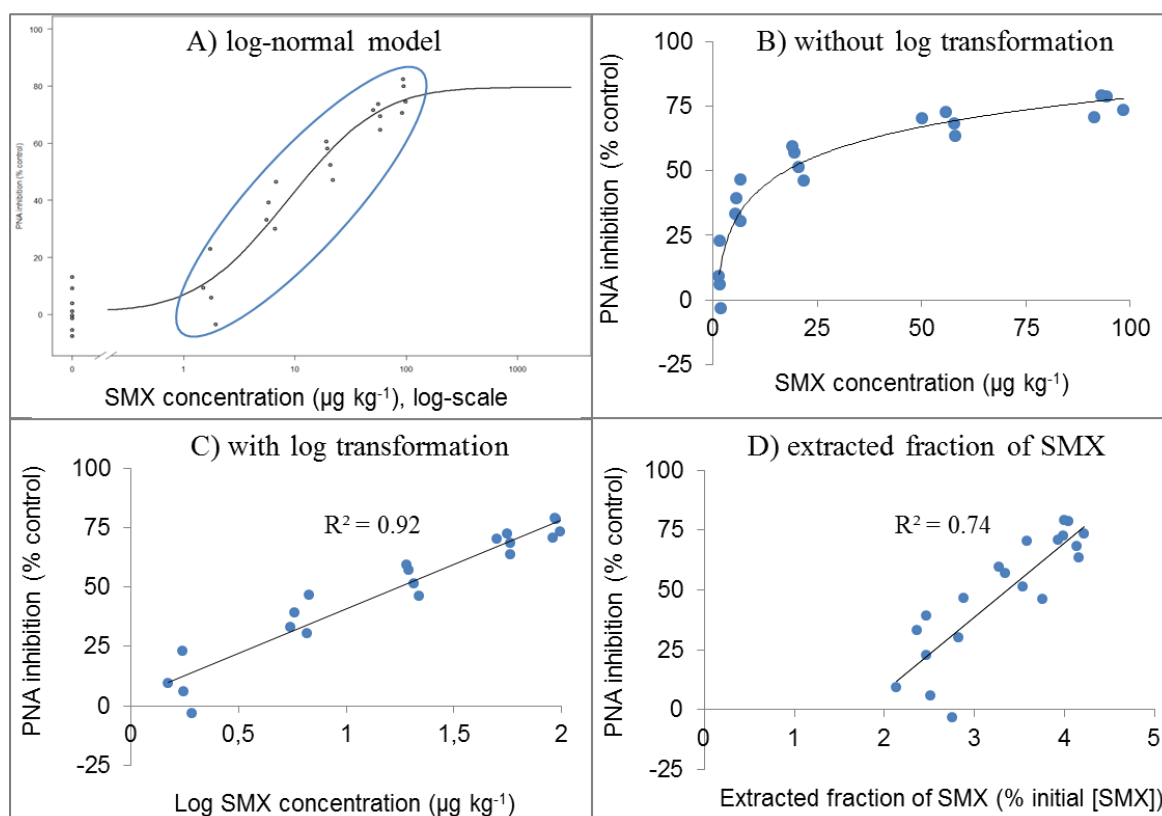


Figure VII.5. Dose-response curves of PNA inhibition (% control) in function of SMX concentration (in µg kg⁻¹ dry soil; in **A**, **B**, **C**) or SMX fraction (% the initial theoretical SMX concentration; in **D**). Logarithm transformation of SMX concentrations was used in **A** and **C**. The examples of dose-response curves given here correspond to the SMX quantification in CaCl₂ extracts after 8 days of incubation.

The regressions between the PNA response and the extracted fractions of SMX are presented in Figure VII.6. The intercept of the regression lines was not zero since the dose-response curves were not linear (as in example in Figure VII.5). At day 8, significant positive correlations were obtained for the four extractants ($R^2 \geq 0.6$, $p < 0.05$), with better correlations obtained with CaCl₂- and HPCD-based extractions ($R^2 = 0.74$ and 0.80 , respectively). The main feature of dose-response curves was respected since the impact of SMX on PNA increased with the measured SMX

concentration. This suggested a clear relationship between the extracted fractions of SMX and the SMX bioavailability in soil/EOM mixtures towards nitrifying microorganisms.

At day 28, significant positive correlations were obtained only for CaCl₂ and HPCD extractants ($R^2 = 0.79$ and 0.82 , respectively; $p < 0.05$). The PNA inhibition was not correlated to the SMX fractions measured in solvent and EDTA extracts. The lack of correlation for EDTA- and solvent-based extractions could be due the too “strong” extraction of SMX after 28 days at the low initial SMX doses ([SMX] ×1, ×3, ×10). In fact, both extraction mechanisms were not considered as specific for the extraction of available SMX, the solvent-based extraction being as exhaustive as possible, and the EDTA solution being less specific of free-dissolved and easily desorbed SMX extraction from soil samples than CaCl₂ and HPCD solutions (Chapters IV and V).

At day 84, there was no correlation between SMX fractions measured with solvent-based extraction and PNA inhibition. The impacts on PNA were still high while the measured SMX fractions were so low that the positive correlations were significant for the three soft extracting solutions ($R^2 = 0.57-0.79$, $p < 0.05$). The correlation with EDTA extractant was the highest but four points (corresponding to [SMX] ×3) were excluded from the linear domain of the regression line (Figure VII.6).

The soft extraction was a better chemical method than the solvent-based extraction that probably extracted a fraction of SMX more strongly adsorbed on soil/compost mixtures, compared to soft extracting solutions. In conclusion, the fraction of SMX desorbed with the aqueous solutions corresponded to the SMX molecules freely-dissolved in soil/compost solution and easily-desorbed from soil/compost mixture, with respect to the soft extraction based on the desorption equilibrium. Hence, this work highlighted that both the CaCl₂- and HPCD-based extractions could be retained as chemical method to assess the SMX bioavailability regarding nitrifying microorganisms in soils amended with compost. The correlations between the PNA inhibition and the available fraction of SMX were slightly better for HPCD- compared to CaCl₂-based extraction. Moreover, the HPCD-based extraction is currently included in a standardization procedure for the assessment of environmental availability of organic contaminants (ISO/DIS 16751, 2015). In some studies concerning the biodegradation of organic contaminants, the HPCD solution was used to assess their bioavailability for soil bacteria (e.g., pesticides, PAHs; Harmsen et al., 2005). Considering the structure of HPCD molecules, the encapsulation of organic contaminants (e.g., antibiotics) in the cavity via hydrophobic interactions could simulate the absorption of the contaminant through the membrane of bacteria cells.

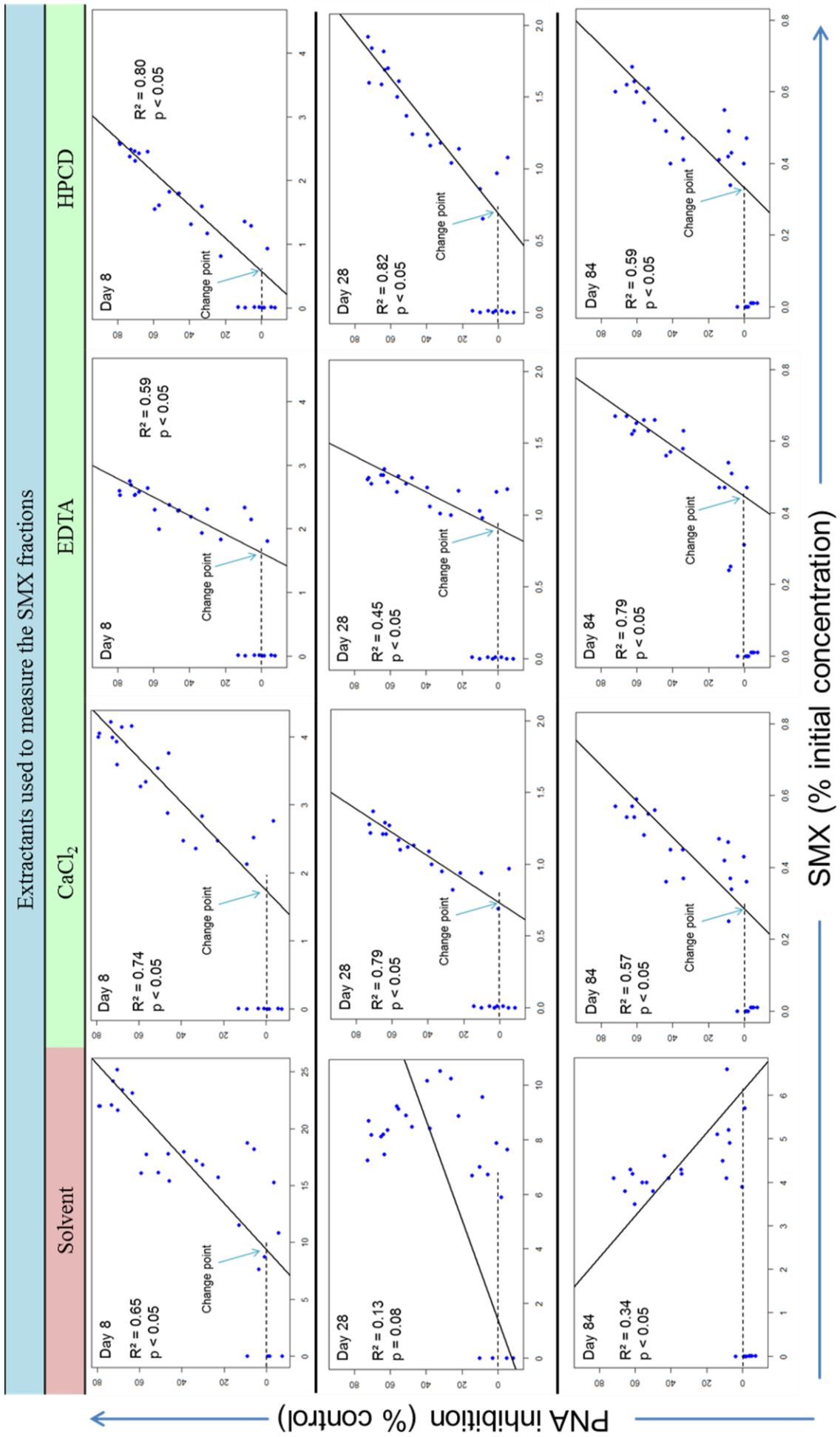


Figure VII.6. Linear regression between the inhibition of PNA (% control; y axis) and fractions of SMX (% initial concentration; x axis). Pearson correlation was tested and the p-value was < 0.05 for all regressions (except for solvent-based extraction at day 28, even after excluding the four points at zero).

VII.5. Conclusion

The microbial exposure to sulfamethoxazole in compost-amended soils was assessed by two indicators: the first indicator was biological, with the measure of the impact of sulfamethoxazole on potential nitrification activity (PNA); and the second one was chemical, with the measure of the antibiotic fraction by soft extraction or by solvent-based extraction. Considering the increase of PNA inhibition with the SMX concentration in compost-amended soils, we considered the extracted fraction of SMX assessed by different extraction mechanisms engaged by solutions of CaCl₂, EDTA, cyclodextrin solutions or organic solvent. Since the best correlations between PNA inhibition and SMX fraction were obtained when soft extractions were performed, we assumed that the soil microorganisms were exposed to SMX molecules both in soil solution and easily-desorbed from soils. Indeed, the soft extraction was based on a desorption equilibrium, contrary to the solvent-based extraction that was the most exhaustive as possible.

Among the three aqueous solutions, CaCl₂ and 2-hydroxypropyl- β -cyclodextrin (HPCD) solutions were retained to assess the antibiotic bioavailability with regard to the relationships between the extracted fractions of SMX and its impact on microbial activity in soils. In fact, the extraction mechanisms of these solutions could be more “specific” of soluble and easily-desorbed molecules in soils compared to the EDTA solution. Moreover, the solution of cyclodextrin could be preferred considering its potential biomimetic mechanism of extraction. Both aqueous solutions should further tested for the bioavailability assessment of antibiotics.

The potential nitrification activity could be used as a biological indicator of toxicological effects on short/medium-term (e.g., 7 and 28 days). For long-term effects of antibiotics (e.g., 84 days), another biological indicator should be selected, more representative of the potential adaptation of soil microorganisms (e.g., accelerated biodegradation, antibiotic resistance genes). Considering the impacts of antibiotics in soils following the soil amendment, our results suggested that environmentally relevant SMX concentrations, i.e., below 100 $\mu\text{g kg}^{-1}$ dry soil, could be at risk for soil nitrification, with a transient impact on the nitrogen mineralization, mainly in soil rich in ammonium. This may encompass the refining of information on predicted environmental concentrations (PEC) and predicted non-effect concentrations (PNEC) respectively related to the environmental fate and the effects of antibiotics with further considerations of their environmental availability.

Acknowledgments

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VII. Supplementary material

Table VII.S1

Distribution of ^{14}C -activity (% initial ^{14}C -activity) during the incubation of soil/compost and soil/manure mixtures with ^{14}C -SMX or ^{14}C -CIP (n=3), firstly extracted by aqueous solutions (EDTA, HPCD, CaCl₂ or Borax).

Incubation time (days)	Available fraction (EDTA)			Sum of available and solvent-extractable fraction			Non-extractable fraction			Mineralized fraction		
	SMX	CIP	% initial ^{14}C -activity	SMX	CIP	% initial ^{14}C -activity	SMX	CIP	% initial ^{14}C -activity	SMX	CIP	% initial ^{14}C -activity
Soil/compost mixtures	0	57.3 ± 1.5	6.3 ± 0.3	65.9 ± 2.4	32.8 ± 0.9	16.9 ± 0.3	64.2 ± 3.2	0	0	0	0	0
	7	31.8 ± 1.1	5.6 ± 0.5	53.9 ± 2.6	29.2 ± 2.7	35.9 ± 1.1	63.2 ± 5.3	0.58 ± 0.19	0.16 ± 0.01	0.58 ± 0.19	0.16 ± 0.01	0.16 ± 0.01
	28	31.5 ± 0.7	5.6 ± 0.7	54.3 ± 0.3	23.9 ± 0.7	38.7 ± 3.0	65.7 ± 0.8	2.05 ± 0.13	0.26 ± 0.04	2.05 ± 0.13	0.26 ± 0.04	0.26 ± 0.04
Soil/manure mixtures	0	51.3 ± 0.2	3.9 ± 0.2	62.0 ± 3.3	29.0 ± 0.7	28.5 ± 1.8	67.0 ± 1.9	0	0	0	0	0
	7	36.9 ± 2.7	15.4 ± 3.6	44.2 ± 0.9	34.5 ± 3.4	47.7 ± 2.4	56.8 ± 5.8	0.70 ± 0.02	0.19 ± 0.02	0.70 ± 0.02	0.19 ± 0.02	0.19 ± 0.02
	28	32.4 ± 0.9	13.6 ± 1.2	40.2 ± 0.7	28.8 ± 1.3	60.3 ± 1.6	61.6 ± 1.0	2.15 ± 0.04	0.35 ± 0.01	2.15 ± 0.04	0.35 ± 0.01	0.35 ± 0.01
Incubation time (days)	Available fraction (HPCD)			Sum of available and solvent-extractable fraction			Non-extractable fraction			Mineralized fraction		
	SMX	CIP	% initial ^{14}C -activity	SMX	CIP	% initial ^{14}C -activity	SMX	CIP	% initial ^{14}C -activity	SMX	CIP	% initial ^{14}C -activity
Soil/compost mixtures	0	60.7 ± 1.9	0.7 ± 0.01	74.3 ± 2.1	32.4 ± 1.2	14.6 ± 0.1	64.1 ± 3.0	0	0	0	0	0
	7	17.1 ± 0.3	0.4 ± 0.04	45.7 ± 3.4	26.4 ± 1.0	49.1 ± 1.9	68.6 ± 7.8	0.58 ± 0.19	0.16 ± 0.01	0.58 ± 0.19	0.16 ± 0.01	0.16 ± 0.01
	28	11.1 ± 0.3	0.3 ± 0.03	42.7 ± 2.2	22.7 ± 0.5	48.7 ± 1.9	69.8 ± 4.1	2.05 ± 0.13	0.26 ± 0.04	2.05 ± 0.13	0.26 ± 0.04	0.26 ± 0.04
Soil/manure mixtures	0	63.0 ± 2.0	2.0 ± 0.1	69.2 ± 0.7	30.6 ± 0.9	11.8 ± 0.3	64.3 ± 2.1	0	0	0	0	0
	7	24.7 ± 1.9	10.1 ± 1.1	38.2 ± 1.0	32.6 ± 1.0	55.6 ± 4.2	64.1 ± 0.8	0.70 ± 0.02	0.19 ± 0.02	0.70 ± 0.02	0.19 ± 0.02	0.19 ± 0.02
	28	14.0 ± 1.0	11.8 ± 1.8	29.7 ± 1.8	29.6 ± 2.1	53.9 ± 3.8	59.7 ± 3.4	2.15 ± 0.04	0.35 ± 0.01	2.15 ± 0.04	0.35 ± 0.01	0.35 ± 0.01

**Table VII.S1
(continued)**

	Incubation time	Available fraction	Sum of available and solvent-extractable fraction	Non-extractable fraction	Mineralized fraction
		(CaCl ₂)	SMX	SMX	SMX
% initial ¹⁴ C-activity					
Soil/compost mixtures	0	30.9 ± 1.3	71.9 ± 1.8	22.3 ± 1.3	0
	7	10.4 ± 0.5	49.9 ± 1.8	48.2 ± 2.7	0.58 ± 0.19
	28	6.5 ± 0.1	46.0 ± 0.32	50.5 ± 2.0	2.05 ± 0.13
Soil/manure mixtures	0	39.7 ± 2.3	63.1 ± 0.7	32.1 ± 3.2	0
	7	18.3 ± 2.4	41.1 ± 3.1	61.5 ± 1.6	0.70 ± 0.02
	28	7.7 ± 0.1	29.3 ± 1.3	61.8 ± 0.4	2.15 ± 0.04
% initial ¹⁴ C-activity					
	Incubation time	Available fraction	Sum of available and solvent-extractable fraction	Non-extractable fraction	Mineralized fraction
		(Borax)	CIP	CIP	CIP
% initial ¹⁴ C-activity					
Soil/compost mixtures	0	3.2 ± 0.3	35.4 ± 1.1	57.9 ± 2.5	0
	7	1.9 ± 0.8	28.2 ± 1.7	64.7 ± 51.5	0.16 ± 0.01
	28	1.0 ± 0.04	22.6 ± 2.8	69.2 ± 5.6	0.26 ± 0.04
Soil/manure mixtures	0	7.2 ± 0.3	38.3 ± 1.0	56.7 ± 2.2	0
	7	16.2 ± 2.8	38.2 ± 0.8	54.1 ± 0.5	0.19 ± 0.02
	28	9.2 ± 4.0	28.7 ± 2.9	62.1 ± 0.3	0.35 ± 0.01

VIII. Discussion générale et perspectives

Au cours de ce travail, des méthodes chimiques d'extractions douces avec des solutions aqueuses ont été développées afin d'estimer la disponibilité environnementale des antibiotiques, principalement le sulfaméthoxazole (sulfonamide) et la ciprofloxacine (fluoroquinolone), dans des sols le plus souvent amendés par des matières fertilisantes d'origine résiduaire (MAFOR). L'analyse bibliographique (Chapitre I) a permis de souligner que l'adsorption des antibiotiques sur les phases minérales et/ou organiques des MAFOR gouverne ensuite leur disponibilité dans les sols amendés et les effets sur les micro-organismes du sol, en lien avec leur biodisponibilité. Aucune méthode universelle n'existe pour estimer la biodisponibilité des contaminants dans les sols car celle-ci dépend aussi de l'organisme considéré dont les caractéristiques anatomiques et le comportement conditionnent son exposition. Ceci implique que, lorsqu'une méthode chimique est mise en œuvre, elle doit être complétée par des méthodes biologiques (bioaccumulation, toxicité, adaptations, biodgradation).

La démarche de ce travail a été de comprendre en premier lieu quels facteurs peuvent influencer la disponibilité environnementale (Lanno et al., 2004) des antibiotiques dans les sols lorsqu'elle est estimée avec des extractions douces. Les mécanismes d'adsorption des antibiotiques à la fois sur les MAFOR et les sols ont été pris en compte pour sélectionner des extractants aqueux le plus à même de mimer le passage des antibiotiques plus ou moins adsorbés vers la solution du sol. Dans la plus grande partie de ce travail, les antibiotiques ont été apportés sur les MAFOR avant de les incorporer dans le sol. Même si les antibiotiques apportés n'ont pas subi le traitement des MAFOR tel que le compostage, cette procédure permettait de respecter au mieux les conditions de contamination des sols au champ par les résidus d'antibiotiques (Boxall et al., 2012).

La suite de la démarche a été de mettre au regard des fractions d'antibiotiques obtenues par les extractions douces, dites fractions disponibles, des effets sur les micro-organismes du sol ; ceci revenait à analyser comment la méthode d'extraction permettait d'estimer la biodisponibilité des antibiotiques. Deux grands types d'effets ont été considérés, avec d'une part la biodégradation des antibiotiques par des micro-organismes pré-adaptés, et d'autre part, la toxicité des antibiotiques sur l'activité de communautés microbiennes du sol.

Cette étude visait donc dans son ensemble à proposer une méthode d'extraction douce pour estimer la biodisponibilité d'antibiotiques dans les sols amendés. Les différents résultats obtenus au cours de ce travail sont discutés ici en reprenant les différentes questions initialement posées au début de la thèse. Des perspectives sont aussi présentées.

1) Quelles sont les fractions d'antibiotiques extraites par les méthodes chimiques d'extractions douces en fonction des mécanismes d'adsorption des antibiotiques et des mécanismes d'extraction mis en jeu?

Les solutions de CaCl_2 , EDTA, Borax et cyclodextrine permettent d'extraire des fractions disponibles différentes suivant l'antibiotique considéré, ciprofloxacine ou sulfaméthoxazole, et suivant le type de MAFOR apportée, fumier de ferme ou compost de déchets verts et de boue de station d'épuration (Chapitres II et III). L'utilisation d'antibiotiques radiomarqués au ^{14}C permet de suivre l'évolution des fractions disponibles au cours du temps dans les sols amendés avec les MAFOR préalablement contaminées, et de relier cette évolution au devenir des antibiotiques dans les sols (biodégradation, formation de résidus de moins en moins extractibles). Les fractions disponibles extraites peuvent être expliquées à partir des mécanismes d'adsorption des deux antibiotiques sur les sols et les MAFOR et des mécanismes d'extraction des solutions aqueuses mis en jeu lors des extractions douces. Les sulfonamides et les fluoroquinolones sont pris en exemples dans les Figures VIII.1 et VIII.2.

La solution de CaCl_2 ($0,01 \text{ mol L}^{-1}$) permet d'extraire les molécules dissoutes dans la solution du sol ainsi qu'une fraction de molécules faiblement adsorbées sur les constituants de la matrice solide (mélange sol/MAFOR) par échange ionique. Les antibiotiques anioniques peuvent s'adsorber par attraction électrostatique au niveau des cations présents à la surface des phases organique et/ou minérale des matrices solides. Par échange ionique, le sulfaméthoxazole anionique, à un pH de 7, est désorbé au détriment de l'interaction avec les cations de la matrice solide, ce qui permet son extraction (Figure VIII.1). Cependant, la formation de complexes cationiques entre la charge négative de la ciprofloxacine (zwitterion à un pH de 7), les cations divalents Ca^{2+} et les charges négatives des surfaces solides peuvent limiter son extraction (Figure VIII.2). Pour cette raison, la composition des solutions salines peut être adaptée en remplaçant les sels de cations divalents par ceux de cations monovalents (par exemple, KCl) dans les équilibres de désorption des fluoroquinolones ou des tétracyclines qui peuvent aussi former des complexes cationiques (Ter Laak et al., 2006).

Les cations divalents présents à la surface des matrices solides sont chélatés par l'EDTA en solution ($0,1 \text{ mol L}^{-1}$, pH 7), ce qui permet de désorber les antibiotiques chargés négativement qui interagissent avec ces cations (Figures VIII.1 et VIII.2). De plus, la formation de complexes entre l'EDTA et la charge positive de la ciprofloxacine sous forme de zwitterion peut améliorer son extraction (Ferhi et al., 2016), et par compétition, l'EDTA peut limiter la complexation cationique des fluoroquinolones à la surface des matrices solides (Figure VIII.2). Une quantité importante de matière organique est co-extraite par la solution d'EDTA ; les molécules d'antibiotiques

potentiellement adsorbées sur ces matières organiques peuvent également être extraites. Compte tenu de la spéciation ionique des antibiotiques en fonction du pH et des mécanismes d'extraction de l'EDTA décrits précédemment, cette solution pourrait être utilisée pour cibler la fraction disponible d'un grand nombre d'antibiotiques dans les sols amendés par les MAFOR. Par exemple, la solution d'EDTA a permis d'estimer les fractions disponibles d'un autre sulfonamide, la sulfaméthazine, et d'un macrolide, l'érythromycine (Chapitre VI).

La solution de 2-hydroxypropyl- β -cyclodextrine (HPCD, 0.05 mol L⁻¹) et la solution de Borax (tétraborate de sodium ; 0,2 mol L⁻¹) permettent de solubiliser les antibiotiques par l'intermédiaire d'interactions hydrophobes avec les groupements hydrophobes à l'intérieur des molécules cages (HPCD) ou des micelles (Borax) (Figures VIII.1 et VIII.2). Toutefois, du fait de la taille et de la structure tridimensionnelle des molécules de HPCD, les antibiotiques dans les matrices solides doivent être assez facilement accessibles à la cyclodextrine pour permettre leur extraction. Cette condition a globalement été respectée pour l'extraction de sulfonamides tels que le sulfaméthoxazole (Chapitre III), mais par exemple, l'accessibilité de la ciprofloxacine a pu être très limitée dans le mélange sol/compost, ce qui a conduit à un faible niveau d'extraction de l'antibiotique par la solution de HPCD. Etant donné l'adsorption possible des fluoroquinolones sur les substances humiques (Aristilde et Sposito, 2010) et des tétracyclines en bordure des feuillets d'argiles (Aristilde et al., 2016), l'extraction des antibiotiques de ces deux familles par une solution de cyclodextrine doit être testée au préalable car de tels sites d'adsorption risquent d'être peu accessibles aux cyclodextrines. Quant à la solution de Borax, des niveaux d'extraction satisfaisants ont été obtenus pour plusieurs antibiotiques de familles différentes, la ciprofloxacine, la sulfaméthazine et l'érythromycine (Chapitres II et VI). Par son pH alcalin (pH 9), la solution de Borax permet de solubiliser davantage ces antibiotiques en jouant sur leur ionisation (Figures VIII.1 et VIII.2).

Sulfonamides : structure commune à pH 7		
<p style="text-align: center;">Interactions électrostatiques avec les charges positives des surfaces solides</p> <p style="text-align: center;">(1) ↑</p> <div style="display: flex; justify-content: space-around; align-items: center;"> <div style="text-align: center;"> <p>Groupement R : Impliqués dans l'adsorption, interactions hydrophobes et/ou interactions polaires</p> <p>(2) ←</p> </div> <div style="text-align: center;"> <p>(2)</p> </div> <div style="text-align: center;"> <p>Liaisons covalentes avec la matière organique</p> <p>← (2)</p> </div> </div> <p style="text-align: center; border: 1px solid black; padding: 2px;">Mécanisme majoritaire d'adsorption</p>		
Solution d'extraction	Mécanisme d'extraction	Type d'interactions sur lesquelles joue l'extraction
CaCl ₂ (0,01 mol L ⁻¹ pH 4,5)	Désorption, échange ionique	Interactions électrostatiques (1)
EDTA (0,1 mol L ⁻¹ pH 7)	Chélation de cations	Interactions électrostatiques (1) : extraction des molécules initialement associées aux cations de la surface solide
HPCD (0,05 mol L ⁻¹ pH 7)	Solubilisation	Solubilisation dans les molécules cages par l'intermédiaire d'interactions hydrophobes (2)
Borax (0,2 mol L ⁻¹ pH 9)	Solubilisation et alcalinisation	Solubilisation dans les micelles par l'intermédiaire d'interactions hydrophobes (2)

Figure VIII.1. Structure chimique des sulfonamides, types d'interactions impliquées dans l'adsorption, mécanismes d'extraction des solutions aqueuses.

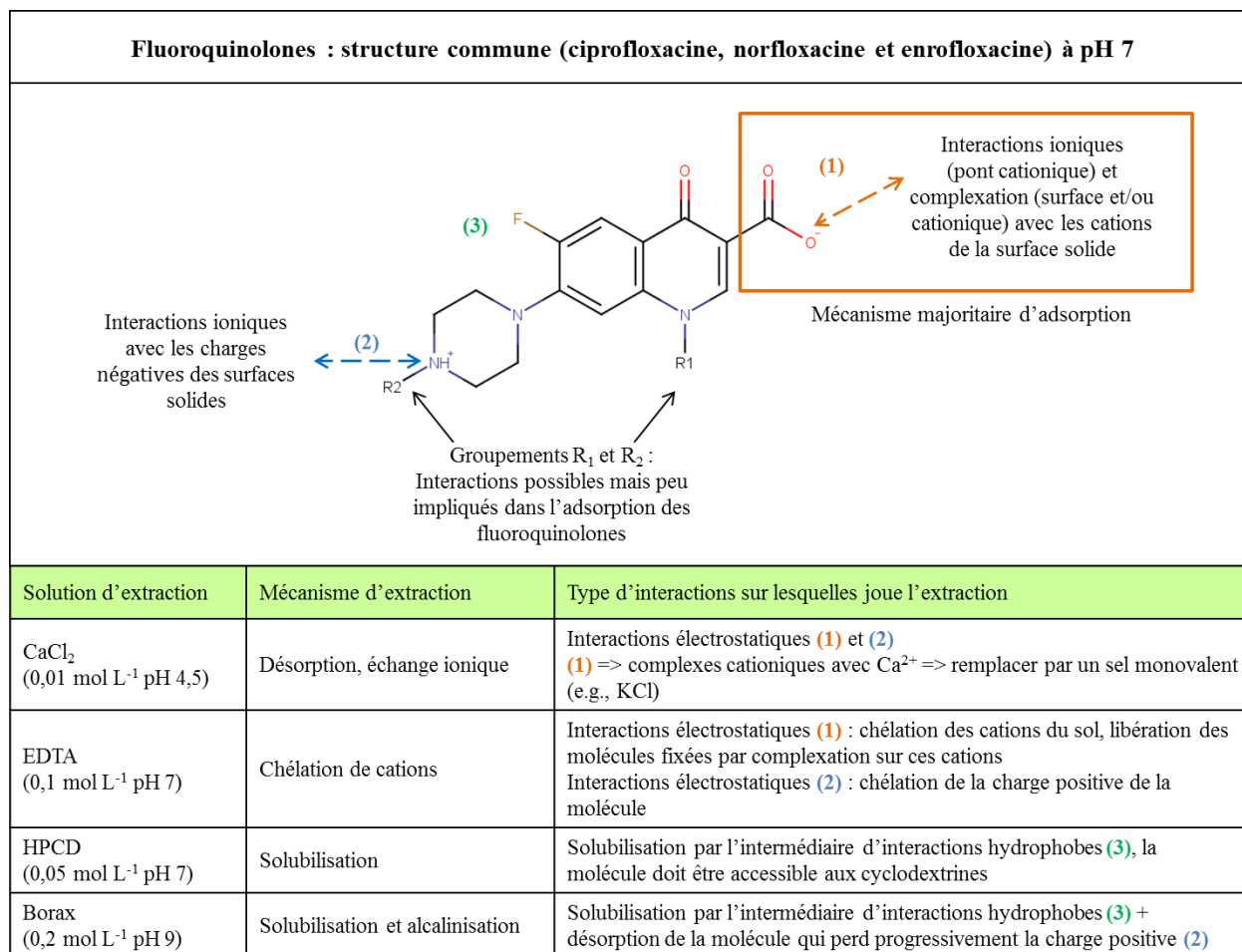


Figure VIII.2. Structure chimique des fluoroquinolones, types d'interactions impliquées dans l'adsorption, mécanismes d'extraction des solutions aqueuses.

De façon générale, les principaux mécanismes d'adsorption sur des constituants minéraux et organiques varient peu d'un antibiotique à un autre au sein d'une même famille (Figures VIII.1 et VIII.2 ; Figueroa-Diva et al., 2010). Les interactions données sur ces Figures ne sont pas exhaustives puisque d'autres types d'interactions peuvent être établies avec les constituants des phases solides, comme par exemple des liaisons électrostatiques par l'intermédiaire des électrons libres des cycles aromatiques, ou encore des liaisons hydrogènes ou des forces de Van der Waals. Etant donné que toutes ces interactions peuvent être établies pour l'ensemble des antibiotiques, il est essentiel de tenir compte des interactions les plus discriminantes qui interviennent dans les différents mécanismes d'adsorption des antibiotiques. Par conséquent, nos conclusions concernant la spécificité des différents extractants vis-à-vis de certains mécanismes d'adsorption ou de certains sites réactifs des MAFOR et des sols pourraient être étendues à d'autres sulfonamides et d'autres fluoroquinolones. Cependant, il est parfois nécessaire de tenir compte des différents groupes fonctionnels portés par les antibiotiques d'une même famille, notamment pour les sulfonamides. En effet, comme le montre la Figure VIII.1, ces groupements peuvent jouer un rôle plus ou moins important dans les propriétés physico-chimiques contrôlant les interactions des sulfonamides qui

s'adsorbent majoritairement sur la matière organique (Thiele-Bruhn et al., 2004 ; Figueroa-Diva et al., 2010). Pour les fluoroquinolones, les groupements fonctionnels de substitution peuvent être engagés dans des interactions non spécifiques, de type liaisons hydrogènes, forces de Van der Waals, moins stables que les interactions ioniques et de complexation engagées par les autres groupements de la molécule ; ces antibiotiques s'adsorbent donc majoritairement par échange cationique, complexation de surface et complexation cationique, les groupes fonctionnels jouant peu sur l'adsorption de ces molécules (Figure VIII.2).

2) En quoi la nature des MAFOR (composantes organique et minérale) influence-t-elle, initialement et dans le temps, la disponibilité des antibiotiques dans les sols amendés?

La nature des phases porteuses des antibiotiques au sein de la MAFOR et les mécanismes d'adsorption impliqués sont essentiels pour expliquer la disponibilité et le devenir des antibiotiques dans les sols amendés. Les résultats de cette thèse montrent que la qualité de la matière organique des MAFOR, notamment sa composition biochimique et sa biodégradabilité, influence la disponibilité des antibiotiques (Chapitres II et III). En particulier, la plus grande instabilité de la matière organique du fumier par rapport à celle du compost peut entraîner une libération de la ciprofloxacine, initialement adsorbée sur le fumier et davantage extraite par les solutions aqueuses après 7 jours (Chapitre II). Il est aussi important de considérer la composante minérale et plus globalement les propriétés physico-chimiques des MAFOR dans le devenir des antibiotiques. Le pH des MAFOR apportées influence la disponibilité de la ciprofloxacine, du sulfaméthoxazole et de l'érythromycine dans les sols (Chapitres II, III, VI). Au pH du fumier plus alcalin que celui du compost, la proportion d'antibiotiques anioniques est plus importante dans le fumier par rapport à celle dans le compost. Comme l'adsorption de la ciprofloxacine, du sulfaméthoxazole et de l'érythromycine est plus faible lorsque le pH augmente (Kim et al., 2004 ; Vasudevan et al., 2009 ; Lertpaitoonpan et al., 2009), les antibiotiques sont globalement plus disponibles dans les sols amendés par le fumier par rapport à des les sols amendés par le compost (pour ciprofloxacine et sulfaméthoxazole) ou des sols non amendés (pour l'érythromycine). Le pH étant globalement stable dans les mélanges sol/MAFOR au cours des incubations (Chapitres II, III, IV), ce facteur peut influencer la disponibilité des antibiotiques dans les sols à long terme. De la même façon, l'apport de MAFOR sur le sol permet de stabiliser le pH de celui-ci ; la disponibilité des antibiotiques varie en fonction de leurs constantes de dissociation acide (pK_a).

D'autres paramètres, tels que les teneurs en argiles et en oxydes métalliques, se rapportant à la composante minérale de la MAFOR (cas des composts de boue de station d'épuration), ainsi que

la capacité d'échange cationique peuvent influencer la disponibilité des antibiotiques. L'adsorption de certains antibiotiques, comme les fluoroquinolones ou les tétracyclines, peut en effet varier en fonction des valeurs de ces paramètres, comme cela a été montré dans les sols (Figuro-Diva et al., 2010). Concernant les sulfonamides, la composante minérale des matrices solides jouerait un moindre rôle dans leur adsorption en comparaison de la composante organique (Thiele-Bruhn et al., 2004), en particulier dans les matrices riches en matière organique telles que les MAFOR ou les sols amendés par celles-ci (Kahle et Stämme, 2007a ; Kahle et Stämme, 2007b).

Comme les antibiotiques sont introduits dans les sols via l'épandage des MAFOR, il est essentiel de ne pas négliger les effets de leurs propriétés sur la disponibilité des antibiotiques dans les sols et sur les formes chimiques disponibles (formes libres ou complexées ; voir le point 4). Nous avons montré que la matière organique co-extraite par les solutions aqueuses était moins complexe dans les extraits de mélanges sol/fumier par rapport aux extraits de mélanges sol/compost (Chapitre IV), ce qui a permis de mettre en évidence une fraction disponible de sulfaméthoxazole supérieure dans les sols amendés par le fumier.

Cependant, d'autres facteurs comme le pH, influencent fortement l'extraction aqueuse du sulfaméthoxazole, comme celle de la ciprofloxacine.

Les propriétés des solutions d'extraction comme le pH doivent être adaptées en fonction des MAFOR. Par exemple, si le pH de la MAFOR est plus alcalin que celui du sol, le pH de la solution d'extraction doit être proche de celui de la MAFOR considérée pour estimer la disponibilité maximale des antibiotiques dans les sols amendés. Par exemple, une extraction avec une solution de Borax (0.2 mol L^{-1} , pH 9) peut être utilisée (Chapitre II). Si la MAFOR a un pH proche de celui du sol, le pouvoir tampon du sol permet d'obtenir le bon pH de la solution aqueuse, par exemple, lors d'une extraction par une solution de CaCl_2 (0.01 mol L^{-1}) dont le pH est autour de 4,5.

Il paraît nécessaire de compléter ce travail en s'intéressant aux phases porteuses inorganiques présentes dans certaines MAFOR (par exemple, boues de station d'épuration). Certaines fractions granulométriques des composts peuvent être enrichies en minéraux et en métaux (Houot et al., 2009). Nous avons souligné le rôle des cations pouvant engager des liaisons de complexation ou des liaisons ioniques avec des molécules comme les fluoroquinolones. Une meilleure caractérisation de la fraction inorganique des matrices (minéralogie, granulométrie, surface spécifique) serait nécessaire pour mieux expliquer certains processus d'immobilisation.

3) Un modèle simulant les évolutions conjointes de la matière organique et des fractions d'un polluant organique peut-il être utilisé dans le cas des antibiotiques?

Les résultats de cette thèse montrent qu'un modèle couplant la dynamique de la matière organique dans le sol et celle du polluant organique, tous deux apportés par la MAFOR (COP-Soil version Brimo et al., 2016b), peut être utilisé pour le sulfaméthoxazole et son métabolite acétylé *N*-acétylsulfaméthoxazole (Chapitre III). L'évolution de la disponibilité des deux composés au cours du temps a été bien simulée dans les sols amendés par le compost ou par le fumier. La composition biochimique de la matière organique de la MAFOR apportée a été prise en compte pour décrire la minéralisation de celle-ci et pour modéliser l'évolution des fractions minéralisée, disponible, faiblement et fortement adsorbées du sulfaméthoxazole et de son métabolite dans le sol. Le modèle permet de simuler l'évolution des différentes fractions en fonction de l'évolution de la matière organique au cours du temps, en tenant compte de l'affinité des sulfonamides pour la matière organique. Quel que soit l'extractant doux utilisé (CaCl₂, EDTA, HPCD), la fraction disponible des molécules diminue au cours du temps en lien avec la formation de résidus non-extractibles d'origine chimique et/ou physique ; l'évolution de ces deux fractions semble être le résultat de la biodégradation des matières organiques les plus accessibles dans la MAFOR. De plus, la faible minéralisation du sulfaméthoxazole et du *N*-acétylsulfaméthoxazole est liée au co-métabolisme, plus important dans les sols amendés par le fumier et aussi responsable d'une plus grande formation de métabolites et de résidus non-extractibles biogéniques. En conclusion, l'utilisation de ce modèle couplé a permis de démêler des processus liés entre eux et à l'origine de l'évolution de la répartition du sulfaméthoxazole et de son métabolite dans les différentes fractions disponible, adsorbée, liée ou minéralisée au cours du temps. Il a aussi montré qu'il est possible de simuler leur devenir sur la base des fractions disponibles extraites par les différents extractants.

En perspective, le modèle doit être davantage testé avec d'autres MAFOR et d'autres antibiotiques. L'affinité que peuvent avoir les antibiotiques pour certaines composantes de la fraction minérale des MAFOR doit être prise en compte car celle-ci peut influencer la répartition des antibiotiques dans les MAFOR et leur devenir dans les sols. Certaines caractéristiques telles que le pH de la matrice et les constantes de dissociation acide (pK_a) des antibiotiques doivent être prises en compte dans le modèle pour considérer la spéciation ionique des antibiotiques en fonction du pH.

4) Quels sont les effets matrices au moment de la quantification des antibiotiques dans les extraits? La présence de matière organique dissoute dans les extraits permet-elle d'expliquer les interactions entre les molécules et les matrices solides qui peuvent limiter leur disponibilité?

Les effets matrices dans les extraits CaCl₂, EDTA et HPCD ont été déterminés lors de l'utilisation des solutions aqueuses pour extraire la fraction disponible de sulfaméthoxazole et de trois produits de transformation potentiels dans les sols amendés par les MAFOR (Chapitre IV). En considérant leurs mécanismes d'extraction respectifs, les effets matrices sont plus importants dans les extraits EDTA en lien avec la concentration en matière organique co-extraite qui est supérieure à celle présente dans les extraits CaCl₂. Les effets matrices des extraits CaCl₂ et EDTA peuvent être corrigés grâce à l'ajout d'un étalon interne deutéré (sulfaméthoxazole-d₄) au moment de l'injection SPE-UHPLC-MS-MS. En revanche, les effets matrices lors de l'extraction avec la solution de HPCD sont très importants et conduisent à la suppression totale du signal. Une purification QuEChERS (Bourdat-Deschamps et al., 2014) des extraits est donc nécessaire ; les effets matrices et les rendements de purification peuvent être corrigés grâce à l'ajout de l'étalon interne avant la purification.

L'application de la méthode analytique pour le suivi des fractions disponibles dans les sols amendés par le compost ou le fumier et la caractérisation des matières organiques co-extraites par les solutions CaCl₂, EDTA et HPCD par la spectrométrie de fluorescence-3D (Chapitre IV), ont montré que :

i) La matière organique co-extraite par la solution d'EDTA est plus complexe que celle co-extraite par les solutions de CaCl₂ ou de HPCD. Dans les extraits EDTA, la matière organique dissoute contribue aux effets matrices en spectrométrie de masse plus importants en comparaison aux extraits CaCl₂ ; ainsi qu'à la plus faible quantité de SMX libre dissout dans les extraits. La nécessité d'une purification des extraits HPCD n'est pas forcément liée à la présence de matière organique complexe dans ces extraits mais plutôt à la quantité importante de carbone organique et/ou la présence des molécules de cyclodextrine.

ii) la matière organique co-extraite est moins complexe dans les extraits des mélanges sol/fumier par rapport aux extraits des mélanges sol/compost. Ceci semble expliquer la plus grande disponibilité du sulfaméthoxazole dans les sols amendés par le fumier.

iii) en lien avec l'humification de la matière organique dans les mélanges sol/MAFOR au cours de l'incubation, plus la matière organique évolue vers des formes plus condensées, moins le sulfaméthoxazole est disponible.

L'approche analytique alliant l'utilisation du ^{14}C -radiomarquage et l'analyse par la SPE-UPLC-MS-MS a abouti à des mesures de disponibilités différentes pour le sulfaméthoxazole dans les mélanges sol/MAFOR. Pour des concentrations initiales proches, les fractions disponibles du sulfaméthoxazole sont plus élevées en considérant la radioactivité mesurée dans les extraits aqueux par rapport à celles déduites des concentrations quantifiées par SPE-UHPLC-MS-MS (Chapitres III et IV). Des matières colloïdales organiques et/ou minérales sont co-extraites par les solutions aqueuses. Toutes les molécules marquées au ^{14}C présentes dans les extraits, qu'elles soient libres dissoutes et/ou associées aux matières colloïdales, peuvent être quantifiées ; et elles peuvent correspondre à la fois à la molécule mère de l'antibiotique, mais aussi aux potentiels produits de transformation également extraits. La méthode analytique de SPE-UHPLC-MS-MS permet de ne quantifier que les molécules libres dissoutes dans les extraits et de façon spécifique puisque les molécules mères et les produits de transformation sont quantifiés séparément. Ainsi, au sein de la fraction disponible extraite par les trois solutions, une partie des molécules du sulfaméthoxazole étaient associées aux colloïdes et/ou à la matière organique co-extraite (Chapitre IV).

De façon plus large, l'extraction aqueuse des fractions disponibles des antibiotiques entraîne dans les extraits la présence de molécules libres et/ou de molécules plus ou moins associées aux colloïdes minéraux et/ou organiques co-extraits selon l'extractant utilisé. Dans l'estimation de la disponibilité environnementale, il est essentiel de ne pas négliger ces molécules associées aux colloïdes car ceux-ci peuvent favoriser le transport des antibiotiques dans le sol (Xing et al., 2015). Les antibiotiques adsorbés sur la phase solide du sol peuvent contribuer à l'exposition microbienne et exercer un stress toxique pour les micro-organismes du sol (Chander et al., 2005 ; Peng et al., 2015). Par conséquent, il pourrait en être de même pour les molécules associées aux colloïdes dans la solution du sol mais il reste difficile d'estimer la proportion réellement biodisponible de la fraction colloïdale.

L'utilisation de la technique analytique SPE-UPLC-MS-MS ne permet pas la quantification des molécules associées aux colloïdes dans les extraits, puisque seules les molécules libres dissoutes sont quantifiées dans les extraits injectés. Cette limite analytique dépend de la quantité de matières colloïdales co-extraites : relativement peu dans les extraits CaCl_2 et beaucoup dans les extraits EDTA. La purification des extraits HPCD avant leur injection peut permettre de désorber une partie des molécules et ainsi de ne doser que les molécules sous formes libres.

Dans les extraits, un moyen de savoir si les molécules libres et/ou les molécules associées aux colloïdes sont bioactives serait de réaliser des tests écotoxicologiques avec des bioessais comme le test Microtox[®] (Backhaus et Grimme, 1999). Quack et al. (2005) ont montré que la tétracycline dans des extraits de sols était moins toxique que l'antibiotique en solution aqueuse en

réalisant des analyses HPLC-MS-MS en parallèle de tests de toxicité avec un bioessai bactérien. La tétracycline serait moins bioactive car partiellement adsorbée sur des composants du sol, qui influencent également son extractabilité.

Ces bioessais sont intéressants même s'ils sont délicats à mettre en œuvre car la matrice de l'extrait en elle-même peut être toxique pour l'organisme testé. Par exemple, l'EDTA chélate les cations qui sont indispensables aux réactions enzymatiques et au fonctionnement physiocellulaire. Il faut donc procéder par étapes successives, en testant la toxicité de : i) l'antibiotique en solution aqueuse ; ii) l'extrait seul sans antibiotiques ; iii) la toxicité de l'extrait contenant à la fois les molécules d'antibiotique dissoutes et associées aux colloïdes organiques et/ou minéraux ; iv) la toxicité de l'extrait filtré, la filtration de l'extrait permettant d'éliminer les matières colloïdales. Toutefois, de tels tests écotoxicologiques réalisés sur des extraits ne permettent pas réellement d'estimer la biodisponibilité de contaminants dans les sols (Chapitre I). En revanche, ce genre de test peut contribuer à l'identification des molécules responsables de la toxicité globale d'un extrait, et ainsi de distinguer la part de la toxicité de la molécule mère et/ou des produits de transformation.

5) En quoi la composition et les propriétés physico-chimiques des sols influencent-elles la disponibilité des antibiotiques?

Les effets matrices liés aux propriétés des sols ont été étudiés dans le cas d'extractions exhaustives de composés pharmaceutiques (Salvia et al., 2013) : des facteurs tels que le pH, la teneur en argiles, la teneur en matière organique et la CEC influencent les rendements d'extraction. De la même façon, les facteurs influençant la sorption des antibiotiques dans les sols ont beaucoup été étudiés (Lertpaitoonpan et al., 2009 ; Figuero-Diva et al., 2010 ; Maszkowska et al., 2015). Dans le Chapitre V, les résultats des extractions douces réalisées sur des sols présentant des propriétés pédologiques différentes et ne recevant pas de MAFOR ont montré que la disponibilité du sulfaméthoxazole dépend de la teneur en matière organique, de la texture et de la capacité d'échange cationique (CEC) du sol. Plus il y a de matière organique dans le sol, et de la même façon, plus la CEC et la teneur en argiles sont importantes, moins le sulfaméthoxazole est disponible. Cependant, ces corrélations ont été établies seulement pour les extractions avec les solutions de CaCl_2 et de HPCD, et non avec celle d'EDTA ; ceci a mis en évidence un mécanisme d'extraction moins spécifique de l'extractant EDTA vis-à-vis des molécules de sulfaméthoxazole dans la solution du sol et faiblement adsorbées sur la phase solide.

En considérant que les résidus d'antibiotiques peuvent être libérés notamment lors de la dégradation de la matière organique des MAFOR dans les sols, il semble important de déterminer la

disponibilité environnementale des résidus d'antibiotiques qui conditionne leur devenir environnemental et qui dépend des propriétés des sols. Un nombre suffisant de sols pour couvrir une large gamme de propriétés (pH, teneur en matière organique ou en argiles) devrait être utilisé afin d'estimer la disponibilité des antibiotiques dans chacun des sols en fonction de plusieurs niveaux de concentrations en antibiotiques dans l'objectif de hiérarchiser les facteurs les plus influents. Par exemple, le mécanisme principal d'adsorption des sulfonamides étant les interactions avec la matière organique (Figure VIII.1), la disponibilité environnementale de ces antibiotiques doit être étudiée selon des gradients de teneur en matière organique, de pH. De la même façon, le mécanisme principal d'adsorption des fluoroquinolones dans le sol étant la complexation ionique (Figure VIII.2), la disponibilité environnementale de ces molécules doit être étudiée notamment selon des gradients de teneurs en oxydes métalliques, en argile, de pH. Lorsque la disponibilité d'un antibiotique dans le sol est étudiée, il est essentiel de faire varier, au mieux, les paramètres physico-chimiques un par un car les paramètres peuvent être corrélés entre eux, comme la CEC et les teneurs en matière organique et en argiles.

6) Les antibiotiques sont-ils dégradés dans les sols après l'application de MAFOR ?

La formation de produits de transformation dans les sols amendés a permis d'estimer indirectement la disponibilité des antibiotiques pour leur biotransformation. Les processus de biodégradation des antibiotiques dans les sols dépendent de leur biodisponibilité, elle-même influencée par leur capacité d'adsorption sur les matrices solides. Dans le Chapitre II, au bout d'un mois d'incubation, la fraction de ciprofloxacine minéralisée était trop faible pour dire que la minéralisation de la molécule était réelle ; cette fraction minéralisée peut correspondre à la présence d'impuretés dans la solution de ciprofloxacine. Girardi et al. (2011) et Mougin et al. (2013) ont également obtenu des taux de minéralisation de moins de 1% au bout de trois mois d'incubation. De plus, quelques analyses HPLC ont pu être réalisées mais n'ont pas pu confirmer la présence de produits de transformation de la ciprofloxacine. Les fluoroquinolones peuvent être dégradées dans des matrices aqueuses ou dans les sédiments (Lin et al., 2010), mais les processus abiotiques tels que la photodégradation semblent être majoritairement responsables de la dégradation des fluoroquinolones (Van Doorslaer et al., 2014). La biodégradation des fluoroquinolones a aussi été observée lors du traitement des eaux usées ou dans des biofilms issus de filtres pour l'eau potable (Dorival-García et al., 2013 ; Liao et al., 2016). Dans les sols, plusieurs études ont montré que les fluoroquinolones étaient biodégradées par quelques espèces bactériennes ou plus souvent par des champignons (Martens et al., 1996 ; Chen et al., 1997 ; Parshikov et al., 1999 ; Parshikov et al., 2000 ; Marengo et al., 2001 ; Parshikov et al., 2001). Cependant, en raison de la forte adsorption des

fluoroquinolones sur les matrices solides, la biodégradation dans les sols reste très faible et les fluoroquinolones ont tendance à persister (Golet et al., 2003 ; Picó and Andreu, 2007 ; Van Doorslaer et al., 2014).

Contrairement aux fluoroquinolones, les sulfonamides peuvent être relativement disponibles dans les sols amendés ou non, pour être biodégradés. Même si les sulfonamides semblent difficilement biodégradés, ces antibiotiques peuvent subir différentes transformations jusqu'à la formation de produits de transformation voire la minéralisation, dans les sols et les solutions aqueuses, par des processus à la fois biologiques et physico-chimiques (Halling-Sørensen et al., 1998 ; Baran et al., 2006 ; Sukul et al., 2008 ; Schwarz et al., 2010 ; Sittig et al., 2014 ; Topp et al., 2013). Dans nos expérimentations (Chapitre III), environ 2% du sulfaméthoxazole ont été minéralisés dans les mélanges sol/MAFOR au bout d'un mois d'incubation. Les analyses HPLC de certains extraits ont révélé la présence de produits de transformation non identifiés. Afin de les identifier, la formation de produits de transformation potentiels tels que l'aniline, le *N*-acétylsulfaméthoxazole et le 3-amino-5-méthylisoxazole, a été suivie au cours de l'incubation de trois mois (Chapitre IV). Le *N*-acétylsulfaméthoxazole a été rapidement formé et quantifié dans la fraction disponible extraite juste après l'incorporation de la MAFOR dans le sol ; puis la concentration dans la fraction extraite a diminué au cours du temps de la même façon que celle du sulfaméthoxazole. De plus, les produits de transformation du sulfaméthoxazole tels que le *N*-acétylsulfaméthoxazole peuvent être retransformés en leur composé parent (Su et al., 2016 ; Chapitres III et IV). Le 3-amino-5-méthylisoxazole a également été détecté en fin d'incubation. Dans la littérature, les produits de transformation issus de la modification du sulfaméthoxazole en position para conservent une activité antibactérienne et l'effet d'un mélange de produits de transformation peut être additif (Majewski et al., 2014). Il est donc essentiel de considérer les produits de transformation dans l'évaluation des impacts des antibiotiques dans les sols, en testant la toxicité des produits de transformation seuls et aussi en mélanges vis-à-vis des micro-organismes.

Par ailleurs, des produits de transformation non identifiés ont été détectés dans les extraits de sols dans des expérimentations de biodégradation avec l'érythromycine et la sulfaméthazine (Chapitre VI). Ces produits de transformation étaient observés aussi bien dans les sols où les antibiotiques étaient introduits directement en solution ou via l'apport d'un fumier. Même si la biodégradation des composés parents permet de réduire l'exposition des micro-organismes à ces antibiotiques, ces derniers restent exposés aux produits de transformation qui pourraient exercer une pression de sélection.

7) Une méthode chimique d'extraction permet-elle d'estimer la biodisponibilité des antibiotiques vis-à-vis des micro-organismes ? Deux types d'effets ont été considérés pour cette question : la biodégradation des antibiotiques par des micro-organismes adaptés (7a) et les effets toxiques des antibiotiques sur l'activité des communautés nitrifiantes des sols (7b).

7a) Biodégradation des antibiotiques par des micro-organismes adaptés

Lors d'une exposition à long terme à des doses permettant d'exercer des pressions de sélection suffisantes, les micro-organismes du sol peuvent s'adapter à la présence des antibiotiques en développant un métabolisme spécifique qui permet de dégrader l'antibiotique, pour en tirer un avantage compétitif (résistance, acquisition de nutriments). Il est alors question de biodégradation dite accélérée dans les sols régulièrement exposés aux antibiotiques en comparaison à un sol ne recevant pas d'antibiotiques ou exposé à des doses trop faibles pour induire une pression de sélection (Topp et al., 2013 ; Topp et al., 2016). Cependant, le phénomène d'adaptation des micro-organismes n'est pas équivalent d'un antibiotique à l'autre, à concentrations égales. L'antibiotique doit en effet avant tout être biodisponible vis-à-vis des micro-organismes afin que les processus d'adaptation soient mis en jeu. Les résultats du chapitre VI ont montré que la biodégradation accélérée était observée à condition que l'antibiotique soit extrait lors des extractions douces (solutions de Borax ou d'EDTA). En effet, la ciprofloxacine, fortement adsorbée dans les sols, était peu extraite par les solutions aqueuses, donc peu disponible et non minéralisée au cours de l'incubation, contrairement à l'érythromycine et la sulfaméthazine. Pour ces deux antibiotiques, les extractions aqueuses ont mis en évidence une fraction chimiquement disponible, et potentiellement biodisponible pour les micro-organismes capables de biodégradation, et ces deux antibiotiques étaient rapidement biodégradés dans les sols adaptés. Mis à part le fait que la ciprofloxacine ne soit pas disponible pour la biodégradation, d'autres hypothèses peuvent expliquer ce résultat : cet antibiotique pourrait ne pas être sélectionné par les micro-organismes en tant que source de nutriments (carbone, azote), et/ou les micro-organismes n'auraient pas acquis le métabolisme spécifique. Cependant, des produits de transformation peuvent être formés même si la ciprofloxacine n'est pas minéralisée.

Les résultats ont par ailleurs montré que l'adaptation des micro-organismes semble spécifique vis-à-vis d'une molécule d'antibiotique au sein d'une même famille. En perspective, il serait intéressant de tester davantage l'hypothèse de l'adaptation spécifique des micro-organismes d'un sol pour la biodégradation d'un sulfonamide (par exemple, la sulfaméthazine) vis-à-vis d'autres composés de la même famille pour déterminer les capacités de biodégradation croisée. Si cela se vérifie, étant donné que l'adsorption varie d'un sulfonamide à un autre (Thiele-Bruhn et al.,

2004), il serait envisageable de tester l'hypothèse de la relation entre la disponibilité des sulfonamides et la biodégradation. Une autre approche pour tester la relation entre disponibilité environnementale et biodisponibilité microbienne (via la biodégradation) serait de sélectionner plusieurs sols présentant des propriétés physico-chimiques différentes, de les inoculer avec une souche bactérienne isolée qui est capable de biodégrader la sulfaméthazine (*Microbacterium sp* ; Topp et al., 2013), de doper ensuite ces sols avec la sulfaméthazine afin d'estimer sa disponibilité dans chaque sol et de suivre sa biodégradation. En particulier, ces sols pourraient présenter un gradient de matière organique, l'adsorption des sulfonamides étant prioritairement influencée par celle-ci (Figueroa-Diva et al., 2010 ; Thiele-Bruhn et al., 2004).

Les fractions disponibles pouvant constituer un indicateur de l'exposition chronique des micro-organismes, une autre perspective serait de tester la pertinence des techniques d'extractions douces au champ pour déterminer quelle fraction disponible des antibiotiques peut induire l'acquisition de capacité de biodégradation microbienne accélérée.

7b) Toxicité des antibiotiques sur l'activité des communautés nitrifiantes du sol

Les résultats préliminaires obtenus avec des solutions aqueuses présentant différents pouvoirs d'extraction (Chapitres II à IV) ont permis de choisir certaines d'entre elles afin d'estimer la biodisponibilité du sulfaméthoxazole et de la ciprofloxacine dans les sols amendés par les MAFOR (Chapitre VII). L'adsorption de la ciprofloxacine plus forte dans le mélange sol/compost permet de réduire sa biodisponibilité et empêcher toute toxicité sur l'activité de nitrification. En revanche dans le mélange sol/fumier, même faiblement disponible, la ciprofloxacine peut entraîner un effet sur la nitrification. Il en était de même pour le sulfaméthoxazole dans les deux types de mélange sol/MAFOR, même si des effets différents ont été observés. La méthode d'extraction avec des solvants organiques, qui se veut exhaustive, ne semble pas adaptée pour évaluer la biodisponibilité (Alexander, 2000). En effet, la fraction de ciprofloxacine extraite avec les mélanges à base d'acétonitrile est relativement élevée dans les mélanges sol/compost alors qu'aucun effet n'est observé même à des doses plus élevées (Chapitre VII-Partie A). De plus, si une corrélation entre les pourcentages d'inhibition de l'activité nitrifiante dans les sols amendés par le compost (relations doses-réponses ; Partie B) et les fractions de sulfaméthoxazole extraites avec le solvant organique est établie au début de l'incubation (8 jours), cette corrélation n'est pas maintenue au cours de l'incubation (28 et 84 jours). La fraction totale extractible au solvant surestime l'exposition des micro-organismes aux antibiotiques dans les sols. Seules les molécules dans la solution du sol et facilement désorbées de la matrice solide semblent être biodisponibles vis-à-vis des communautés nitrifiantes. Les mécanismes d'extraction mis en jeu par les solutions CaCl_2 et HPCD permettent

d'obtenir cette fraction, contrairement à la solution d'EDTA ou au solvant organique qui permet de désorber davantage des molécules plus fortement adsorbées ; ce qui peut conduire à une mauvaise estimation des fractions biodisponibles. De plus, ces relations sont établies avec la fraction de sulfaméthoxazole libre extraite et ne concernent que le composé majoritaire qui est la molécule mère. Au regard de ces résultats, les solutions de CaCl_2 (sulfaméthoxazole) ou de HPCD (sulfaméthoxazole et ciprofloxacine) pourraient être choisies. La solution de Borax n'avait pas été sélectionnée pour mesurer la disponibilité du sulfaméthoxazole mais étant donné le mécanisme d'extraction proche de celui de l'HPCD, son utilisation pour estimer la disponibilité d'antibiotiques peut être envisagée.

En perspective, le choix d'un extractant pourrait en particulier être appuyé par une expérimentation au cours de laquelle les effets des antibiotiques sur les micro-organismes seraient étudiés dans différents sols présentant une gamme de propriétés physico-chimiques (par exemple un gradient de teneurs en matière organique ou en argiles). En effet, l'adsorption des antibiotiques étant influencée par ces propriétés, la disponibilité environnementale et donc la biodisponibilité en dépendent. Cette expérimentation permettrait d'autre part de s'affranchir du facteur temps qui, dans le cas du suivi de paramètres écotoxicologiques comme la mesure d'effet sur une activité microbienne, apporte des facteurs confondants dans l'étude de la biodisponibilité. En effet, comme expliqué dans le Chapitre VII (Partie B), la résilience des communautés modifie la sensibilité initiale des communautés, indépendamment de l'évolution des fractions disponibles qui conditionnent l'exposition.

D'autre part, il est essentiel de tester la toxicité des résidus d'antibiotiques en mélanges, car ceux-ci peuvent avoir un effet agoniste, synergique ou antagoniste sur les réponses des micro-organismes (Backhaus et al., 2000 ; Brosche et Backhaus, 2010). L'estimation de la biodisponibilité des antibiotiques par les extractions douces pourrait permettre d'expliquer les différences de réponses observées et de comprendre si certaines molécules peuvent être en compétition sur des sites d'adsorption.

Pour estimer la biodisponibilité des antibiotiques dans les sols à long terme, l'abondance des gènes de résistance à un antibiotique pourrait aussi être un bon indicateur de pressions de sélection. L'augmentation d'antibiorésistances à différents antibiotiques dont le sulfaméthoxazole et la ciprofloxacine a été montrée dans des sols irrigués par des eaux usées (Dalkmann et al., 2012). La mesure de l'abondance des gènes de résistance permettrait de confronter leur dynamique à celle de la disponibilité des antibiotiques estimée par différents extractants, ce qui pourrait permettre comme pour la biodégradation, d'identifier des seuils de stress sélectionnant l'adaptation des micro-organismes dans les sols.

Ainsi, les méthodes chimiques développées au cours de cette thèse pourraient être utilisées pour estimer la biodisponibilité des antibiotiques dans les sols amendés par les MAFOR. Les tendances dessinés pour les comportements des deux molécules étudiées, la ciprofloxacine et le sulfaméthoxazole, et représentatives de deux familles d'antibiotiques majeures, pourraient être généralisées à d'autres antibiotiques. L'outil TyPol (Servien et al., 2014 ; Mamy et al., 2015) pourrait permettre de déterminer les propriétés des molécules prépondérantes dans le contrôle de leur biodisponibilité et le déterminisme d'effets sur les micro-organismes.

D'autre part, des connaissances complémentaires sont à acquérir sur les effets des propriétés des MAFOR et des sols sur la disponibilité environnementale et la biodisponibilité des antibiotiques dans les sols. Il est important de hiérarchiser l'importance de ces propriétés physico-chimiques afin de les intégrer au mieux dans des outils de modélisation. Ces connaissances permettraient de contribuer à l'évaluation des risques dans un contexte de recyclage des MAFOR à grande échelle, en particulier pour identifier des sols plus à risque que d'autres en termes de transfert des résidus d'antibiotiques vers les eaux souterraines et d'impacts sur les micro-organismes avec l'acquisition d'antibiorésistances.

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Devenir et biodisponibilité des antibiotiques entrant dans les sols agricoles lors du recyclage des matières fertilisantes d'origine résiduaire

Mots-clés : Antibiotique ; sol ; devenir ; biodisponibilité ; extraction chimique ; impact

Résumé : Des antibiotiques sont introduits de façon chronique dans les sols lors de l'épandage des matières fertilisantes d'origine résiduaire (MAFOR), telles que le fumier ou les boues de station d'épuration. La dissémination de ces polluants dans l'environnement présente un risque à long terme pour la santé en raison notamment de l'apparition de résistances aux antibiotiques. L'origine et les propriétés des MAFOR peuvent influencer le devenir ultérieur des antibiotiques dans les sols. Ce devenir et les effets des antibiotiques sur les organismes vivants dépendent de leur (bio)disponibilité. Peu de données existent sur la (bio)disponibilité des antibiotiques dans les sols car elles sont conditionnées par le développement de méthodes d'extraction et d'analyse des molécules à de faibles teneurs, dans des matrices organo-minérales complexes. Ce travail de recherche se focalise sur les liens entre nature des MAFOR, devenir et biodisponibilité des antibiotiques dans les sols, et impacts sur les micro-organismes du sol. Des méthodes chimiques ont été développées pour estimer la (bio)disponibilité et le devenir de deux antibiotiques (ciprofloxacine et sulfaméthoxazole) et de certains produits de transformation dans les sols amendés par des MAFOR et incubés au laboratoire. Un modèle a été utilisé pour décrire le devenir du sulfaméthoxazole et de son métabolite principal dans les sols en fonction de l'évolution de la matière organique de la MAFOR ; ceci a permis de montrer que le co-métabolisme pouvait être à l'origine de la biodégradation des molécules et de la formation de résidus non-extractibles. Enfin, ces données chimiques ont été confrontées à des mesures biologiques : la (bio)disponibilité des antibiotiques mesurée à l'aide de différentes extractions aqueuses a été reliée à leur biodégradation par des micro-organismes adaptés dans les sols ou à leur toxicité sur les activités microbiennes nitrifiantes des sols. Ce travail a permis d'obtenir des résultats originaux qui pourront être utiles pour une évaluation des risques liés à la dispersion des antibiotiques dans l'environnement.

Behavior and bioavailability of antibiotics introduced in agricultural soils through the recycling of exogenous organic matter

Keywords: Antibiotic; soil; behavior; bioavailability; chemical extraction; impact

Abstract: Antibiotics are chronically introduced in soils through the application of exogenous organic matter (EOM) such as manure or sewage sludge. The environmental dissemination of these pollutants presents a long-term risk for health particularly due to the acquisition of antibiotic resistances. The origin and the properties of EOM can influence the antibiotic behavior in soils. This behavior and the potential impact on living-organisms depends on the antibiotic (bio)availability. Few data about the antibiotic bioavailability in soils exist and increasing knowledge is conditioned by the development of extraction and analytical methods for molecules at low concentrations in complex organomineral matrices. This research work focuses on the link between the MAFOR type, the behavior and the bioavailability of antibiotics in soils, and their impacts on soil microorganisms. Chemical methods were developed to assess the (bio)availability and the behavior of two antibiotics (sulfamethoxazole and ciprofloxacin) and some transformation products in EOM-amended soils incubated in controlled conditions. A modelling approach was used to describe the behavior of sulfamethoxazole and its main metabolite in soils as a function of the organic matter evolution; the modelling results suggest that co-metabolism was responsible for the molecule mineralization and the formation of biogenic non-extractable residues. Finally chemical data were confronted to biological measures: the (bio)availability of antibiotics estimated through different aqueous extractions was related to their biodegradation by adapted soil microorganisms or to their toxicity on soil microbial activities. Original results were obtained in this work and will be useful to assess the risk related to the antibiotic dispersion in the environment.

